Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

A RE-EXAMINATION OF THE ACETONATION OF AN EQUILIBRIUM MIXTURE OF D-ALTROSE AND 1,6-ANHYDRO-β-D-ALTROPYRANOSE AND A SYNTHESIS OF 1,2:5,6-DI-*O*-ISOPROPYLIDENE-β-D-MANNOFURANOSE

J. S. BRIMACOMBE* AND (MISS) P. A. GENT
Chemistry Department, P. O. Box 363, The University, Birmingham 15 (Great Britain)
(Received May 27th, 1969)

ABSTRACT

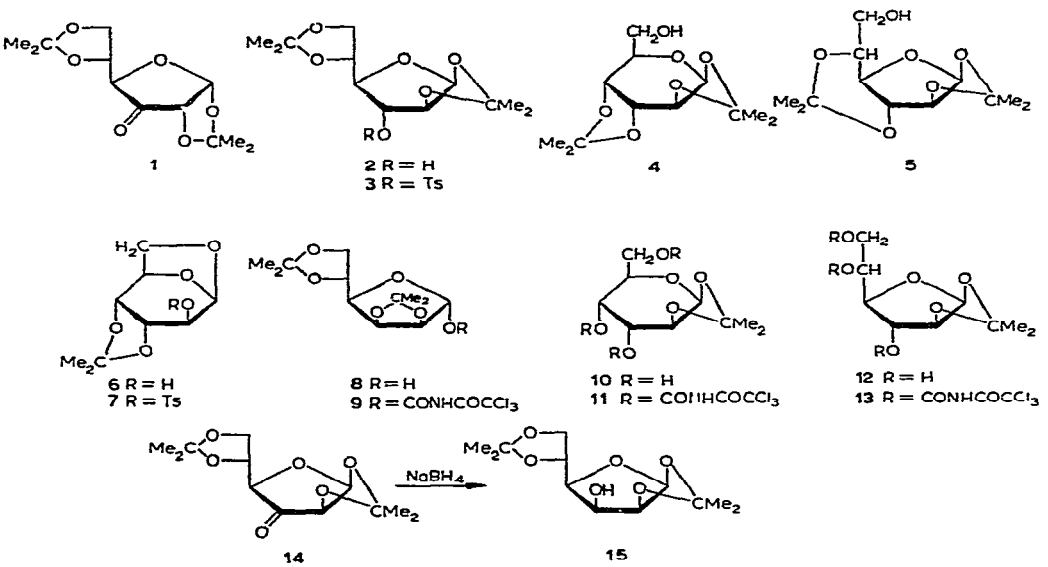
Acid-catalysed acetonation of an equilibrium mixture of D-altrose and 1,6-anhydro-β-D-altropyranose was shown by g.l.c. to yield 1,6-anhydro-3,4-*O*-isopropylidene-β-D-altropyranose (**6**, 60%), 1,2:5,6-di-*O*-isopropylidene-β-D-altrofuranose (**2**, 23%), and 1,2:3,4-di-*O*-isopropylidene-β-D-altropyranose (**4**, 17%). Evidence is presented in support of the structure **4** for the latter diacetal which has been obtained in crystalline form. Oxidation of the diacetal **2** with acetic anhydride–methyl sulphoxide afforded 1,2:5,6-di-*O*-isopropylidene-β-D-*arabino*-hexofuranos-3-ulose (**14**), which was reduced stereospecifically, with sodium borohydride, to 1,2:5,6-di-*O*-isopropylidene-β-D-mannofuranose (**15**).

INTRODUCTION

The ketone **1**, which is obtained[1–3] by oxidation of 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose, has proved to be a versatile intermediate in the synthesis of a number of sugars of biological interest. Syntheses of 3-acetamido-3-deoxy-D-glucose[3], 3-acetamido-3-deoxy-D-galactose[4], 3-amino-3-deoxy-D-xylose[3], 3-amino-3-deoxy-L-arabinose[5], 3-deoxy-3-fluoro-D-glucose[6], 3-deoxy-3-fluoro-D-galactose[7], D-allose[8], and D-gulose[9] have been initiated from the ketone **1**. The general procedures adopted in the foregoing syntheses should be applicable to other 1,2:5,6-di-*O*-isopropylidene-hexofuranos-3-uloses, and, in this connection, we sought to prepare the ketone **14** by oxidation of 1,2:5,6-di-*O*-isopropylidene-β-D-altrofuranose (**2**). The diacetal **2** is obtained, *inter alia*, by acid-catalysed acetonation of D-altrose[10] or the equilibrium mixture of D-altrose and 1,6-anhydro-β-D-altropyranose resulting from acid hydrolysis of methyl 4,6-*O*-benzylidene-α-D-altropyranoside[11]. The structure **2** is reasonably founded[10] on the unreactivity of the diacetal towards oxidation by alkaline permanganate. The latter acetonation also yielded 1,6-anhydro-3,4-*O*-isopropylidene-β-D-altropyranose (**6**) and a second, syrupy diacetal to which the structure 1,2:3,4-di-*O*-isopropylidene-β-D-altropyranose (**4**) was tentatively assigned[11], since, on oxidation with alkaline permanganate, it afforded a carboxylic acid derivative. However, it was pointed out[11] that the structure **5**, although less likely, would also fit the available

*To whom enquiries should be addressed at The Chemistry Department, The University, Dundee DD1 4HN, Scotland.

evidence. We now present evidence in favour of the structure **4** and report on a synthesis of 1,2:5,6-di-*O*-isopropylidene-β-D-mannofuranose (**15**) from diacetal **2**.



DISCUSSION

The diacetal **2** was prepared according to the literature procedures[10,11] and was isolated from the acid-catalysed acetonation of D-altrose by chromatography on silica gel. A comprehensive analysis of the products of acetonation was not attempted in this case, but the percentage composition of the acetals formed by acetonation of D-altrose–1,6-anhydro-β-D-altropyranose was given as **6** (60%), **2** (23%), and **4** (17%) by g.l.c. Chromatography of these products on silica gel afforded a fraction containing both the diacetals **2** and **4**, together with a second fraction which yielded 1,6-anhydro-3,4-*O*-isopropylidene-β-D-altropyranose (**6**) (characterised as the crystalline 2-toluene-*p*-sulphonate **7**)[11]. 1,2:5,6-Di-*O*-isopropylidene-β-D-altrofuranose (**2**) could be crystallised directly from the mixture of diacetals, whereafter preparative g.l.c. of the mother liquors was used to separate 1,2:3,4-di-*O*-isopropylidene-β-D-altropyranose (**4**), which was obtained in crystalline form. Comparison of the physical constants (see Experimental) of the crystalline diacetal **4** with those reported[11] for a syrupy preparation suggested that the latter was probably heavily contaminated with the strongly laevorotatory acetal **6**.

The chemical evidence[10] for the structure of diacetal **2** was confirmed by mass spectrometry. In addition to the highest mass peak at *m/e* 245 (M − 15), the mass spectrum of **2** exhibited a prominent peak at *m/e* 101 (M − 159) which is characteristic[12] in these compounds for fragmentation of the C-4–C-5 bond. The mass spectrum of the isomeric diacetal **4** contained a peak at *m/e* 245 (M − 15), and it also exhibited other mass peaks (see Experimental) that were compatible with[12], but not definitive for, the structure assigned.

More-compelling evidence for the structure **4** accrued from n.m.r. spectroscopy of the monoacetals formed on graded, acid hydrolysis; complete, acid hydrolysis liberated D-altrose as the only reducing sugar. Attempts to selectively remove one of the isopropylidene groups by brief treatment of diacetal **4** with hot 1% nitric acid in ethyl acetate gave a fairly complex product mixture, although these conditions selectively removed[13] the 3,4-acetal group from 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose. Treatment of diacetal **4** with 80% acetic acid for 2 h at 50° gave a small proportion of a monoacetal fraction that was isolated by chromatography on silica gel. N.m.r. spectroscopy (see Fig. 3) and g.l.c. of this fraction showed that it consisted of two monoacetals in the ratio of *ca.* 4:1. The major component (*A*) was readily distinguished from 1,2-*O*-isopropylidene-β-D-altrofuranose[14] (**12**) by n.m.r. spectroscopy (*cf.* Figs. 2 and 3) and by comparison of the derived tris-*O*-(trimethylsilyl) derivatives by g.l.c. A choice between other possible structures for *A* was based on n.m.r. spectroscopy of the tris(trichloroacetylcarbamate) which was rapidly formed, *in situ*, by the addition of a slight excess of trichloroacetyl isocyanate[15] to a solution of *A* in deuterioacetone. Characteristic downfield shifts of carbinol protons are observed[16] upon formation of the trichloroacetylcarbamoyl derivative, and related shifts have been of immense value in assigning structures to partially esterified carbohydrates[17]. The effect on the n.m.r. spectrum of adding trichloroacetyl isocyanate to a solution of 2,3:5,6-di-*O*-isopropylidene-α-D-mannofuranose (**8**) in deuteriochloroform is clearly seen from Fig. 1. The H-1 resonance of **8** appears at τ 4.64 as a narrow doublet ($J_{H,OH}$ 2.5 Hz) due to coupling with the hydroxylic proton. Conversion into the trichloroacetylcarbamate **9** removes this coupling, and the H-1 resonance now appears as a singlet at τ 3.82, a downfield shift of *ca.* 0.8 p.p.m.

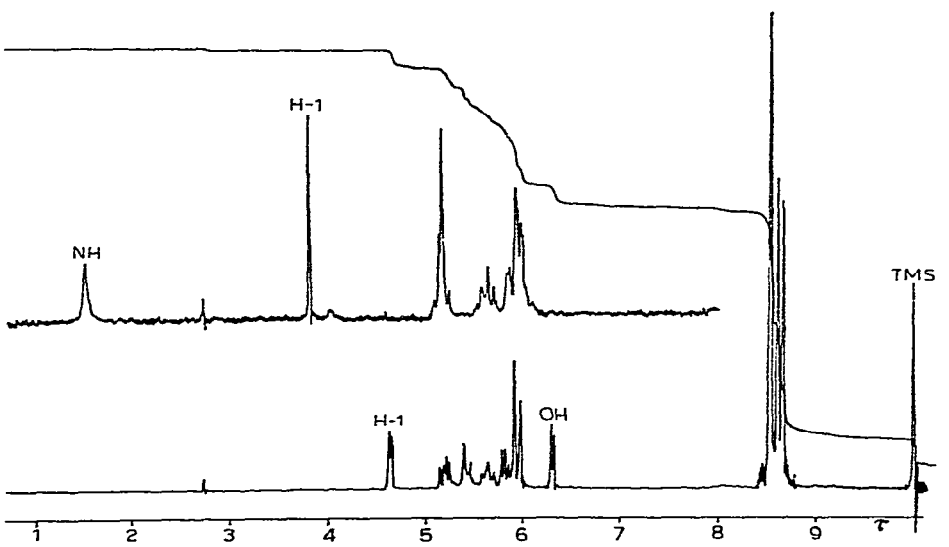


Fig. 1. The n.m.r. spectrum (100 MHz, $CDCl_3$) of 2,3:5,6-di-*O*-isopropylidene-α-D-mannofuranose (**8**), and the partial spectrum of the derived trichloroacetylcarbamate (**9**).

By contrast, the H-1 resonance observed as a narrow doublet at τ 4.20 in the n.m.r. spectrum of the 1,2-acetal **12** suffers only a minor shift on formation of the tris-(trichloroacetylcarbamoyl) derivative **13** (see Fig. 2). Marked shifts to lower field can be noted, however, for other resonances in the spectrum, which is now amenable to analysis. Treatment of monoacetal *A* with trichloroacetyl isocyanate caused (Fig. 3)

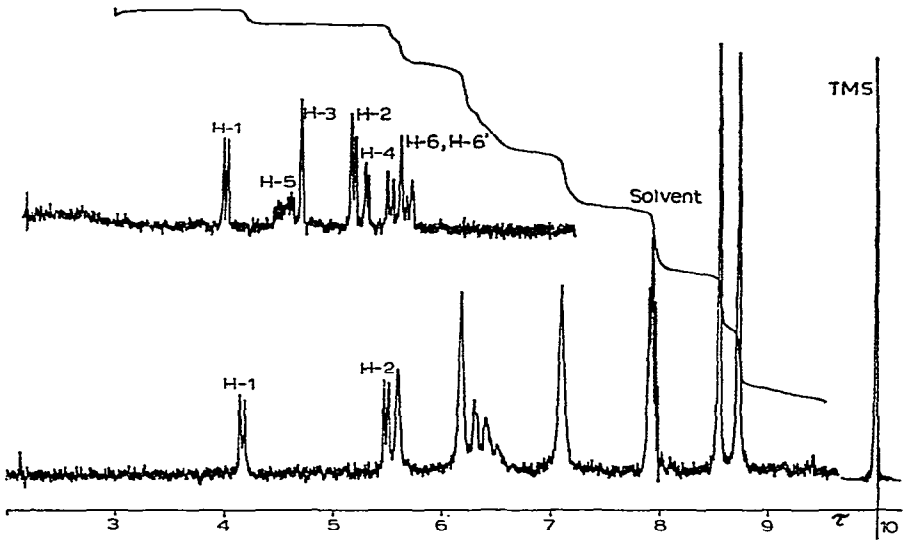


Fig. 2. The n.m.r. spectrum (100 MHz, Me_2CO-d_6) of 1,2-*O*-isopropylidene-β-D-altrofuranose (**12**) and the partial spectrum of the tris(trichloroacetylcarbamoyl) derivative (**13**). The coupling constant obtained for **13** were $J_{1,2}$ 4; $J_{2,3} \sim J_{3,4}$ <0·5; $J_{4,5}$ 2·5; $J_{5,6}$ 5; $J_{5,6'} \sim$10 Hz.

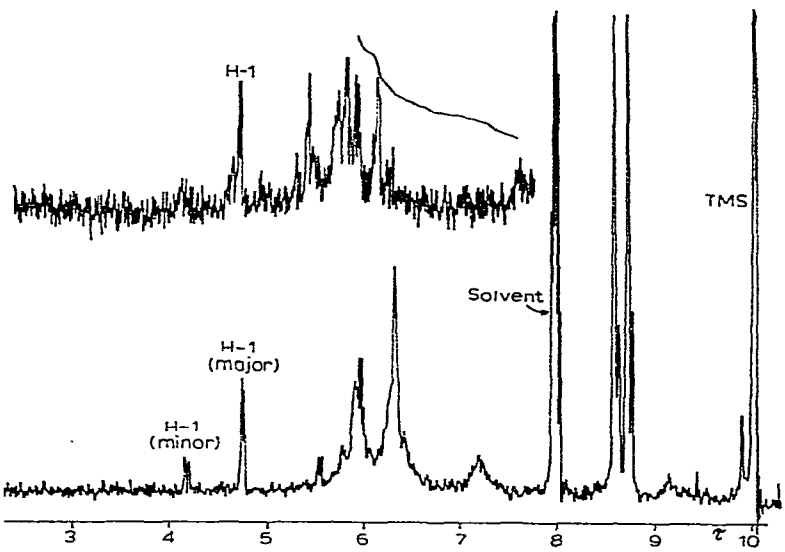


Fig. 3. The n.m.r. spectra (100 MHz, Me_2CO-d_6) of the monoacetals resulting from graded hydrolysis of diacetal **4** before and after (inset) the addition of trichloroacetyl isocyanate.

no change in the chemical shift of the H-1 resonance, which appears as a narrow doublet ($J_{1,2}$ *ca.* 2 Hz) centred at τ 4.75. This implies that the acetal group must involve C-1 and C-2, and hence that *A* is 1,2-*O*-isopropylidene-β-D-altropyranose (**10**). This information, combined with the chemical evidence[11], precludes structures other than **4** for the original diacetal.

The diacetal **2** was oxidised with acetic anhydride–methyl sulphoxide[18] to give, principally, 1,2:5,6-di-*O*-isopropylidene-β-D-*arabino*-hexofuranos-3-ulose (**14**), which gave 1,2:5,6-di-*O*-isopropylidene-β-D-mannofuranose (**15**) on reduction with sodium borohydride in aqueous ethanol. The stereospecificity of this reduction parallels that observed[3,19] with the ketone **1**, and, in each case, the approach of the nucleophile to the carbonyl group is clearly favoured from an *exo*-direction with respect to the trioxabicyclo[3.3.0]octane ring-system. The structure of diacetal **15** was proved by elemental analyses, by the formation of D-mannose (characterised as the phenylhydrazone[20]) on complete acid hydrolysis, and from the presence of a peak at *m/e* 101 (M − 159) in its mass spectrum[12]. The mass spectrum of **15** was generally similar to that of the isomeric diacetal **8**, but it was significant that the latter spectrum contained a more intense peak at *m/e* 243 (M − 17) due to the attachment of the hydroxyl group to C-1 rather than to C-3. This difference was also apparent[21] in the mass spectra of the isomeric D-talofuranose diacetals having 2,3:5,6- and 1,2:5,6-arrangements of the isopropylidene groups. These results lend weight to a suggestion of De Jongh and Biemann[12] that differences of this sort may prove useful in assigning structures to isomeric di-*O*-isopropylidenehexofuranoses.

EXPERIMENTAL

Thin-layer chromatography (t.l.c.) was performed on Kieselgel G, and detection was effected with vanillin–sulphuric acid[22]. Paper chromatography was performed on Whatman No. 1 paper by downward irrigation with ethyl acetate–pyridine–water (8:2:1, solvent *a*) or butyl alcohol–pyridine–water (6:4:3, solvent *b*), and detection with aniline hydrogen phthalate[23]. Analytical g.l.c. was carried out by using a Pye 104 gas chromatograph, and preparative separations were effected on a Pye 105 gas chromatograph (column, 30′ × 3/8″); a column packing of 10% silicon ester-30 on Celite was used in both cases, at the stated temperature. Infrared spectra were recorded with a Perkin–Elmer 257 spectrometer, and, unless otherwise indicated, n.m.r. spectra were obtained with a Perkin–Elmer R-14 spectrometer for deuteriochloroform solutions with tetramethylsilane as internal reference. Mass spectra were measured with an A.E.I. MS9 spectrometer using a direct-insertion technique. Trimethylsilylations were achieved by using bis(trimethylsilyl)acetamide according to the manufacturer's instructions.

Acetonation of D-*altrose.* — A solution of syrupy D-altrose[24] (0.72 g) in dry acetone (50 ml) containing anhydrous copper(II) sulphate (3.6 g) and conc. sulphuric acid (0.05 ml) was shaken for 48 h at room temperature, and the reaction mixture was then processed as described by Steiger and Reichstein[10]. A partial separation

of the diacetal fraction was obtained by chromatography of the syrupy products (0.88 g) on silica gel (elution with 15% of acetone in toluene). Two recrystallisations from ether–light petroleum (b.p. 40–60°) gave the diacetal **2** (0.13 g), m.p. 89–90°, $[\alpha]_D$ +25° (*c* 0.49, acetone); lit., m.p. 89°, $[\alpha]_D$ +28.3° (*c* 2, acetone) (ref. 10); m.p. 87–88°, $[\alpha]_D$ +28.7° (*c* 1.04, acetone) (ref. 11). The derived 3-toluene-*p*-sulphonate[14] **3**, prepared in the usual manner, had m.p. 101–102° (from ethanol), $[\alpha]_D$ +29° (*c* 0.5, chloroform). The mass spectrum of diacetal **2** showed prominent peaks at *m/e* 245 (M−15), 187, 159, 127, 101, 59, and 43, in agreement[12] with the structure originally assigned[11]. N.m.r. data: τ 4.10 (1-proton doublet, $J_{1,2}$ 4 Hz, H-1); 8.50, 8.58, 8.65, 8.68 (singlets, each 3 protons, isopropylidene groups).

Acetonation of an equilibrium mixture of D-*altrose and 1,6-anhydro-β-*D-*altropyranose.* — A mixture[11] of the title compounds (18 g) was shaken with acetone (280 ml) containing conc. sulphuric acid (1.6 ml) for 24 h at room temperature, whereafter the solution was neutralised with conc. ammonia, and solids were filtered off. Evaporation of the solvent left a syrup which was extracted with ether (6 × 50 ml), and the combined extracts were evaporated to give the products (13.8 g). G.l.c. (column temperature, 151°) showed that the mixture contained **6** (60%), **2** (23%), and **4** (17%), with retention times of 13.2, 25.8, and 21.6 min, respectively. A portion (6 g) of the mixture was chromatographed on silica gel (elution with ethyl acetate–hexane, 2:1) to yield a crystalline mixture of the diacetals **2** and **4** (1.65 g) and, after washing the column with acetone, the acetal **6** (*ca.* 3.4 g). The latter compound was characterised as the crystalline 2-toluene-*p*-sulphonate **7** which had m.p. 178–178.5°, $[\alpha]_D$ −153° (*c* 0.57, chloroform); lit.[11], m.p. 176–177°, $[\alpha]_D$ −150.8° (*c* 2.24, chloroform). The remainder of the original acetonation products was chromatographed in the same way to give a total of 5.5 g of diacetals **2** and **4**. Fractional crystallisation from ether–light petroleum (b.p. 60–80°) gave diacetal **2** (1.8 g), m.p. and mixed m.p. 87–88°, $[\alpha]_D$ +29° (*c* 0.7, acetone). G.l.c. of the mother liquors at this stage revealed the predominance of diacetal **4**. Concentration of the mother liquors left a crystalline residue (3.1 g) which was dissolved in ether to give an approximately 50% solution, which was subjected, in several aliquot portions (200 μl), to preparative g.l.c. (column temperature, 182°; nitrogen flow-rate, 120 ml/min). Collection of the appropriate fractions (retention time, 1.7 h) afforded a crystalline material which was sublimed at 15 mmHg to give diacetal **4** (0.2 g), m.p. 64–65°, $[\alpha]_D$ +68° (*c* 0.96, chloroform), ν_{max} 1379 (CMe_2) and 3507 cm^{-1} (OH) (Found: C, 55.1; H, 7.8. $C_{12}H_{20}O_6$ calc.: C, 55.4; H, 7.7%). The mass spectrum contained characteristic[12] peaks at *m/e* 245 (M−15), 187, 171, 127, 113, 100, 99, 85, 71, 69, 59, and 43. N.m.r. data: τ 4.73 (1-proton doublet, $J_{1,2}$ 2.5 Hz, H-1); 8.49, 8.55, 8.62, 8.65 (singlets, each 3 protons, isopropylidene groups). Newth and Wiggins[11] reported $[\alpha]_D$ −13.1° (*c* 1.3, chloroform) for a syrupy preparation which was probably contaminated with the acetal **6**.

Hydrolysis of diacetal **4** in a small quantity of methanol containing an equal volume of N sulphuric acid on a boiling water-bath for 2 h liberated D-altrose (chromatographic identification in solvent *a*) as the only reducing component.

1,2-O-Isopropylidene-β-D-altrofuranose (**12**). — This compound, m.p. 129–130°, $[\alpha]_D$ +23° (*c* 0.64, methanol), was prepared as described by Newth[14] who reported m.p. 125–126°, $[\alpha]_D$ +25.8° (*c* 1.01, methanol). The n.m.r. spectrum of monoacetal **12** in deuterioacetone and that of the tris(trichloroacetylcarbamate) **13** (prepared by the addition of a slight excess of trichloroacetyl isocyanate[15]) are shown in Fig. 2.

Graded hydrolysis of 1,2:3,4-di-O-isopropylidene-β-D-altropyranose (**4**). — A solution of diacetal **4** (0.16 g) in 80% acetic acid (5 ml) was heated for 2 h at 50°, after which time t.l.c. (methanol–ethyl acetate, 9:1) revealed the presence of the free sugar, a monoacetal fraction, and unreacted material. The solvents were removed, and the residue was chromatographed on silica gel (elution with methanol–ethyl acetate, 19:1) to give a crystalline fraction (30 mg), m.p. 128–129° (from ethyl acetate), containing monoacetal **10** and a second monoacetal, presumably 3,4-*O*-isopropylidene-D-altropyranose, in the ratio of *ca.* 4:1; further recrystallisations failed to remove the minor component (Found: C, 49.1; H, 7.0. $C_9H_{16}O_6$ calc.: C, 49.1; H, 7.3%). The monoacetals **10** and **12** were distinguished by g.l.c. (column temperature, 190°) of their tris-*O*-(trimethylsilyl) derivatives which had retention times of 18.4 and 21.6 min, respectively. The m.p. of the crystalline fraction containing **10** was depressed to 113–117° on admixture with compound **12**, and the X-ray powder photographs of the two materials showed them to be different.

The n.m.r. spectrum (deuterioacetone) of the mixture of monoacetals resulting from graded hydrolysis of diacetal **4** is shown in Fig. 3, together with part of the spectrum after the addition of trichloroacetyl isocyanate.

1,2:5,6-Di-O-isopropylidene-β-D-mannofuranose (**15**). — A solution of diacetal **2** (0.31 g) in methyl sulphoxide (24 ml) and acetic anhydride (3 ml) was stirred for 15 h at room temperature, whereupon t.l.c. (acetone–toluene, 1:4) showed that all of the starting material had reacted. The solvents were removed under diminished pressure at 50° to give a syrup which was distilled to give the ketone **14** (0.22 g), b.p. 102–108° (bath)/0.7 mmHg, $[\alpha]_D$ −25° (*c* 0.9, acetone), ν_{max} 1778 (C = O) and 1375 cm^{-1} (CMe_2). T.l.c. showed that this material contained a small proportion of a faster-moving component, but it was used in subsequent experiments without further purification (Found: C, 55.1; H, 6.7. $C_{12}H_{18}O_6$ calc. : C, 55.8; H, 7.0%).

Sodium borohydride (0.2 g) was gradually added to the ketone **14** (0.2 g) in 75% aqueous methanol (5 ml) and, on complete addition, the solution was kept for 1.5 h at room temperature. It was then extracted with chloroform (3 × 5 ml), and the combined extracts were washed with water (5 ml) and dried ($MgSO_4$). G.l.c. (column temperature, 172°) of the trimethylsilyl ether of the product (retention time, 22.8 min) showed that negligible amounts of diacetal **2** were formed; trimethylsilylation of **2** gave a product having a retention time of 20.4 min. Removal of the solvent left a syrupy residue (0.19 g) which still contained a faster-moving impurity. Preparative t.l.c. [acetone–toluene (1:4) as the mobile phase] and distillation gave the pure diacetal **15** (0.12 g), b.p. 120–125°(bath)/0.05 mmHg, which crystallised on standing. The crystalline material had m.p. 51–53.5° [from ether–light petroleum (b.p. 40–60°)], $[\alpha]_D$ +3 ±1° (*c* 1.27, acetone), ν_{max} 3500 (OH) and 1377 cm^{-1} (CMe_2) (Found:

C, 55.1; H, 7.8. $C_{12}H_{20}O_6$ calc.: C, 55.4; H, 7.7%). N.m.r. data: τ 4.33 (1-proton doublet, $J_{1,2}$ 4 Hz, H-1); 5.30–6.30 (6 protons, H-2–H-6); 8.41, 8.56, 8.60, 8.63 (singlets, each 3 protons, isopropylidene groups). The mass spectrum of **15** exhibited peaks at *m/e* 245 (M − 15), 187, 159, 127, 113, 101, 85, 59, and 43, in accord[12] with the structure assigned.

Hydrolysis of **15** (80 mg) in 50% aqueous methanol (5 ml) for 5 h at 66° with an excess of Amberlite IR-120 (H^+) afforded D-mannose (*ca.* 50 mg) (chromatographic identification, solvent *b*) as the only reducing sugar. D-Mannose phenylhydrazone, prepared in the usual way[20], had m.p. 195–196° (dec.), and its X-ray powder photograph was indistinguishable from that of an authentic sample; lit.[20] m.p. 199–200°.

ACKNOWLEDGMENTS

The authors thank Professor M. Stacey, C. B. E., F. R. S., for his interest, Dr. E. F. Mooney for measuring the n.m.r. spectra, and Dr. J. R. Majer for obtaining the mass spectra. The Science Research Council is gratefully acknowledged for the award of a studentship (to P. A. G.).

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

THE STRUCTURE OF *Acacia campylacantha* GUM*

D. M. W. ANDERSON AND A. C. MUNRO
Department of Chemistry, The University, Edinburgh EH9 3JJ (Great Britain)

(Received May 28th, 1969)

ABSTRACT

Acacia campylacantha trees exude a polysaccharide containing D-galactose (54%), L-arabinose (29%), L-rhamnose (8%), D-glucuronic acid (7%), and 4-*O*-methyl-D-glucuronic acid (2%). Linkage analysis gives 3-*O*-α-D-galactopyranosyl-L-arabinose, 3-*O*-β-L-arabinopyranosyl-L-arabinose, 3-*O*-β-L-arabinofuranosyl-L-arabinose, 3-*O*-β-D-galactopyranosyl-D-galactose, and 6-*O*-β-D-galactopyranosyl-D-galactose. The *O*-methyl derivative of the gum was examined, after methanolysis, by g.l.c.; 2,3,4-tri-*O*-methyl-L-rhamnose, 2,3,5- and 2,3,4-tri-, and 2,5-di-*O*-methyl-L-arabinose, 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-, and 2,4-di-*O*-methyl-D-galactose, and 2,3,4-tri- and 2,3-di-*O*-methyl-D-glucuronic acid were detected. Degraded-gum *A*, obtained after controlled, acid hydrolysis, was examined by linkage and methylation analysis, and was subjected to a Smith-degradation. The whole gum was subjected to five successive Smith-degradations; the polysaccharides obtained after each degradation were examined by methylation and linkage analysis. The structural evidence suggests that *A. campylacantha* gum is based on a branched galactan core, to which are attached uronic acid, rhamnose, and unusually short chains containing not more than three arabinose residues. *Acacia campylacantha* gum is broadly similar in structure to the gums from *A. senegal* and *A. laeta*, although differences in fine structure can be distinguished.

INTRODUCTION

Acacia campylacantha Hochst. ex A. Rich. [the "claw" *Acacia*[1], syn. *A. suma* (non Kurz.), syn. *A. polyacantha* Willd. subsp. *campylacantha* (Hochst. ex A. Rich.) Brenan] is one of the species in Series 5 (Vulgares), sub-series 1 (Gerontogeae spiciflorae) of Bentham's classification[2] of the genus. Of the *Acacia* species studied so far, those closest botanically[3] to *A. campylacantha* are *A. senegal* (L.) Willd.[4] and *A. laeta* R. Br. ex Benth.[5].

This Paper gives the results of a detailed study of *A. campylacantha* gum, and its structural features are compared with those of *A. senegal* and *A. laeta* gums.

*This is Part 34 of the Series: Studies of Uronic Acid Materials. Part 33: Ref. 8.

TABLE I

DATA FOR *A. campylacantha* GUM AND ITS DEGRADATION PRODUCTS

Polysaccharide	*Yield* (%)	$[\alpha]_D$ (*degrees*)	$\overline{M}_n$ $\times 10^3$	*Constituent sugars* (%)					*Periodate* (*mmoles/g*) *reduced*	*Formic acid* (*mmoles/g*) *released*
				Gal	*Ara*	*Rha*	*GluA*	*4-O-Me-GluA*		
A. campylacantha gum	97	−12	135	54	29	8	7	2	6.43	1.64 (98 h)
Degraded-gum *A*	36[a]	− 1	6.4	82	5	—	13	—	12.1	—
Degraded-gum *B*	18	+ 2	—	n.d.	—	—	n.d.	—	—	—
Polysaccharide I	43[a]	−28	44	78	19	—	3	—	4.91	3.28 (120 h)
Polysaccharide II	28[a]	+14	14	97	3	—	—	—	4.83	1.88 (48 h)
Polysaccharide III	44[a]	+35	10.5	100	0	—	—	—	2.34	1.62 (48 h)
Polysaccharide IV	70[a]	+20	9.1	100	—	—	—	—	2.58	2.78 (48 h)
Polysaccharide V	68[a]	+19	8.3	100	—	—	—	—	2.20	0.69 (48 h)

[a]Yields are corrected for the amounts of material withdrawn in aliquots to monitor the progress of the degradations.

Analytical data for a different specimen of *A. campylacantha* gum have been published[6], and the acidic components of the gum have been identified[7].

RESULTS AND DISCUSSION

The crude gum was completely soluble in water. After filtration, dialysis, and freeze-drying, the gum polysaccharide, which had the sugar composition shown in Table I, was examined by zone electrophoresis on Millipore Phoroslides[8], by ion-exchange chromatography on DEAE-cellulose[9], and by ultracentrifugation. None of these experiments indicated any sharp discontinuity in the properties of the system of heteropolymolecular species involved. In contrast, examination of the polysaccharide by molecular-sieve chromatography on a column of "Sepharose 4B" appeared to indicate the presence of two components[11]. The major component was eluted

TABLE II

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED *A. campylacantha* GUM

Relative retention times (T) *of methyl glycosides*[a]		R_G	*O-Methyl sugar identified*
Column (i)	*Column* (ii)	*Solvent* (c)	
0.49	0.44	1.01	2,3,4-Tri-*O*-methyl-L-rhamnose
0.58, 0.71	0.51, 0.61	0.98	2,3,5-Tri-*O*-methyl-L-arabinose
1.01	0.83	0.77	2,3,4-Tri-*O*-methyl-L-arabinose
1.80, 3.27	1.27, (2.22)	0.80	2,5-Di-*O*-methyl-L-arabinose
1.69	1.66	0.88	2,3,4,6-Tetra-*O*-methyl-D-galactose
3.76, 4.21	3.01, 3.49	0.72	2,4,6-Tri-*O*-methyl-D-galactose
6.50	5.16	0.72	2,3,4-Tri-*O*-methyl-D-galactose
—	—	0.54	2,6-Di-*O*-methyl-D-galactose
14.7, 16.6	9.7, 11.1	0.52	2,4-Di-*O*-methyl-D-galactose
—	—	0.32	2-*O*-Methyl-D-galactose
2.29, 3.01	(2.22), 2.71	—	2,3,4-Tri-*O*-methyl-D-glucuronic acid[b]
7.7, 9.5	5.9, 6.9	—	2,3-Di-*O*-methyl-D-glucuronic acid[b]
1.16, 1.44	0.97, 1.15	—	Unknown components

[a]Figures in parenthesis indicate *T* values of incompletely resolved components. [b]As methyl ester methyl glycoside.

within the effective sieving range of the column; a minor component of high molecular weight was eluted at the void volume. The latter component is not, however, present in the crude gum, and there is evidence[12] that it is a molecular aggregation artefact, arising during freeze-drying or subsequent storage of the purified polysaccharide. Similar behaviour has been observed for other gum exudates[8]. It therefore appears that *A. campylacantha* gum is heteropolymolecular and analogous to *A. senegal*[4] and *A. laeta*[5] gums.

The analytical data obtained for the gum polysaccharide were similar to those obtained previously for a different specimen[6]. Light-scattering measurements gave $\bar{M}_W = 312{,}000$, and molecular-sieve chromatography, on a precalibrated "Bio-Gel P300" column[10], indicated $\bar{M}_n \sim 135{,}000$.

The gum was methylated, and, after methanolysis, examination by g.l.c. showed (Table II) the presence of 2,3,4-tri-*O*-methyl-L-rhamnose; 2,3,5- and 2,3,4-tri-, and 2,5-di-*O*-methyl-L-arabinose; 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-, and 2,4-di-*O*-methyl-D-galactose; and 2,3,4-tri-, and 2,3-di-*O*-methyl-D-glucuronic acid. The g.l.c. chromatograms were similar to those obtained for the methanolysed *O*-methyl derivatives of *A. senegal*[4] and *A. laeta*[5] gums.

Controlled hydrolysis of the gum by 0.01N sulphuric acid gave degraded-gum *A* (see Table I) together with fragments having the same paper-chromatographic mobility as 3-*O*-α-D-galactopyranosyl-L-arabinose, 3-*O*-β-L-arabinopyranosyl-L-arabinose, and, in small proportion, 3-*O*-β-L-arabinofuranosyl-L-arabinose. Since all three disaccharides were released under relatively mild conditions, the reducing arabinose residues may have been present in the furanose form. These disaccharides are ubiquitous in Series 5 of the *Acacia* genus[3] and have been characterised rigorously for *A. senegal*[4] and *A. laeta*[5] gums.

During mild, acid hydrolysis, the specific rotation of *A. campylacantha* gum changed from −12 to −1°; this is similar to the changes reported for the gums from *A. senegal*[4] (−31.5 to −11°) and *A. laeta*[5] (−42 to −38°). Molecular-sieve chromatography of degraded-gum *A* indicated $\bar{M}_n$ ~6,400, and this is similar to the values reported for the corresponding degraded gums from *A. senegal*[4] (4,800) and *A. laeta*[5] (5,700). The mere removal of arabinose and rhamnose from the periphery of the molecule by controlled, acid hydrolysis could not give a degraded gum of such low molecular weight; nor does the degradation appear to result from internal, acid-labile, arabinofuranose units, since acid hydrolysis of borohydride-reduced, degraded-gum *A* gave galactitol but no arabinitol. For *A. senegal*[4], *A. laeta*[5], *A. arabica*[16], *A. drepanolobium*[17], and *A. seyal*[18] gums, it has been proposed that the drastic decrease in molecular weight implies that certain galactopyranosidic bonds must be unusually sensitive to mild conditions of hydrolysis. From the data given in Table I, the *A. campylacantha* gum molecule contains, on average, ~500 galactose residues; assuming the gum to have a branched galactan core, the decrease in $\bar{M}_n$ from 13,500 to 6,400 (*ca.* 32 galactose residues) for degraded-gum *A* could be caused by random hydrolysis of only 3% of the original galactopyranosidic bonds. The presence of a small proportion of galactose in the diffusate from mild, acid hydrolysis suggests that there is some random hydrolysis of galactopyranosidic bonds. Furthermore, on this statistical basis, a galactan of $\bar{M}_n$ 30,000 can give galactans of $\bar{M}_n$ 4,600 and 3,600 from 3% and 4% hydrolysis respectively; for a galactan of $\bar{M}_n$ 3,000,000, the corresponding products have $\bar{M}_n$ 5,400 and 4,000, respectively. This concept may explain the marked similarity in the values of $\bar{M}_n$ for degraded-gum *A* of the *Acacia* species studied to date, despite the wide variation in the values of $\bar{M}_W$ for the whole gums, *e.g.*, *A. senegal*[4,19] $\bar{M}_W$ 0.6×10^6, degraded-gum *A* $\bar{M}_n$ ~4,800; *A. arabica*[16,19] $\bar{M}_W$ 2.3×10^6, degraded-gum *A* $\bar{M}_n$ ~5,400; *A. nubica*[19,26] $\bar{M}_W$ 0.87×10^6, degraded-gum *A* $\bar{M}_n$ ~5,730; *A. drepanolobium*[17,19] $\bar{M}_W$ 0.95×10^6 degraded-gum *A* $\bar{M}_n$ ~5,700; *A. laeta*[5,19] $\bar{M}_W$ 0.72×10^6, degraded-gum *A* $\bar{M}_n$ ~5,700; *A. seyal*[18,19] $\bar{M}_W$ 0.85×10^6, degraded-gum *A* $\bar{M}_n$ ~4,900.

Partial, acid hydrolysis of degraded-gum *A* gave the β-(1→3)- and β-(1→6)-linked D-galactobioses. Methylated, degraded-gum *A*, on methanolysis and hydrolysis, gave the *O*-methyl sugars shown in Table III. The presence of 2,6-di- and 2-*O*-methyl-D-galactose is ascribed to undermethylation. The *O*-methyl sugars identified indicate the presence of terminal, non-reducing arabinopyranose, arabinofuranose, galactose, glucuronic acid and its 4-*O*-methyl analogue, and 3-*O*-, 6-*O*-, and 3,6-di-*O*-substituted galactopyranose residues. The ratio of 2,3,4-tri- to 2,4,6-tri-*O*-methyl-D-galactose (5:2) is higher than that observed for other species (3:2 and 1:1 for degraded-gum *A* from *A. senegal*[4] and *A. laeta*[5], respectively).

TABLE III

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED, DEGRADED-GUM *A*

Relative retention times (T) *of methyl glycosides*		R_G	*Approx. molar proportions*	O-*Methyl sugar identified*
Column (i)	*Column* (ii)	*Solvent* (c)		
0.58, 0.72	0.47, 0.65	0.97	0.5	2,3,5-Tri-*O*-methyl-L-arabinose
0.97	0.85	0.78	trace	2,3,4-Tri-*O*-methyl-L-arabinose
1.67	5.25	0.87	3	2,3,4,6-Tetra-*O*-methyl-D-galactose
3.78, 4.23	3.02, 3.46	0.72	2	2,4,6-Tri-*O*-methyl-D-galactose
6.43	5.25	0.72	5	2,3,4-Tri-*O*-methyl-D-galactose
—	—	0.55	trace	2,6-Di-*O*-methyl-D-galactose
14.6, 16.5	9.9, 11.4	0.52	2	2,4-Di-*O*-methyl-D-galactose
—	—	0.34	1.5	2-*O*-Methyl-D-galactose
2.26, 2.97	2.12, 2.73	—	2.5	2,3,4-Tri-*O*-methyl-D-glucuronic acid[a]

[a]As methyl ester methyl glycoside.

TABLE IV

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED, DEGRADED-GUM *B*

Relative retention times (T) *of methyl glycosides*		R_G	*Approx. molar proportions*	O-*Methyl sugar identified*
Column (i)	*Column* (ii)	*Solvent* (c)		
1.68	1.66	0.78	2	2,3,4,6-Tetra-*O*-methyl-D-galactose
3.71, 4.15	2.99, 3.44	0.40	8	2,4,6-Tri-*O*-methyl-D-galactose
—	—	0.49	1	2,3,6-Tri-*O*-methyl-D-galactose
6.38	5.28	0.34	0.5	2,3,4-Tri-*O*-methyl-D-galactose
—	—	0.18	trace	2,6-Di-*O*-methyl-D-galactose
14.3, 16.4	9.8, 11.3	0.11	2	2,4-Di-*O*-methyl-D-galactose
—	—	0.05	1.5	2-*O*-Methyl-D-galactose
2.20	2.14, 2.73	—	trace	2,3,4-Tri-*O*-methyl-D-glucuronic acid[a]

[a]As methyl ester methyl glycoside.

An indication of the distribution of the β-(1→3) and β-(1→6) linkages in the branched D-galactan core of the polysaccharide was obtained by a study of degraded-gum *B*, obtained by Smith degradation[20] of degraded-gum *A*. Partial, acid hydrolysis

of degraded-gum *B* gave the β-(1→3)- and, in trace amount, the β-(1→6)-D-galactobioses. Examination of methylated, degraded-gum *B* indicated the presence of the *O*-methyl sugars shown in Table IV. The presence of 2,3,6-tri-, 2,6-di-, and 2-*O*-methyl-D-galactose is probably due to incomplete methylation at the axial 4-position of galactopyranose. Since all of the uronic acid residues in degraded-gum *A* are present as terminal, non-reducing units, the identification of trace amounts of uronic acid in degraded-gum *B* probably results from incomplete periodate oxidation of the uronic acid in degraded-gum *A*. This effect has been reported before[4], and it is also suspected[16] that not all of the uronic acid residues are to be found on the periphery of *Acacia* gum polysaccharides.

A. campylacantha gum was subjected to five successive Smith-degradations[20], giving polysaccharides I–V; their compositions are shown in Table I. The first degradation would be expected to remove all of the uronic acid, but small amounts of glucuronic acid were observed in polysaccharide I; the incomplete oxidation may arise through steric hindrance[21].

On methanolysis, the *O*-methyl derivatives of polysaccharide I gave the mixture of methyl glycosides shown in Table V. The original rhamnose and arabinopyranose were therefore completely removed by the first Smith-degradation. Mention has already been made of the presence of some uronic acid.

TABLE V

METHYLATION DATA AND RELATIVE PROPORTIONS OF *O*-METHYL SUGARS PRESENT IN METHYLATED POLYSACCHARIDES I–V

	Polysaccharides				
	I	*II*	*III*	*IV*	*V*
Weight of polysaccharide used (mg)	290	304	253	198	44
Weight of product (mg)	252	163	141	104	not isolated
$[\alpha]_D$ *of product (degrees)*	−47	−6	+10	−6	—
OMe of product (%)	39.2	36.4	38.7	38.9	—
O-Methyl sugars					
2,3,5-Tri-*O*-methyl-L-arabinose	3	0.5	trace	—	—
2,5-Di-*O*-methyl-L-arabinose	trace	trace	—	—	—
2,3,4,6-Tetra-*O*-methyl-D-galactose	1.5	4	2	1	0.5
2,4,6-Tri-*O*-methyl-D-galactose	5	5	6	8	8
2,3,6-Tri-*O*-methyl-D-galactose	—	0.5	0.5	0.5	0.5
2,3,4-Tri-*O*-methyl-D-galactose	1	1	0.5	trace	trace
2,6-Di-*O*-methyl-D-galactose	1	trace	1.5	1.5	2
2,4-Di-*O*-methyl-D-galactose	3	3	1	0.5	trace
2-*O*-Methyl-D-galactose	1	0.5	1	0.5	trace
2,3,4-Tri-*O*-methyl-D-glucuronic acid	0.5	—	—	—	—

The second Smith-degradation removed some galactose and arabinose, and the residual uronic acid. Examination of methylated polysaccharide II indicated that all but a trace of the arabinose was present as terminal, non-reducing arabinofuranose.

Polysaccharide III was shown, by examination of the methylated product to contain only a trace amount (<1%) of terminal, non-reducing arabinofuranose. Polysaccharides IV and V were galactans whose *O*-methyl derivatives gave the same methyl galactosides as polysaccharides I–III (Table V).

Partial, acid hydrolysis of polysaccharides I–V gave, in each case, 6-*O*-*β*-D-galactopyranosyl-D-galactose and 3-*O*-*β*-D-galactopyranosyl-D-galactose. The amounts of the former galactobiose decreased from polysaccharide II–V, in which it was detected in trace amount only.

Polysaccharides I–V were examined by molecular-sieve chromatography on a precalibrated "Bio-Gel P300" column[10]; the approximate values obtained for $\bar{M}_n$ are shown in Table I. This degradation pattern is similar to those reported for *A. senegal*[4] and *A. laeta*[5] gums, except that the decrease in $\bar{M}_n$ after the second degradation is much greater for *A. campylacantha*.

The identification of 2,3,4,6-tetra- and 2,4-di-*O*-methyl-D-galactose, and the small amounts of the *β*-D-(1→6)-linked galactobiose indicate that polysaccharide V is a branched galactan made up of *β*-(1→3)-D-galactose chains joined mutually by *β*-D-(1→6) linkages. The relatively low intrinsic viscosity of the gum, indicative of a globular-type molecule[22], and the spherical particles observed by electron microscopy, give further support for a highly branched, compact structure. The branched galactan framework of *A. campylacantha* gum therefore appears to be similar to that of *A. senegal*[4] and *A. laeta*[5] gums, the main difference being the lower relative proportion of *β*-D-(1→6) to *β*-D-(1→3) linkages in the latter. Fig. 1 shows one possible structural representation of part of the branched galactan framework of *A. campylacantha* gum.

Since only three Smith-degradations were required to remove all of the arabinose, the arabinose chains in *A. campylacantha* gum cannot contain more than three arabinose residues; these are the shortest, arabinose-containing side chains of any

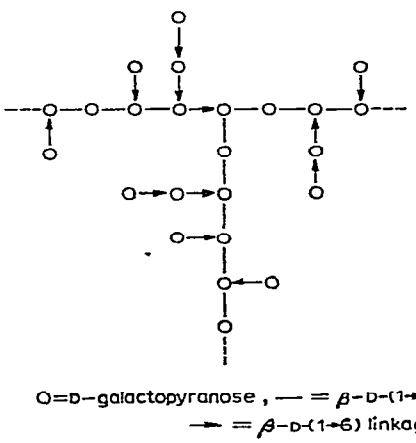

Fig. 1. Possible structural fragment for the galactan framework of *A. campylacantha* gum; O = D-galactopyranose; — denotes *β*-D-(1→3) linkage, → denotes *β*-D-(1→6) linkage.

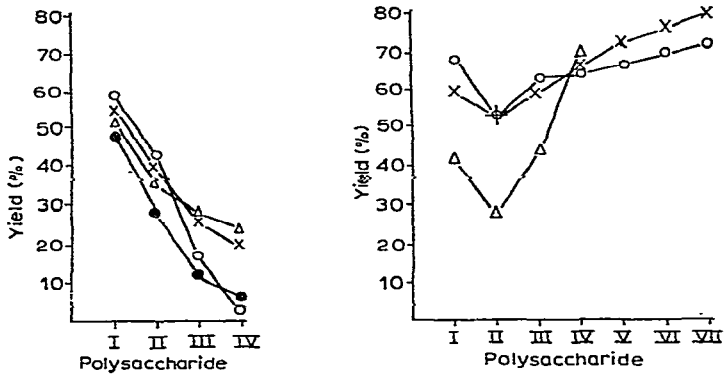

Fig. 2. Yields of polysaccharides I–V for some *Acacia* gum exudates: (*a*) Species in Bentham's Series 4, Gummiferae (△, *A. nubica*[26]; ×, *A. drepanolobium*[17]; ●, *A. seyal*[18]; ○, *A. arabica*[16]); (*b*) Species in Bentham's Series 5, Vulgares[2] (○, *A. senegal*[4]; ×, *A. laeta*[5]; △, *A. campylacantha*).

species of *Acacia* gum studied so far (*cf. A. senegal*[4], 4 arabinose units; *A. laeta*[5], 5 units). From the oligosaccharides in the diffusate after mild, acid hydrolysis, these side chains may include the following fragments:

L-Ara*f*-(1→
L-Ara*p*-(1→
β-L-Ara*f*-(1→3)-L-Ara*f*-(1→
β-L-Ara*p*-(1→3)-L-Ara*f*-(1→
α-D-Gal*p*-(1→3)-L-Ara*f*-(1→
R-(1→3)-β-L-Ara*f*-(1→3)-L-Ara*f*-(1→

where R = D-Gal*p*-; L-Ara*p*-; L-Ara*f*-.

The evidence obtained in this study therefore suggests that *A. campylacantha* gum molecules possess a branched galactan framework of the type depicted in Fig. 1, to which are attached unusually short arabinose-containing side chains, uronic acid, and rhamnose. Although most of the β-(1→6)-linked D-galactose chains in *A. senegal*[4] and *A. laeta*[5] gums are terminated by uronic acid, the higher proportion of β-(1→6)-linked D-galactose chains, and the lower uronic acid content in *A. campylacantha* gum indicates that only some of its β-(1→6)-D-galactose chains can be terminated by uronic acid.

The structural similarity between *A. campylacantha*, *A. senegal*[4], and *A. laeta*[5] gums can be illustrated by comparing the respective yields of the polysaccharides obtained from successive Smith-degradations. Fig. 2 shows the data available for all of the *Acacia* gums studied in a comparable manner by this technique. The plots appear to be characteristic of the nature of the galactan frameworks involved; the similarity between *A. campylacantha*, *A. laeta*[5], and *A. senegal*[4] (members[2] of Bentham's Series 5, Vulgares) and their difference from *A. arabica*[16], *A. seyal*[18], *A. drepanolobium*[17], and *A. nubica*[26] (members[2] of Bentham's Series 4, Gummiferae) is striking.

This study therefore gives support to the indications[3] that gum chemistry can contribute usefully to plant taxonomy; it has also shown that, although *A. campylacantha* gum is broadly similar in structure to the gums from *A. senegal*[4] and *A. laeta*[5], differences in fine structure can be distinguished.

EXPERIMENTAL

Analytical methods. — The standard analytical methods have been described[8]. Paper chromatography was carried out on Whatman No. 1 paper by using the organic phase of the following solvent systems (v/v); (*a*) benzene–butyl alcohol–pyridine–water (1:5:3:3); (*b*) acetic acid–ethyl acetate–formic acid–water (3:18:1:4); (*c*) butyl alcohol–ethanol–water (4:1:5); (*d*) ethyl acetate–pyridine–water (10:4:3); (*e*) ammonia (sp. gr. 0.880)–butanone–water (1:200:17); (*f*) butyl alcohol–ethanol–0.1M hydrochloric acid (1:10:5, *cf.* ref. 8); (*g*) acetic acid–butanone–water (1:9:1, saturated with boric acid). R_G values of *O*-methyl sugars refer to the distance migrated relative to 2,3,4,6-tetra-*O*-methyl-D-glucose in solvent (*c*). G.l.c. (Chromatograph Type S3A, fitted with flame-ionisation detectors; Gas Chromatography Ltd.) of mixtures of *O*-methyl sugars was carried out at nitrogen flow-rates of *ca.* 100 ml/min on columns of (*i*) 15% by weight of butane-1,4-diol succinate polyester on 60–70 mesh Celite (120 × 0.5 cm) at 175°, and (*ii*) 15% by weight of ethylene glycol adipate polyester on 60–70 mesh Celite (75 × 0.5 cm) at 160°. Retention times (*T*) are given relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside.

Galactose and arabinose in the Smith-degraded products (polysaccharides I–V) were estimated as the trimethylsilyl ethers of galactitol and arabinitol. The polysaccharides (20 mg) were hydrolysed [N sulphuric acid (10 ml) for 7.5 h at 100°], and the hydrolysates were neutralised with barium carbonate, filtered, deionised with Amberlite IR-120 (H^+) resin, and adjusted to *ca.* 10 ml. Sodium borohydride (0.25 g) was added, and after 1 h at room temperature, excess of borohydride was destroyed by the addition of Amberlite IR-120 (H^+) resin, and borate was eliminated by several distillations with methanol; the residues were then taken up in water, deionised, and again treated with methanol to ensure complete removal of borate. The resulting syrups were dried at 60° for 10 min *in vacuo* to ensure complete removal of water. Hexamethyldisilazane (0.2 ml), chlorotrimethylsilane (0.2 ml), and pyridine (2 ml) were added; the solutions were shaken for 10 min, kept for 30 min, and then centrifuged. The centrifugate was examined by g.l.c., using a column (100 × 0.5 cm) of 3% by weight of SE53 on 60–70 mesh Celite, at 160° with a nitrogen flow-rate of *ca.* 100 ml/min. Ratios of galactitol to arabinitol were estimated from the peak areas obtained on g.l.c., by comparison with a calibration graph.

Unless otherwise stated, polysaccharides were methylated successively with methyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide. Methanolyses were carried out under reflux for 12 h with dry, 5%, methanolic hydrogen chloride.

Origin and purification of A. campylacantha *gum.* — Gum nodules from

Acacia campylacantha Hochst. trees, growing at El Ain Forest Reserve near El Obeid, were collected in January 1961 by the Gum Research Officer to the Republic of the Sudan.

The gum (125 g) was dissolved in water (4 l), and the solution was filtered and dialysed; the polysaccharide was isolated as the freeze-dried product (122 g), $[\alpha]_D$ −12° [Found: N, 0.37; OMe, 0.29; uronic acid (by decarboxylation), 9; D-galactose, 54; L-arabinose, 29; L-rhamnose, 8%]. All sugar percentages are for the "anhydro forms", and are corrected for moisture and protein contents.

A portion of the gum (2 g) was exhaustively electrodialysed against distilled water, and freeze-dried. The resulting, acidic polysaccharide had equivalent weight 1760, corresponding to 10% of uronic anhydride, if all acidity arises from uronic acid groups.

Ultracentrifugation (1% solution in M sodium chloride, 44,770 r.p.m.) of the purified gum gave a single, symmetric boundary. The gum migrated as a single band during electrophoresis on strips (18 × 5 cm) of cellulose acetate film in both 0.05M ammonium carbonate (pH 8.9) and 0.1M acetate (pH 4.7) buffers, at field strengths of 18.8 volts/cm for 2.5 h. Polysaccharide bands were located by a modification of the periodate–rosaniline hydrochloride method[24]. The gum also migrated as a single band on thin-layer electrophoresis in 0.05M ammonium carbonate (pH 8.9) and 0.05M borate (pH 9.2) buffers[8].

The gum was chromatographed[9] on a column (30 × 1.5 cm) of DEAE-cellulose buffered at pH 4.1 by 0.02M acetate. Gradient elution with sodium chloride (0.0–0.5M) gave a single, symmetric peak. Chromatography of the purified gum on a "Sepharose 4B" column[11] gave a pattern which indicated the presence of two components; the crude gum was found to show only the major component, however, and there is evidence[12] that the other apparent component of high molecular weight is an artefact that arises during the purification or storage of the gum. The purified gum was chromatographed on a precalibrated[10] "Bio-Gel P300" column (47 × 5 cm); elution with M sodium chloride gave a single peak close to the void volume of the column. Because of the method of calibration used, the approximate value of 135,000 for $\bar{M}_n$ cannot be regarded as an absolute value.

The gum (0.0003% aqueous solution) was examined by electron microscopy, on a "Formavar" coated grid, shadow-cast with chromium (50 mg) at an angle of 30° and distance of 10 cm, with a General Electric EM6 electron microscope. Photographs taken at 40,000 diameters showed spherical particles of 10–15 nm diameter.

Physical measurements. — The gum had a limiting-viscosity number, in M sodium chloride, of 15.8 $cm^3.g^{-1}$ (Ubbelohde dilution viscometer at 27°). Light-scattering measurements, using a SOFICA photogoniodiffusometer, gave $\bar{M}_W$ = 312,000.

The neutral and acidic components. — The gum (0.2 g) was hydrolysed (N sulphuric acid for 7.5 h at 100°). The solution was cooled, neutralised with barium carbonate, deionised with Amberlite IR-120 (H^+) resin, concentrated, and fraction-

ated on a column (30 × 1.5 cm) of Duolite A-4 resin in the formate form. Elution with water (0.5 l) and then with 5% formic acid (0.5 l) gave the neutral and acidic fractions, respectively. After concentration to a syrup, the neutral fraction was chromatographed in solvents (*a*), (*b*), (*c*), and (*d*), which showed the presence of three components, identical with authentic galactose, arabinose, and rhamnose. The acidic fraction was concentrated and then examined in solvent (*b*). Two components were observed (R_{Gal} 0.63 and 0.21), identical in solvent (*b*) with 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose and 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose. Small amounts of galactose and of D-glucurono-(6→3)-lactone were also detected. The two aldobiouronic acids had been characterised previously[7] in a different specimen of *A. campylacantha* gum.

Partial, acid hydrolysis of the gum. — The polysaccharide (0.1 g) was hydrolysed [0.5N sulphuric acid (10 ml) for 1 h at 100°]. The hydrolysate was cooled, neutralised with barium carbonate, deionised with Amberlite IR-120 (H^+) resin, and chromatographed in solvents (*a*) and (*d*). Galactose, arabinose, rhamnose, and three neutral disaccharides were found. The latter had the mobilities of 6-*O*-β-D-galactopyranosyl-D-galactose, R_{Gal} 0.29 [solvent (*a*)], 0.36 [solvent (*d*)]; 3-*O*-β-D-galactopyranosyl-D-galactose, R_{Gal} 0.48 (*a*), 0.51 (*d*); and 3-*O*-α-D-galactopyranosyl-L-arabinose, R_{Gal} 0.72 (*a*), 0.76 (*d*), pink spot with aniline oxalate.

Preparation of degraded-gum A. — Purified *A. campylacantha* gum (20.2 g, dry weight) was dissolved in 0.01N sulphuric acid (1 litre) and heated on a boiling water-bath. Aliquots (10 ml) of the solution were withdrawn at intervals and filtered, and the extent of hydrolysis was determined from flow-time measurements in an Ubbelohde viscometer. After 15 h, a precipitate, presumed to be denatured protein, appeared in the solution. After 96 h, the solution was cooled, neutralised with barium carbonate, deionised, concentrated, and dialysed against several changes of distilled water for 72 h (the diffusate being retained for study below). Degraded-gum *A* (6.6 g) was isolated as the freeze-dried product (see Table I).

The diffusate from the dialysis of degraded-gum *A* was concentrated to a syrup (7.7 g). Portions were chromatographed in solvents (*a*), (*b*), and (*d*). Arabinose, rhamnose, small amounts of galactose, and three neutral disaccharides were detected: these had the paper-chromatographic mobilities of 3-*O*-α-D-galactopyranosyl-L-arabinose [R_{Gal} 0.71, solvent (*a*); 0.51 (*b*); 0.78 (*c*)]; 3-*O*-β-L-arabinofuranosyl-L-arabinose [R_{Gal} 1.25, (*b*)]; and 3-*O*-β-L-arabinopyranosyl-L-arabinose [R_{Gal} 0.82 (*a*), 0.74 (*b*), and 0.91 (*c*)].

Examination of degraded-gum A. — Degraded-gum *A* (1.0 g) was dissolved in water (200 ml), and sodium borohydride (800 mg) was added. After 24 h at room temperature, further borohydride (200 mg) was added. After a further 6 h, the solution was dialysed, and the reduced degraded-gum was isolated as the freeze-dried product (850 mg). A portion was hydrolysed (N sulphuric acid, 7 h, 100°); paper chromatography (solvent *g*) indicated a trace of galactitol, but no arabinitol.

Degraded-gum *A* gave a brown, aqueous solution that could not be used for light-scattering measurements; molecular-sieve chromatography[10] on a precalibrated

"Bio-Gel P300" column indicated $\bar{M}_n$ ~6,400. The borohydride-reduced, degraded-gum *A* gave a clear solution, for which $\bar{M}_W$ = 9,400.

Methylation of degraded-gum A. — Degraded-gum *A* (373 mg) was methylated to give a product (275 mg), $[\alpha]_D$ −14° (*c* 0.5, chloroform) (Found: OMe, 39.3%). A portion of this was methanolysed, and examined by g.l.c.; the *O*-methyl sugars identified are listed in Table III. Hydrolysis of the methyl glycosides, followed by paper chromatography with solvents (*c*) and (*e*), indicated the presence of 2,6-di- and 2-*O*-methyl-D-galactose, in addition to those *O*-methyl sugars already identified as their methyl glycosides.

Smith degradation of degraded-gum A. — Degraded-gum *A* (2.02 g) was dissolved in water (50 ml), and 0.5M sodium metaperiodate solution (50 ml) was added. After 96 h in darkness at room temperature, the polysaccharide had reduced 12.1 mmoles of periodate/g. The reaction was stopped by the addition of ethylene glycol (4 ml), and the solution was dialysed for 2 days. Sodium borohydride (1.0 g) was added, and after 30 h at room temperature, the solution was dialysed for a further 2 days. The solution was made N with respect to sulphuric acid, and the polyalcohol was hydrolysed for 48 h at room temperature. The solution was then neutralised with barium carbonate, filtered, de-ionised with Amberlite resin IR-120 (H^+), and dialysed against distilled water for 2 days. Degraded-gum *B* was isolated as the freeze-dried product (350 mg, 18%), $[\alpha]_D$ +2° (*c* 0.25).

Partial, acid hydrolysis and methylation of degraded-gum B. — Degraded-gum *B* was hydrolysed [0.5N sulphuric acid (10 ml), 1 h, 100°], and the solution was cooled, neutralised with barium carbonate, filtered, deionised with Amberlite IR-120 (H^+) resin, and concentrated. Paper-chromatographic examination in solvents (*a*) and (*d*) indicated the presence of galactose, and two neutral disaccharides having the mobilities of 6-*O*-β-D-galactopyranosyl-D-galactose and 3-*O*-β-D-galactopyranosyl-D-galactose (minor and major components, respectively).

Degraded-gum *B* was methylated, and, after methanolysis, the methyl glycosides recorded in Table IV were identified by g.l.c. Hydrolysis of the methyl glycosides, followed by paper chromatography in solvents (*c*) and (*e*), indicated the presence of 2-6-di- and 2-*O*-methyl-D-galactose, in addition to those *O*-methyl sugars already identified by g.l.c. of their methyl glycosides.

Methylation of A. campylacantha *gum.* — The gum (312 mg) was methylated to give a product (261 mg), $[\alpha]_D$ −35° (*c* 1.0, chloroform) (Found: OMe, 39.7%, not raised on further attempted methylation). After methanolysis, the methyl glycosides shown in Table II were identified by g.l.c. Hydrolysis of the methyl glycosides, followed by paper chromatography in solvents (*c*) and (*e*), indicated the presence of small amounts of 2,6-di- and 2-*O*-methyl-D-galactose, in addition to the *O*-methyl sugars already identified by g.l.c. of their methyl glycosides.

Preparation, partial, acid hydrolysis, and methylation of Polysaccharide I. — *A. campylacantha* gum (60 g, dry weight) was dissolved in water (1500 ml), 0.25M sodium metaperiodate (1500 ml) was added, and the solution was kept in darkness at room temperature. The oxidation was followed by measuring the release of formic

acid with time, by titrating aliquots (1 ml) with 0.0104N sodium hydroxide (Methyl Red indicator). After 100 h, the amount of periodate reduced was found by back titration with sodium arsenite[25]. The reaction was stopped by the addition of ethylene glycol (30 ml), and the solution was dialysed against running tap-water for 48 h. Sodium borohydride (15 g) was added, and the mixture was kept for 30 h at room temperature and then dialysed for a further 48 h. The solution was made N with respect to sulphuric acid by the addition of 4N acid, and the polyalcohol was hydrolysed for 48 h at room temperature. After dialysis against running tap-water for 48 h, the Smith-degradation product (polysaccharide I) was isolated as the freeze-dried product (see Table I).

After partial, acid hydrolysis [0.5N sulphuric acid (10 ml), 1 h, 100°] of polysaccharide I (30 mg), paper chromatography indicated the presence of galactose, arabinose, and two neutral disaccharides having the mobilities of 3-*O*-β-D-galactopyranosyl-D-galactose and 6-*O*-β-D-galactopyranosyl-D-galactose (minor and major components, respectively).

Polysaccharide I (290 mg) was methylated (see Table V). Methanolysis of a portion of the product, followed by examination of the mixture of methyl glycosides by g.l.c., gave the results shown in Table V. Hydrolysis of the methyl glycosides, followed by paper chromatography in solvents (*c*) and (*e*), indicated the presence of 2-*O*-methyl-D-galactose, in addition to those *O*-methyl sugars identified as their methyl glycosides by g.l.c.

Preparation of the sequential Smith-degradation products, Polysaccharides II–V. — The following weights of the sequence of Smith-degradation products were periodate-oxidised, borohydride-reduced, and hydrolysed, and the corresponding products were recovered, all as already described for Polysaccharide I. Polysaccharide I (24.3 g) gave Polysaccharide II (6.4 g); II (5.26 g) gave III (2.14 g); III (1.21 g) gave IV (0.77 g); and IV (264 mg) gave V (173 mg). The percentage yields, specific rotations, estimated value for $\bar{M}_n$, and sugar composition of polysaccharides II–V are given in Table I.

Partial, acid hydrolysis of Polysaccharides II–V. — After hydrolysis (0.5N sulphuric acid, 1 h, 100°), paper chromatography indicated the presence of 3-*O*-β-D-galactopyranosyl-D-galactose (major component) and 6-*O*-β-D-galactopyranosyl-D-galactose (minor component, trace only in Polysaccharide V) in each of these polysaccharides.

Methylation of Polysaccharides II–V. — Polysaccharides II–V were methylated (polysaccharide V with silver oxide and methyl iodide in *N*,*N*-dimethylformamide); data for the products are shown in Table V. Methanolysis of a portion of each *O*-methyl derivative, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table V. For Polysaccharide II, hydrolysis of the methyl glycosides, followed by paper chromatography in solvents (*c*) and (*e*), indicated the presence of 2,6-di- and 2-*O*-methyl-D-galactose, in addition to those *O*-methyl sugars identified by g.l.c.

ACKNOWLEDGMENTS

We thank the Science Research Council for a maintenance award (to A. C. M.). We are grateful to Messrs. Rowntree & Co. Ltd. (York) and Laing-National Ltd. (Manchester) for financial support.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

THE ISOLATION OF 1,2-*O*-ETHYLENE DERIVATIVES OF D-GLUCOSE FROM *O*-(2-HYDROXYETHYL)STARCH

H. C. SRIVASTAVA, K. V. RAMALINGAM, N. M. DOSHI, AND A. S. CHAUDHARI
Ahmedabad Textile Industry's Research Association, Ahmedabad-9 (India)

(Received March 26th, 1969; in revised form, June 12th, 1969)

ABSTRACT

Acid hydrolysis of a commercial sample of *O*-(2-hydroxyethyl)starch (D.S., 0.1) gave, in addition to the expected 2-*O*-, 3-*O*-, and 6-*O*-(2-hydroxyethyl)-D-glucoses, three isomeric anhydro derivatives of 2-*O*-(2-hydroxyethyl)-D-glucose (**1**). These have been identified as 1,2-*O*-ethylene-α-D-glucofuranose (**2**), 1,2-*O*-ethylene-β-D-glucopyranose (**3**), and 1,2-*O*-ethylene-α-D-glucopyranose (**4**). The formation of 1,2-*O*-ethylene-D-glucose takes place by intramolecular glucosidation of **1**.

INTRODUCTION

By employing the Smith-degradation technique[1,2] for the structural analysis of polysaccharides, it has been shown[3] that, in a commercial sample of *O*-(2-hydroxyethyl) derivatives of maize starch (D.S., 0.1), O-2 is substituted to the extent of 84%; the remaining ether groups are located mainly at O-6 with only negligible substitution at O-3. To confirm these findings, another method of determining the distribution of *O*-(2-hydroxyethyl) groups, involving acid hydrolysis followed by quantitative chromatographic separation of the hydrolysis products, was investigated. In the course of this study, we have isolated, in addition to the expected 2-*O*-, 3-*O*-, and 6-*O*-(2-hydroxyethyl) derivatives of D-glucose, three isomeric anhydro derivatives of 2-*O*-(2-hydroxyethyl)-D-glucose (**1**) (*cf.* ref. 3*b*). We now report on the structural characterization of the anhydro derivatives of **1**.

RESULTS AND DISCUSSION

From the mixture of sugars produced on hydrolysis of *O*-(2-hydroxyethyl) derivatives of maize starch, D-glucose was removed by fermentation with baker's yeast. The residual mixture of non-fermentable sugars was fractionated by chromatography on a cellulose column to give four crystalline compounds (*A*, *B*, *C*, and *D*; Table I).

Compound *A* could not be located on the paper chromatogram by such spray reagents (for reducing sugars) as *p*-anisidine hydrochloride[4] or silver nitrate–sodium hydroxide[5], but was detected by the periodate–benzidine reagent[6]. The non-reducing character of *A* was confirmed by its lack of reaction with Fehling's solution. Molecular

TABLE I

PROPERTIES OF COMPOUNDS ISOLATED FROM THE HYDROLYSATE OF *O*-(2-HYDROXYETHYL)STARCH

Compound	R_G	*M.p.* (*degrees*)	$[\alpha]_D^{30}$ (*water*) (*degrees*)
A	2.3	215–216	−55
B	1.9	209–210	+54
C	1.9	134–135	+89.7
D	1.5	151–152	+57.2 (equilibrium value)

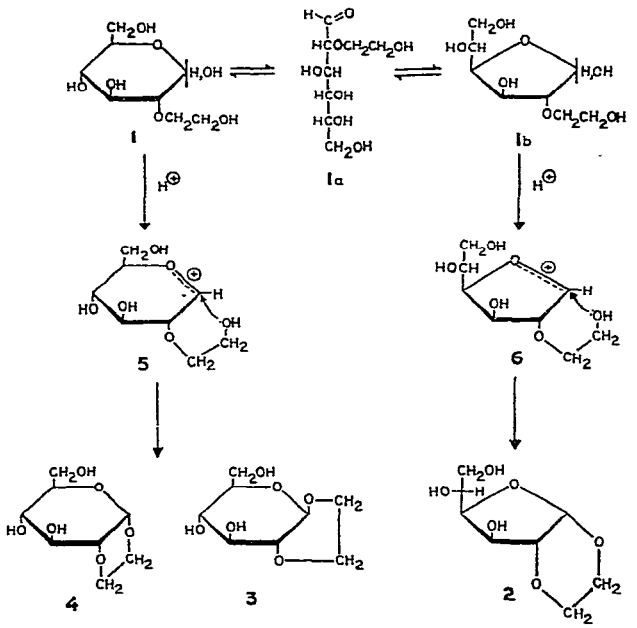
weight (M^+ 206, by mass spectrometry) and elemental analysis of the compound indicated that it was an anhydro derivative of a mono-*O*-(2-hydroxyethyl)hexose. Analysis of the fully acetylated derivative of compound *A* showed that the latter had three free hydroxyl groups. Hydrolysis of the compound with 0.5N sulphuric acid at 95° showed the following changes in specific optical rotation: −50.8 (initial)→ +61.8 (30 min)→ +66.5° (75 min, constant value). Chromatography of the hydrolysate showed the presence of **1**. Upon oxidation with sodium metaperiodate, *A* consumed one mole of the oxidant, with liberation of one mole of formaldehyde, per mole of the compound. No formic acid was produced. The periodate-oxidation data thus indicate an exocyclic, vicinal-diol grouping and a probable furanoid structure for *A*. The infrared spectrum of *A* contained bands of types A, B, C, and D (937, 876, 831, and 788 cm^{-1}, respectively) characteristic of compounds containing a furanoid ring[7]. In addition, strong bands appeared at 895, 946, 994, 1020, 1035, 1084, 1110, and 1150 cm^{-1}. Of these, the absorptions at 895 and those between 1020 and 1150 cm^{-1} are characteristic of the vibrations[8] of a dioxane ring.

On the basis of the foregoing evidence, compound *A* is designated 1,2-*O*-ethylene-D-glucofuranose. Although *A* has a negative optical rotation, it is assigned the α-D configuration **2** in view of the fact that its physical constants (Table I) are almost identical with those given[9] for synthetic 1,2-*O*-ethylene-α-D-glucofuranose (**2**).

As compared to compound *A*, compounds *B* and *C* were isolated only in small proportions. Both *B* and *C* were non-reducing, but could be detected on paper chromatograms by the periodate–benzidine reagent[6]. Hydrolysis of *B* or *C* with N sulphuric acid for 6 h gave **1** (chromatographic evidence). These results suggested that compounds *B* and *C* were also derivatives of **1**, in which the reducing group was blocked but could be regenerated on acid hydrolysis. Compound *B* has been identified as 1,2-*O*-ethylene-β-D-glucopyranose (**3**), which was synthesized by cyclization of 2-chloroethyl β-D-glucopyranoside under alkaline conditions[10,11]. Compound *C* has been tentatively identified as 1,2-*O*-ethylene-α-D-glucopyranose (**4**) from the fact that its physical constants (see Table I) are similar to those reported[12] for this compound.

Compound *D*, which was a major component of the mixture, was identical, in its physical constants and chromatographic and electrophoretic behaviour, to 2-*O*-(2-hydroxyethyl)-D-glucose (**1**)[13,14].

While this work was in progress, Ramnäs and Samuelson[15] reported the identification of **2**, **3**, and **4** in hydrolysates of *O*-(2-hydroxyethyl)cellulose. The major portion of 1,2-*O*-ethylene-D-glucose was found to be present in the α-D-pyranoid form. It is interesting to note that, in the present study, 1,2-*O*-ethylene-D-glucose, isolated from a hydrolysate of *O*-(2-hydroxyethyl)starch, was mainly present in the α-D-furanoid form.



Treatment of **1** with 1% methanolic hydrogen chloride for 8 h gave **2**. When the reaction was monitored by thin-layer chromatography, the conversion **1**→**2** was seen to be almost complete after 15 min, during which time the specific optical rotation had dropped from an initial value of +58.5 to −18.8°. On further heating, a third, as yet unidentified, component (*E*) having chromatographic mobility slightly slower than **4** was detected, and after 3 h, a fourth component appeared, which was probably a mixture of **3** and **4**. When **1** was heated with N sulphuric acid, the only compounds detected on the chromatogram were **1** and **2**, the latter being present in small proportion only. Further heating apparently resulted in the formation of a small proportion of a mixture of **3** and **4**. Compounds **3** and **4** are not very well separated by t.l.c., although **3** has a slightly higher R_F than **4**.

The foregoing results suggest that **2**, **3**, and **4** are all formed from **1**, produced during hydrolysis of *O*-(2-hydroxyethyl) starch, by intramolecular elimination of water between the hydroxyl group at C-1 and the hydroxyl group of the hydroxyethyl function at C-2. It is reasonable to assume that, under the acidic conditions, such cyclizations take place through the intermediacy of carbonium ions **5** and **6**. Höök and Lindberg[9] have shown that, whereas **2**, **3**, and **4** are all formed by treatment of **1**

with hydrogen chloride in *N*,*N*-dimethylformamide, treatment with M sulphuric acid for 18 h gives rise only to **2** in low yield.

It is noteworthy that treatment of **1** with acids in both aqueous and non-aqueous media gives predominantly **2**. Furthermore, from the hydrolysate of *O*-(2-hydroxyethyl)starch, **2** was isolated in relatively much greater quantities than **3** and **4**. These results indicate that, as in the case of the Fischer glycoside synthesis, the furanoid form (**1b**) of **1** reacts first to give the cyclic derivative **2**. However, in contrast with the formation of glycopyranosides from glycofuranosides, **2** is stabilized because of a fused-ring system, and its further conversion into **3** and **4** takes place rather slowly and to a lesser extent.

EXPERIMENTAL

General. — Melting points, determined in thin-walled capillaries, are uncorrected. Solutions were concentrated *in vacuo* at temperatures below 55°. Paper chromatography was performed on Whatman No. 1 paper with butyl alcohol–pyridine–water (6:4:3, v/v). R_G represents rate of movement on paper relative to D-glucose. Thin-layer chromatography (t.l.c.) was carried out on silica gel (National Chemical Lab., Poona) with acetone–hexane (4:1, v/v); detection was effected with conc. sulphuric acid at 120–130° for 15 min. Paper electrophoresis was carried out on Whatman No. 1 paper with 0.1M borate buffer at an applied potential of 500 volts.

Hydrolysis of the O-(2-hydroxyethyl) derivative of maize starch. — The derivative (D.S., 0.1; 125 g) was suspended in 72% (w/w) sulphuric acid (150 ml), and the mixture was kept for 3 h at 30°. The mixture was then diluted to 2.5 l with water, heated on a boiling-water bath for 6 h, neutralized with barium carbonate, and filtered. The filtrate was deionized by ion-exchange resins and evaporated to a syrup (125 g). D-Glucose in the hydrolysate was removed by fermentation with baker's yeast, to leave a syrupy mixture of non-fermentable sugars (12.0 g). A portion (6.22 g) of the mixture was chromatographed on a cellulose column (7 × 69 cm), with butyl alcohol saturated with water, to give the following fractions:

Fraction	*1*	*2*	*3*	*4*	*5*
Yield (g)	0.977	0.720	0.626	1.443	2.026
R_G	2.3	1.9	1.6, 1.5	1.5	1.5, 1.2

Examination of fractions. — (*a*) Fraction 1 crystallized on refrigeration. The crude product (0.6 g) was recrystallized from absolute ethanol to afford 1,2-*O*-ethylene-α-D-glucofuranose (**2**), m.p. 215–216°, $[\alpha]_D^{30}$ −55° (*c* 1, water) (Found: C, 46.2; H, 6.41. $C_8H_{14}O_6$ calc.: C, 46.6; H, 6.84%).

Two treatments of **2** (0.2 g) with pyridine (10 ml) and acetic anhydride (2 ml), in the usual manner, gave the acetate, m.p. 95–96° (from cyclohexane) (Found: C, 50.2; H, 5.97. $C_{14}H_{20}O_9$ calc.: C, 50.6; H, 6.06%).

A solution of **2** (0.1845 g) in sodium metaperiodate (250 ml, 0.02M) was kept in the dark at 10°, and the periodate consumption[16] and liberation of formic acid[17] were determined at intervals. The oxidation was complete in 2 h; 1 mole of the oxidant per mole of **2** was consumed, and no formic acid was produced.

To a solution of **2** (0.01025 g) in water (2 ml), 0.3M sodium metaperiodate (2 ml) and M sodium hydrogen carbonate (2 ml) were added, and the reaction mixture was kept in the dark for 1 h at room temperature (30°). N Sulphuric acid (15 ml) was added, followed by M sodium arsenite (5 ml), the volume was made up to 100 ml, and the formaldehyde content (1.04 moles per mole) was determined by the chromotropic acid method.

(*b*). The syrupy fraction *2* crystallized on storage for a few days, and the crude product (0.06 g) was recrystallized from absolute ethanol to give **3**, m.p. and mixed m.p. 209–210°, $[\alpha]_D^{30}$ +55° (*c* 1, water); lit.[10] m.p. 210–211°, $[\alpha]_D^{21}$ +56° (water). The X-ray diffraction pattern was identical with that of authentic **3**.

The mother liquor, after removal of **3**, from fraction *2* yielded 1,2-*O*-ethylene-α-D-glucopyranose (**4**) (0.12 g), which, after recrystallization, had m.p. 134–135°, $[\alpha]_D^{30}$ +89.7° (*c* 1, water); lit.[12] m.p. 131–132°, $[\alpha]_D^{24}$ +96.8° (water).

(*c*) Paper chromatography of fractions *3*, *4*, and *5* showed that all of them contained 2-*O*-(2-hydroxyethyl)-D-glucose (**1**) (R_G 1.5) as the major component. Fraction *4* crystallized from absolute ethanol to yield **1** (0.56 g, in 2 crops). Recrystallization from absolute ethanol gave **1** having m.p. 151–152°, $[\alpha]_D^{30}$ +57.2° (*c* 1, water, equilibrium value); lit.[13] m.p. 151–153°, $[\alpha]_D$ +55° (water). Compound **1** (0.3 g) was also isolated from fraction 5.

Identification of 3-O- *and* 6-O-(*2-hydroxyethyl*)-D-*glucose.* — The component having R_G 1.6 in fraction *3* was identified as 3-*O*-(2-hydroxyethyl)-D-glucose by comparing its chromatographic and electrophoretic mobility with that of an authentic sample. In a similar manner, the component having R_G 1.2 in fraction 5 was identified as 6-*O*-(2-hydroxyethyl)-D-glucose.

Treatment of 2-O-(*2-hydroxyethyl*)-D-*glucose* (**1**) *with methanolic hydrogen chloride.* — A solution of **1** (0.1 g) in 1% methanolic hydrogen chloride (5 ml) was refluxed for 8 h under anhydrous conditions. The solution was neutralized with silver carbonate and filtered through Celite, and the Celite bed was washed with methanol. The combined filtrate and washings were evaporated, and the residue was recrystallized from absolute ethanol to give compound **2**, m.p. and mixed m.p. 215–216°.

Treatment of 2-O-(*2-hydroxyethyl*)-D-*glucose* (**1**) *with sulphuric acid.* — A solution of **1** (0.1 g) in N aqueous sulphuric acid (5 ml) was heated on a boiling-water bath. Samples were withdrawn at intervals, neutralized by anion-exchange resin, and examined by t.l.c.

ACKNOWLEDGMENTS

This research has been financed, in part, by a grant made by the United States Department of Agriculture, Agricultural Research Service. The authors thank

Dr. N. T. Dweltz, ATIRA, Ahmedabad, for the X-ray analysis, Dr. J. L. Bose, National Chemical Laboratory, Poona, for mass-spectral data, and Dr. G. Srinivasan, Hindusthan Lever Ltd., Bombay, for infrared measurements.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

POLYSACCHARIDES ELABORATED BY *Polyporus fomentarius* (Fr.) AND *Polyporus igniarius* (Fr.)

PART II. WATER-SOLUBLE, ACIDIC POLYSACCHARIDES FROM THE FRUIT BODIES

HÅKAN BJÖRNDAL AND BENGT LINDBERG
Institutionen för organisk kemi, Stockholms Universitet, S-113 27, Stockholm (Sweden)

(Received June 13th, 1969)

ABSTRACT

The structure of two very similar glucuronoglucans, isolated from the fruit bodies of *Polyporus fomentarius* and *Polyporus igniarius*, have been investigated. They both contain a backbone of a branched β-D-glucan, in which the D-glucose residues are connected by (1→3)- and (1→6)-linkages. Chains, containing an average of 4–5 β-(1→4)-linked D-glucuronic acid residues, are β-linked to the 3-position of some D-glucose residues in the backbone.

G.l.c.–mass spectrometry was used for the qualitative and quantitative analysis of the methylated sugars obtained on hydrolyses of the methylated and the methylated–reduced polysaccharide. The reduction was performed with lithium aluminium deuteride, and the methyl ethers derived from D-glucuronic acid residues were distinguished from those derived from D-glucose residues by their mass spectra.

INTRODUCTION

In Part I of this series[1], the isolation of water-soluble polysaccharides from the fruit bodies of *Polyporus fomentarius* and *Polyporus igniarius*, their separation into neutral and acidic polysaccharides, and structural studies on the neutral polysaccharides were reported. In the present paper, structural studies on the acidic polysaccharides are reported.

RESULTS

The acidic polysaccharides were further purified by chromatography on DEAE-Sephadex in the acetate form, using gradient elution with aqueous potassium acetate. The results of the fractionations, under strictly comparable conditions, are given in Fig. 1. A small part of neutral material (A_f and A_i, respectively) was obtained, which on hydrolysis yielded D-glucose, D-galactose, D-mannose, L-fucose, and traces of D-xylose. The acidic fractions (B_f, C_f, D_f and B_i, C_i, D_i, respectively) on hydrolysis yielded D-glucose and D-glucuronic acid.

Fraction C_f had $[\alpha]_{5780}$ −26° and contained 27% of uronic acid residues (carbazole method[2]), with correction for the colour reaction given by the D-glucose.

The corresponding values for C_i were $[\alpha]_{5780}$ −31° and 40% of uronic acid residues. The *B* and *D* fractions had lower and higher contents of uronic acid, respectively, than the *C* fractions. This was expected, as the more acidic polysaccharides should be more strongly adsorbed and eluted at a higher concentration of acetate. Only fractions C_f and C_i were subjected to methylation analysis.

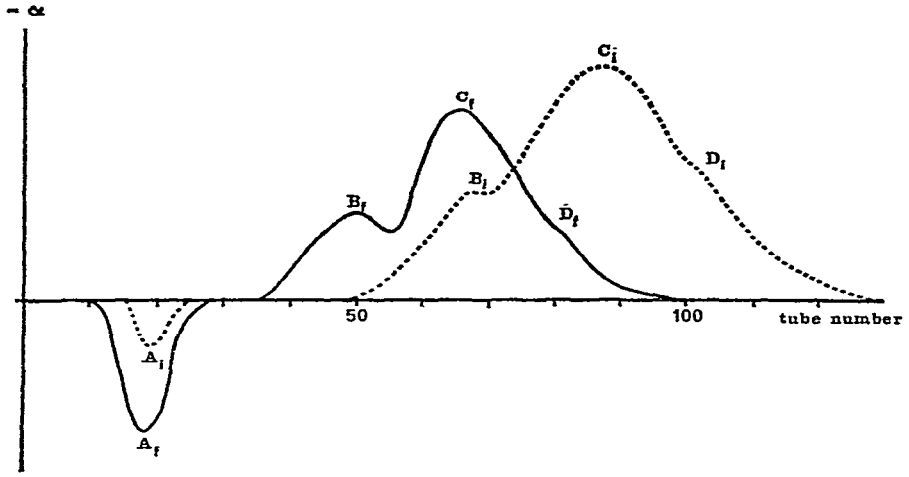

Fig. 1. Fractionation of water-soluble polysaccharides from ———— *P. fomentarius*, — — — — *P. igniarius* on DEAE-Sephadex in the acetate form.

The polysaccharides were methylated by treatment[3] with methylsulphinyl sodium and methyl iodide in methyl sulphoxide. Part of each methylated polysaccharide was hydrolysed, and the resulting mixture of methylated sugars was converted into alditol acetates and analysed by g.l.c.[4]–mass spectrometry[5]. The results are summarised in Table I (columns *A*). The remainder was subjected to treatment with methanolic hydrogen chloride, followed by lithium aluminium deuteride, in order to transform the D-glucuronic acid residues into deuterated D-glucose residues. This product was then hydrolysed, and the methylated sugars in the hydrolysate were investigated as above (Table I, columns *B*). The same components, but in different proportions, were obtained from the two polysaccharides.

The partially methylated alditol acetates, being derived from D-glucose residues, could be unambiguously identified, from their mass spectra, as derivatives of 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-glucose, 2,3,4-tri-*O*-methyl-D-glucose, 2,4-di-*O*-methyl-D-glucose, and 2,3-di-*O*-methyl-D-glucose, respectively. Good agreement between the *T*-values of these compounds and those of the corresponding authentic samples further confirmed their identity.

As the reduction of the methylated polysaccharide was performed with deuteride, the methyl ethers should be deuterated at C-6 ($-CD_2OH$). Mass spectra revealed that all of the 2,3-di-*O*-methyl-D-glucose and part of the 2,3,4-tri-*O*-methyl-D-glucose were deuterated. It should be possible, by comparing the intensities of relevant peaks in the

TABLE I

METHYLATED ETHERS OBTAINED FROM THE HYDROLYSATES OF *A*, METHYLATED GLUCURONOGLUCANS; *B*, METHYL–REDUCED GLUCURONOGLUCANS; *C*, METHYLATED GLUCURONOGLUCANS, RECALCULATED VALUES (SEE THE TEXT)

Sugars	T[a]	*Molar proportion* (%) P. fomentarius			P. igniarius		
		A	B	C	A	B	C
2,3,4,6-Tetra-*O*-methyl-D-glucose	1.00	14	9	9	13	9	9
2,4,6-Tri-*O*-methyl-D-glucose	1.95	17	15	11	19	16	13
2,3,4-Tri-*O*-methyl-D-glucose	2.48	40	37(6)[b]	26	38	33(7)[b]	26
2,4-Di-*O*-methyl-D-glucose	5.10	29	20	19	30	18	21
2,3-Di-*O*-methyl-D-glucose	5.40	—	19	—	—	24	—

[a]Retention times of the corresponding alditol acetate on the ECNSS-M column relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [b]The figures in the brackets represent the 2,3,4-tri-*O*-methyl-D-glucose, derived from D-glucuronic acid residues.

mass spectrum, to determine the proportion of deuterated to undeuterated derivative. For most of the peaks in the mass spectrum of the undeuterated derivative, a smaller peak (2 mass units larger) was evident. These peaks were of sufficient intensity to render the calculation uncertain. The peak at *m/e* 189, however, gave least error. This is a primary fragment, formed by fission between C-3 and C-4 of undeuterated 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol. From the intensities of the peaks at *m/e* 189 and 191, the molar percentages of deuterated 2,3,4-tri-*O*-methyl-D-glucose, derived from terminal D-glucuronic acid residues, was estimated as *ca.* 6% (*P.f.*) and 7% (*P.i.*), respectively. The total percentage of terminal residues is in reasonably good agreement with that of the branched residues (2,4-di-*O*-methyl-D-glucose). The agreement between the content of uronic acid residues found by methylation analysis and by uronic acid determination is satisfactory.

The glucuronoglucan, on graded, acid hydrolysis, yielded a series of acidic oligosaccharides. The same oligosaccharides were obtained from both polysaccharides, as indicated by paper chromatography and paper electrophoresis. The acidic oligosaccharides from the *P. igniarius* glucuronoglucan were fractionated on DEAE-Sephadex in the formate form. Three of the main components were obtained pure (**1**, **2**, **3**, Table II) and were further investigated.

Their low optical rotations indicate that they are β-D-linked. From their chromatographic mobilities, **1** and **3** are acidic disaccharides and **2** is an acidic trisaccharide. Paper electrophoresis indicates that **1** contains one and **2** and **3** two D-glucuronic acid residues. These conclusions were supported by equivalent weight determinations. As glucuronosidic linkages are more resistant to acid hydrolysis than are glucosidic linkages, they may tentatively be identified as a D-GluA-(1→)-D-Glu (**1**), a D-GluA-(1→)-D-GluA-(1→)-D-Glu (**2**), and a D-GluA-(1→)-D-GluA (**3**).

The acidic di- and tri-saccharides were transformed into their methyl ester

methyl glycosides, reduced to neutral di- and tri-saccharides, and subjected to methylation analysis (Table II). From these results, and in conjunction with those discussed above, the acidic di- and tri-saccharides are identified as 3-*O*-(β-D-glucopyranosyluronic acid)-D-glucose (**1**), *O*-(β-D-glucopyranosyluronic acid)-(1→4)-*O*-(β-D-glucopyranosyluronic acid)-(1→3)-D-glucose (**2**), and 4-*O*-(β-D-glucopyranosyluronic acid)-D-glucuronic acid (**3**).

TABLE II

ACIDIC DI- AND TRI-SACCHARIDES FROM PARTIALLY HYDROLYSED *P. igniarius* GLUCURONOGLUCAN (2.2 g)

Oligosaccharide	*Yield (mg)*	$[\alpha]_{5780}$ *(degrees)*	*Equiv. Calc.*	*Found*	R_I^b	M_I^c	*Methylated sugars obtained from the reduced, methylated oligosaccharide*
1	60	+ 5	356	360	1.0	1.0	2,3,4,6-G[c]; 2,4,6-G
2	45	−12	266	290	0.4	1.4	2,3,4,6-G; 2,3,6-G; 2,4,6-G
3	30	− 3	185	200	0.9	1.7	2,3,4,6-G; 2,3,6-G

[a]Relative to oligosaccharide **1**, solvent system *a*. [b]Relative to oligosaccharide **1**, acetate buffer, pH 4. [c]2,3,4,6-G stands for 2,3,4,6-tetra-*O*-methyl-D-glucose, etc.

The other acidic oligosaccharides obtained on graded hydrolysis of the glucuronoglucan, from their paper-chromatographic and paper-electrophoretic mobilities, were higher oligosaccharides, containing several D-glucuronic acid residues.

DISCUSSION

The structural studies reported above establish that the glucuronoglucans from *P. fomentarius* and *P. igniarius* are esssentially β-D-linked and have very similar structures.

The methylation analysis shows that all of the sugar residues are pyranosidic. The D-glucose residues are either terminal or linked to the 3-, 6-, or 3,6-positions. The D-glucuronic acid residues are either terminal or linked to the 4-position.

Assuming that the same proportion of 2,3,4,6-tetra-*O*-methyl-D-glucose is obtained from the hydrolysis of the methylated glucuronoglucan and methylated–reduced glucuronoglucan, the percentages of methylated sugars obtained from the former can be recalculated as molar percentages of the total sugar residues in the polysaccharide (Table I, columns *C*). These percentages, which are probably not very accurate, are closely similar to those obtained from the methylated–reduced glucuronoglucan (Table I, columns *B*) if the percentage of 2,3,4-tri-*O*-methyl-D-glucose is corrected for the portion derived from terminal D-glucuronic acid residues. It seems reasonable to assume that a considerable proportion of the glucuronosidic linkages in the methylated glucuronoglucan were not split during the acid hydrolysis. The results therefore strongly indicate that a major proportion of the D-glucuronic acid residues in the glucuronoglucan are not linked to D-glucose residues but to other D-glucuronic acid residues.

This conclusion is further supported by the results of the partial hydrolysis. The low yield of aldobiouronic acid **1** indicates that few of the glucuronic acid residues are linked to D-glucose residues, in the 3-position. The isolation of **2** and **3** demonstrates the presence of D-glucuronic acid residues, linked to other D-glucuronic acid residues in the 4-position. The presence in the hydrolysate of higher oligosaccharides, rich in D-glucuronic acid residues, indicates the presence of longer chains of D-glucuronic acid residues in the glucuronoglucan.

Branched β-D-glucans, in which the D-glucose residues are connected by (1→3)- and (1→6)-linkages, are common in fungi[6]. The glucuronoglucans from *P. fomentarius* and *P. igniarius* seem to contain a backbone of such a β-D-glucan. Chains, composed on average of 4 to 5 β-(1→4)-linked D-glucuronic acid residues, are attached to some D-glucose residues at the 3-position. As the glucuronoglucan from either fungus could be separated into fractions having different uronic acid contents, a randomness in length and/or frequency of these side chains is indicated. The precise structure of the backbone cannot be deduced from the present results. A solution to this problem must await further studies.

Glucuronoglucans of this type do not seem to have been observed in Nature before. Several *Polyporus* species have been investigated for their polysaccharide content. Two of these, *P. borealis* and *P. annosus* (*Fomes annosus*) also contain glucuronoglucans, which are under investigation.

EXPERIMENTAL

General methods. — Paper electrophoresis was performed on Whatman No. 1 and 3MM papers, using 0.05M sodium acetate buffer of pH 4. Paper chromatography was performed on Whatman No. 1 paper, using the following solvent systems (v/v): (*a*) ethyl acetate–acetic acid–water (3:1:1), (*b*) ethyl acetate–pyridine–water (8:2:1). The components were detected with *p*-anisidine hydrochloride. Other general methods were the same as described[1] in Part I.

Isolation of the acidic polysaccharides. — The dark-coloured, acidic material (4.5 g) from *P. fomentarius* was added to the top of a column (100 × 5 cm) of DEAE-Sephadex A-25 in the acetate form, and eluted with a gradient of potassium acetate (0–2M, 6 l). The fractionation was followed polarimetrically (Fig. 1), and similar fractions were combined and precipitated with ethanol to give fractions A_f–D_f. A hydrolysate of A_f contained D-glucose, D-galactose, D-mannose, L-fucose, and traces of D-xylose, as revealed by paper chromatography (solvent systems *a* and *b*). Hydrolysates of B_f, C_f, and D_f contained D-glucose and D-glucuronic acid. These sugars were isolated, and characterised as penta-*O*-acetyl-D-glucopyranose, m.p. 132–133°, $[\alpha]_{5780}$ +5°; and D-glucofuranurono-6,3-lactone, m.p. 168–172° (undepressed on admixture with an authentic sample), $[\alpha]_{5780}$ +13°. (Because of the small amount available the determination of the optical rotation was not very accurate.)

Fractions B_f and C_f were only slightly discoloured, whereas fraction D_f was light-brown.

The acidic material (8 g) from *P. igniarius* was fractionated by the same method, with similar results.

Fraction C_f (0.9 g) showed $[\alpha]_{5780}$ −26° (*c* 0.5, water) and contained about 27% of uronic acid residues, as estimated by the carbazole method[2]. The corresponding values for fraction C_i (1.3 g) were $[\alpha]_{5780}$ −31° and about 40% of uronic acid residues.

Methylation analyses of the glucuronoglucans. — The polysaccharide (20 mg) was methylated as previously described[1] for the neutral polysaccharides. Half of the methylated glucuronoglucan was hydrolysed by treatment with 0.25M sulphuric acid (5 ml) for 17 h at 100°, and the sugars in the hydrolysate were converted into alditol acetates, and analysed by g.l.c.[4]–mass spectrometry[5]. The results are summarised in Table I, columns *A*. The other half of the methylated glucuronoglucan was refluxed with 2% methanolic hydrogen chloride (5 ml) for 4 h, and the solution was neutralised with silver carbonate, filtered, and concentrated to dryness. The product was then refluxed with lithium aluminium deuteride (0.1 g) in tetrahydrofuran (10 ml) for 4 h. After processing, the reduced product was hydrolysed, and the methylated sugars obtained on hydrolysis were converted into alditol acetates and analysed as above. The results are summarised in Table I, columns *B*.

Graded, acid hydrolysis of the glucuronoglucan. — The combined fractions C_i and D_i (2.2 g) were dissolved in 0.5M sulphuric acid (100 ml) and kept for 6 h at 100°. The hydrolysate was neutralised with barium carbonate and centrifuged, and the precipitate was washed three times with hot water. The combined solutions were treated with Dowex-50 (H^+), concentrated to a small volume, and added to a column (40 × 2.5 cm) of DEAE-Sephadex A-25 in the formate form. Neutral sugars were eluted with water and acidic sugars by a gradient of formic acid (0–8%, 4 l). The fractionation was followed by paper chromatography (solvent system *a*) and paper electrophoresis. Three components **1**, **2**, and **3** (Table II) were obtained pure. The yields given in Table II refer to isolated, pure components. The actual yields should be higher, as the resolution was not complete, and intermediate fractions were discarded. The presence of other components, with low paper-chromatographic but high electrophoretic mobilities, was observed.

Methylation analysis of the acidic oligosaccharides. — Each oligosaccharide (10 mg) was refluxed with 2% methanolic hydrogen chloride (5 ml) for 4 h, and the solution was neutralised with silver carbonate, filtered, and concentrated to dryness. The resulting methyl ester methyl glycoside was treated with sodium borohydride (20 mg) in water (1 ml) for 24 h at room temperature. The solution was then treated with Dowex-50 (H^+), and boric acid was removed by repeated distillation with methanol. The resulting methyl glycoside was subjected to methylation analysis as above. The results are summarised in Table II.

ACKNOWLEDGMENTS

We are indebted to Dr. Aino Käärik, Royal College of Forestry, Stockholm, for classifying the fungi, to Professor Gunnar Widmark, Department of Analytical

Chemistry, Stockholm University, for the mass spectrometry facilities placed at our disposal, to Mrs. Marianne Bagarnäs for her skilful assistance, and to *Cellulosaindustriens stiftelse för teknisk och skoglig forskning samt utbildning* for financial support.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

THE ACETONATION OF D-ALLOSE

J. M. BALLARD AND B. E. STACEY
Department of Chemistry, Sir John Cass College, Jewry Street, London, E. C. 3 (Great Britain)
(Received June 16th, 1969)

ABSTRACT

Acetonation of D-allose gives, as the principal product, 2,3:5,6-di-*O*-isopropylidene-β-D-allofuranose (**1**). The structure of **1** was established by its conversion into D-allono-1,4-lactone following oxidation, and hydrolysis of the resulting di-*O*-isopropylidene-lactone. Acid hydrolysis of reduced **1** gave allitol. Diacetal **1** may also be prepared by acid-catalysed rearrangement of the isomeric 1,2:5,6-diacetal.

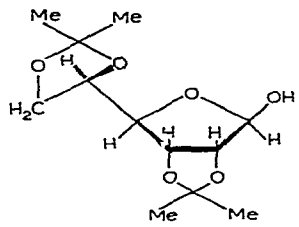
INTRODUCTION

Although the direct acetonation of D-allose has not been investigated experimentally, Mills[1] has predicted from conformational principles that allose would afford the 2,3:5,6- rather than the 1,2:5,6-diacetal; the latter is the only di-*O*-isopropylidene derivative of D-allose reported in the literature and is well known as an intermediate in the conversion of D-glucose into D-allose[2]. An investigation of the reaction between D-allose and acetone was therefore undertaken, as the predicted product was envisaged as a useful intermediate in our study of allitol derivatives and may also find use in the synthesis of nucleosides containing D-allose[3].

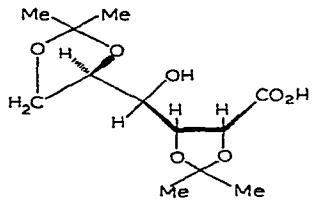
DISCUSSION

Acetonation of D-allose in the presence of anhydrous cupric sulphate and concentrated sulphuric acid gave a major, crystalline product **1**, which was faster moving than 1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose on t.l.c., together with several trace components. Elemental analysis, a positive reaction to Fehling's test (on prolonged heating), and failure to react with both trityl chloride and toluene-*p*-sulphonyl chloride were evidence consistent with the expected structure of the product, namely, 2,3:5,6-di-*O*-isopropylidene-D-allofuranose (**1**). This assignment was supported by the mass spectrum of the compound which showed a very intense peak at *m/e* 101 and a peak at 243 (M−17); the former peak occurs in the mass spectra of both di-*O*-isopropylidene-aldofuranoses and -aldopyranoses[4], which can be distinguished, however, since a characteristic feature of the spectra of di-*O*-isopropylidene-aldofuranoses is the greater intensity of the 101 peak (due[4] to scission of the C-4–C-5 bond) relative to other peaks, *e.g.*, (M−15). The peak at *m/e* 243 was relatively more intense than the same peak in the mass spectrum of 1,2:5,6-di-*O*-

isopropylidene-α-D-allofuranose, indicating[4] the presence of an unsubstituted hydroxyl group at C-1 rather than at C-3.



1



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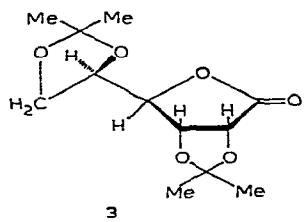

3

The n.m.r. spectrum (concentrated solution in carbon tetrachloride) of acetal **1** showed, *inter alia*, a singlet shifted well downfield at τ 4.77. Progressive dilution of the solution caused this peak to broaden and then to resolve into a doublet (J 7.5 Hz), which collapsed to a singlet on addition of deuterium oxide. These results are fully consistent with the β-D configuration at C-1; the strong coupling between H-1 and the hydroxylic proton[5] is removed on exchanging the latter with deuterium, and a singlet is then to be expected[6] from a *trans* arrangement of H-1 and H-2. The formation of the β-D anomer is to be expected on conformational grounds[1], as it has one *endo* substituent (Me group), whereas the α-D anomer has two (Me and OH groups).

Chemical evidence for the structure **1** was provided by oxidation and reduction. Oxidation of the compound with alkaline permanganate gave a di-*O*-isopropylidene-D-allonic acid (**2**) which, in refluxing 1,2-dimethoxyethane, was lactonised to a compound (**3**) identical with that obtained on acetonation of D-allono-1,4-lactone. Hydrolysis of the di-*O*-isopropylidene-lactone with 50% aqueous acetic acid gave D-allono-1,4-lactone. Reduction of the di-*O*-isopropylideneallose with borohydride gave a syrupy product, presumably 2,3:5,6-di-*O*-isopropylidene-D-allitol, which, on hydrolysis with dilute sulphuric acid, gave crystalline allitol.

The above evidence therefore firmly establishes the identity of the main product from the direct acetonation of D-allose as 2,3:5,6-di-*O*-isopropylidene-β-D-allofuranose (**1**). Similarly substituted products have been obtained from D-gulose[7], D-mannose[8], and D-talose[9]; these results also accord with the predictions of Mills[1] concerning the thermodynamically favoured products.

It was of interest to ascertain whether 1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose showed any tendency to rearrange to the thermodynamically favoured 2,3:5,6-diacetal. Such a rearrangement was observed to occur readily in acidified acetone at room temperature; conversion into the 2,3:5,6-diacetal was almost complete (t.l.c.) within five hours. Two similar rearrangements have been reported. Thus, 1,2:4,5-di-*O*-isopropylidene-D-*ribo*-hexulopyranose rearranges to 1,2:3,4-di-*O*-isopropylidene-D-*ribo*-hexulofuranose[10], and 1,6-anhydro-3,4-*O*-isopropylidene-β-D-talopyranose gives 1,6-anhydro-2,3-*O*-isopropylidene-β-D-talopyranose[11]. However, the present result appears to be the first example of an acetal rearrangement in which the furanoid ring system is present in both the reactant and the product.

EXPERIMENTAL

General. — Solutions were concentrated below 40° under diminished pressure. Melting points were determined on a Kofler block and are uncorrected. Infrared spectra were measured as Nujol mulls on a Perkin–Elmer Model 237 spectrometer. Thin-layer chromatography (t.l.c.) was performed on Kieselgel G (Merck) with (*A*) ethyl acetate–light petroleum (3:1, v/v) or (*B*) ethyl acetate–ethanol–water (10:3:2, v/v). Spots were detected with sulphuric acid in ethanol (5%) and heating.

Mass spectra were obtained by using a direct-insertion system with an AEI MS-902 mass spectrometer at 70 eV and a source temperature of 260°. N.m.r. spectra were obtained with a JEOL 60 MHz spectrometer for solutions in deuteriochloroform or carbon tetrachloride with tetramethylsilane as internal reference. Optical rotations were measured on a Stanley Photoelectric Polarimeter Serial No. 7. Acetone was dried over calcium chloride before use.

*2,3:5,6-Di-*O*-isopropylidene-β-*D*-allofuranose* (**1**). — D-Allose (1.18 g), anhydrous cupric sulphate (8.5 g), conc. sulphuric acid (0.17 ml, 98% w/w), and acetone (70 ml) were shaken for 62 h at room temperature. The filtered mixture was then neutralised with anhydrous potassium carbonate (5.1 g) and concentrated to an orange syrup (1.34 g). T.l.c. indicated one major component, R_F 0.61 (*cf.* R_F 0.43 for 1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose, which was also present as a minor product), and several slower-moving, trace components. The syrup was taken up in warm light petroleum–ether, treated with charcoal, concentrated, and allowed to cool. The crude product (700 mg), m.p. 57–63°, was again treated with charcoal and recrystallised from light petroleum to afford compound **1** (594 mg, 35%), m.p. 65–67°, $[\alpha]_D^{23}$ −1° (*c* 7.46, water); ν_{max} 1375 (CMe_2) and 3440 cm^{-1} (OH). N.m.r. data: τ 4.77 (1-proton doublet, *J* 7.5 Hz, H-1); 5.25–6.55 (7-proton multiplet); 8.6 (6-proton singlet, CMe_2); *ca.* 8.7 (two 3-proton singlets, incompletely resolved, CMe_2). Addition of deuterium oxide resulted in the collapse of the H-1 doublet at τ 4.77 to a singlet, but the signal due to the hydroxylic proton could not be located with certainty.

Anal. Calc. for $C_{12}H_{20}O_6$: C, 55.3; H, 7.69. Found: C, 55.1; H, 7.55.

*2,3:5,6-Di-*O*-isopropylidene-*D*-allonic acid* (**2**). — A solution of **1** (2.20 g, 8.46 mmoles), 7.5N potassium hydroxide (1.10 ml, 8.25 mmoles), and potassium permanganate (0.930 g, 5.90 mmoles) in water (200 ml) was stirred for 70 h at room temperature[12]. Manganese dioxide was removed by filtration, and the colourless solution was neutralised (to phenolphthalein) with carbon dioxide and concentrated to a syrup (3.03 g). Extraction with cold ethanol gave a syrup (2.92 g) which was treated with the calculated quantity of sulphuric acid (N, 8.8 ml) and extracted immediately with ether (4 × 40 ml). The extract was washed with water (3 × 25 ml), dried (Na_2SO_4), and concentrated to small bulk. Crystallisation ensued to give compound **2** (1.13 g, 51%), m.p. 121–122.5°, $[\alpha]_D^{23}$ −20° (*c* 1.02, water); ν_{max} 1697 (C=O, carboxylic acid), 1380 (CMe_2), and 3235 cm^{-1} (OH).

Anal. Calc. for $C_{12}H_{20}O_7$: C, 52.2; H, 7.24. Found: C, 52.3; H, 7.24.

*2,3:5,6-Di-*O*-isopropylidene-*D*-allono-1,4-lactone* (**3**). — A solution of acid **2**

(506 mg, 1.96 mmoles) in 1,2-dimethoxyethane (25 ml) was heated under reflux[13] for 5 h. T.l.c. then indicated that all of the starting material had been converted into a faster-moving product. The solution was concentrated to a syrup from which several aliquots of light petroleum were evaporated. Crystallisation ensued on the addition of a small volume of ether, followed by a large excess of light petroleum, to give lactone **3** (0.429 g, 92%), m.p. 71–73°, $[\alpha]_D^{23}$ −67° (*c* 2.16, ethanol); ν_{max} 1795 (γ-lactone), 1388 (CMe_2), and 1375 cm^{-1} (CMe_2). N.m.r. data: τ 5.4 (singlet); 5.6–6.2 (multiplet); 8.58 (broad singlet, area indicating two methyl groups with similar chemical shifts); 8.64, 8.70 (two singlets, CMe_2).

Anal. Calc. for $C_{12}H_{18}O_6$: C, 55.8; H, 6.97. Found: C, 55.9; H, 7.04.

Acetonation of D-*allono-1,4-lactone.* — D-Allono-1,4-lactone[14] (300 mg, 1.68 mmoles) was acetonated, as described above, to give an orange syrup (320 mg) which partially crystallised from light petroleum containing a small proportion of ether. A solution of the product in warm, light petroleum was treated with charcoal and filtered. Concentration to small bulk, addition of a few drops of ether, and storage at 0° gave a crystalline product (100 mg, 25%), m.p. 69–73°, which was identical (i.r. spectrum and t.l.c. behaviour) with compound **3** described above.

Acid hydrolysis of lactone **3**. — A solution of **3** (260 mg, 1.08 mmoles) in 50% aqueous acetic acid was heated for 5 h at 60°. T.l.c. then indicated that hydrolysis was complete. The solution was concentrated, and residual acetic acid was removed by repeated co-evaporation with water. The resulting, clear syrup afforded crystalline D-allono-1,4-lactone on trituration with ethanolic ether. Yield, 58 mg (30%); m.p. 125–127°; lit.[15] m.p. 120°. The i.r. spectrum and t.l.c. behaviour (solvent *B*) of the product were identical with those of an authentic sample.

Reduction of acetal **1** *with borohydride.* — Sodium borohydride (500 mg, 8.0 mmoles) was added over 30 min to a stirred, cooled (ice-bath) solution of acetal **1** (2.01 g, 7.74 mmoles) in 70% v/v aqueous ethanol (20 ml). After a further 1.5 h, water (5 ml) was added, and the reaction mixture was extracted with chloroform. The dried (Na_2SO_4) chloroform extract was concentrated to a syrup (2.0 g). T.l.c. (solvent *A*) showed one major product (R_F 0.41), one trace component (R_F 0.55), and some unreacted starting material. Preparative layer chromatography (4 runs), using ethyl acetate–light petroleum (1:5 v/v), gave a pale-yellow syrup (1.09 g). T.l.c. indicated the presence of one major component (R_F 0.35, solvent *A*) and two faster-moving, trace components.

A solution of the syrup (570 mg) in water (15 ml) containing conc. sulphuric acid (0.1 ml) was heated under reflux for 30 min. After neutralisation with barium carbonate, filtration, and concentration, the syrupy residue was triturated with ethanol to give a crude product (290 mg), m.p. 140–147°, which was recrystallised from aqueous ethanol to give allitol (200 mg, 51%), m.p. 149–152°; lit.[16] m.p. 151°; i.r. spectrum identical with that of an authentic sample.

*Acid-catalysed rearrangement of 1,2:5,6-di-*O*-isopropylidene-α-*D*-allofuranose* (**4**). — A solution of **4** (500 mg) in anhydrous acetone (2 ml), acidified with conc. sulphuric acid (0.02 ml), was kept overnight at room temperature. T.l.c. (solvent *A*) then

indicated almost complete conversion of **4** into the faster-moving isomer. After neutralisation with potassium carbonate, filtration, and concentration, a syrup was obtained which afforded a partially crystalline product on trituration with light petroleum. This was purified by preparative layer chromatography, and recrystallisation from light petroleum then gave acetal **1** (140 mg, 28%), m.p. 65–67°; the i.r. spectrum was identical with that of authentic **1**.

ACKNOWLEDGMENTS

The authors thank Dr. R. F. M. White for determining the n.m.r. spectra, the University of London Inter-Collegiate Mass Spectroscopy Service for measuring the mass spectra, and Mr. B. E. Saunderson for performing the microanalyses.

This work was supported by a grant from the British Empire Cancer Campaign for Research.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

PREPARATION OF METHYL-^{14}C D-GLUCURONATE AND NUCLEAR MAGNETIC RESONANCE STUDIES OF ITS MONO- AND DI-O-BENZYLIDENE INTERMEDIATES*

R. H. SHAH
Department of Biology, State University of New York at Buffalo, Buffalo, New York 14214 (U. S. A.)
(Received February 19th, 1969; in revised form, June 20th, 1969)

ABSTRACT

A procedure suitable for the synthesis of methyl-labeled methyl D-glucuronate from D-glucuronic acid was devised. The intermediates prepared during the synthesis, 1,2:3,5-di-*O*-benzylidene-α-D-glucofuranuronic acid and its methyl ester, were determined to exist in an "O-inside" conformation by examination of their n.m.r. spectra. Evidence obtained by n.m.r. and g.l.c. indicated that when a reaction of D-glucuronic acid with benzaldehyde in the presence of anhydrous zinc chloride proceeded for 48 h, the major product was (*1,2-exo-phenyl*)-1,2-*O*-benzylidene-α-D-glucofuranurono-6,3-lactone, along with a small amount of the (1,2-*endo*-phenyl)-isomer.

INTRODUCTION

Although several methods of preparing methyl D-glucuronate (**1**) are described in the literature[1–6], a simple, economic procedure in which methyl-labeled **1** can be prepared from commercially available starting compounds has not been described. Need for such labeled compounds to study uronic acid metabolism in plants prompted this undertaking.

A procedure was devised that was similar to the one used to prepare methyl D-galacturonate in which methyl iodide was used in near-equivalent amount[7]. Esterification of 1,2:3,5-di-*O*-benzylidene-α-D-glucofuranuronic acid (**3**), obtained by benzylidenation of D-glucuronic acid (**2**), was accomplished by methylation of its sodium salt **4** with methyl-^{14}C iodide in *N*,*N*-dimethylformamide. Removal of the benzylidene groups by hydrogenolysis from methyl-^{14}C 1,2:3,5-di-*O*-benzylidene-α-D-glucofuranuronate (**5**) afforded methyl-^{14}C D-glucuronate (**1**).

RESULTS AND DISCUSSION

1,2:3,5-Di-*O*-benzylidene-α-D-glucofuranuronic acid (**3**) was isolated in 10–15% yield from a reaction of **2** with benzaldehyde catalyzed by anhydrous zinc chloride

*Presented, in part, before the Division of Carbohydrate Chemistry, 155th Meeting Amer. Chem. Soc., San Francisco, California, April 1968, Abstracts, p. 21C.

for 6 h. No attempt was made to isolate products other than **3** if any were present. The i.r. spectrum of **3** showed peaks at 1738 cm^{-1} for the carbonyl group and at 700–750 cm^{-1} for the aromatic groups. Papadakis[8] prepared **3** by oxidation of 1,2:3,5-di-*O*-benzylidene-α-D-glucofuranose (**6**) with hypobromite and observed a m.p. of 175°, as compared with that of 195–196° observed in the present study. The positions of the benzylidene groups in Papadakis' compound and in our compound **3** were shown to be identical (see later discussion). It is likely that the lower m.p. observed by Papadakis was due to a mixture of 1,2-*exo*- and 1,2-*endo*-phenyl isomers[9–11].

Neutralization of **3** with an equivalent amount of sodium hydrogen carbonate in aqueous acetone formed sodium 1,2:3,5-di-*O*-benzylidene-α-D-glucofuranuronate (**4**) in an almost quantitative yield. The difference in the i.r. spectra of **3** and **4** was that expected between a carboxylic acid and its metal salt[12]. Crystalline methyl ester **5**, was obtained from **4** in *ca.* 90% yield by treatment with methyl iodide in *N,N*-dimethylformamide for 2 h at 85°. Methylation of **3** with diazomethane also led to the ester **5**. There was no evidence in the i.r. spectrum for the presence of a hydroxyl group in **5**, but peaks at 1728 and 700–750 cm^{-1} indicated the existence of ester carbonyl and aromatic group, respectively. For the preparation of methyl-^{14}C **5**, an approximately 50% excess of **4** was employed to ensure optimum incorporation of the label from methyl-^{14}C iodide. After the reaction, the excess of unreacted **4** was separated from methyl-^{14}C **5** by passing the mixture through a column of silica gel and irrigating the column with chloroform. The sodium salt **4** was retained at the top of the column, while labeled **5** was isolated in a nearly quantitative radiochemical yield from the effluent.

Lithium aluminum hydride converted unlabeled **5** into **6** in 80% yield, m.p. 161–163° and $[\alpha]_D$ +35.7° in pyridine. These values agreed well with those reported in the literature[8,13,14]. The trimethylsilyl derivative, **7**, of **6**, showed in g.l.c. a predominant peak accompanied closely by a negligible minor peak, which quite likely was due to other isomers of **6**. The 6-acetate, **8**, of **6**, obtained in 85% yield by acetylation with acetic anhydride and pyridine, had a m.p. of 145–146° and $[\alpha]_D$ +28.6° in chloroform, which compared favorably with the values reported by Wood *et al.*[14]. On this basis, the benzylidene groups of **6**, and hence of **3**, **4**, and **5**, were assigned the 1,2:3,5-positions. N.m.r. data supported this assignment (see later discussion). In g.l.c., the acetate **8** exhibited one predominant peak, immediately preceded by three minor peaks. Each of these four peaks possibly represents one of the four diastereoisomers theoretically possible when two new asymmetric carbons are created in the formation of **3** from D-glucuronic acid (**2**).

The benzylidene groups of **5** were removed by hydrogenolysis catalyzed by palladium at atmospheric pressure to obtain **1**. On a small scale where *ca.* 0.1 g of **5** was hydrogenolyzed, the removal of benzylidene groups was clean and rapid. Only one product was obtained, chromatographically indistinguishable from authentic[4] methyl D-glucuronate (**1**). Relatively large-scale hydrogenolysis of 1 g of **5** led to the formation of side-products, the amounts of which seemed to increase with an increase

in the catalyst to solvent ratio, although the catalyst to substrate ratio was not higher than that used in small-scale hydrogenolysis. By employing approximately the same catalyst to solvent ratio as had been used in the small-scale hydrogenation, but with a greatly decreased catalyst to substrate ratio, it was possible to eliminate almost completely the formation of side-products, although under these conditions the uptake of hydrogen was considerably slower. Mixtures containing the side-products did not show aromatic absorption in the i.r. spectrum. Further evidence that these side-products did not arise by removal of only one of the two benzylidene groups from **5** was indicated by their stability to further hydrogenolysis. These side-products were not investigated further.

The ester **1** gave a positive reducing sugar test. Its i.r. spectrum, which had peaks at 1780 (shoulder) and 1750–1725 cm^{-1} for the carbonyl and 3500–3200 cm^{-1} for the hydroxyl, but no aromatic absorption, was identical with that of authentic[4] **1**. In three solvents, **1** gave a single spot, the mobility of which on paper-chromatogram was identical with that of authentic **1**.

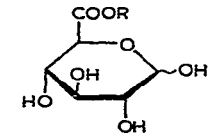
Hydrogenolysis of methyl-^{14}C **5** over palladium-on-carbon yielded a product which co-chromatographed, on a cellulose t.l.c. plate, as a single radioactive spot with authentic[4] **1**. Over-all incorporation of label from methyl-^{14}C iodide into **1** was 95%.

Saponification of unlabeled **1** with aqueous sodium hydroxide, followed by passage through a column of Dowex-50 (H^+), gave a syrup which on paper chromatography revealed three spots having mobilities corresponding to those obtained by similar treatment of D-glucurono-6,3-lactone. The slowest moving component corresponded to D-glucuronic acid (**2**) and the fastest to D-glucurono-6,3-lactone. Although the third product was not identified, D-fructuronic acid, D-mannuronic acid, and L-iduronic acid are likely candidates[15,16].

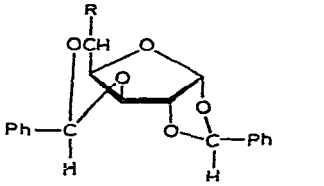
Monobenzylidene glucuronolactones. — When the product of the reaction of **2** with benzaldehyde, catalyzed by zinc chloride, was studied after 48 h (in contrast to the 6-h reaction described earlier) the predominant compound was 1,2-*O*-benzylidene-α-D-glucofuranurono-6,3-lactone (**10**), accompanied by small amounts of 1,2-*O*-benzylidene-α-D-glucofuranuronic acid (**19**) and **3**. Partial recovery of **10** and **19** from the mixture was achieved by repeated extractions with water. The residue (fraction A) remaining after removal of water from the combined extracts corresponded to a yield of *ca.* 18% of **10**. Material not extracted with water (fraction B) corresponded to a yield of *ca.* 42% of **10**, and it was examined separately. Fraction A was comprised of nearly equal amounts of the *exo*- and *endo*-phenyl isomers of **10**, whereas fraction B consisted predominantly of *exo*-phenyl **10** (*ca.* 90%), in addition to *ca.* 10% of *endo*-phenyl **10** and a small proportion of **3**. Both fractions contained a trace of the monobenzylidene acid **19**.

The trimethylsilyl and acetyl derivatives **11**, **12**, **15**, **16**, **18**, **22**, and **23** showed two neighboring peaks in g.l.c., which suggests the presence of two isomers of **10** differing only in the configuration of the phenyl group of the 1,2-benzylic carbon. Evidence for the presence of two isomers of **10** was found in the n.m.r. spectrum

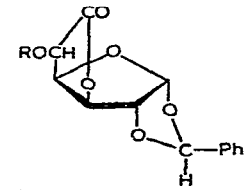
which showed two benzyl proton signals. Assuming that the lactone carbonyl had not reversed the chemical shifts normally expected of the *exo*- and *endo*-benzyl protons, the low- and high-field signals were assigned to the *endo*- and *exo*-benzyl protons, respectively[9,10].



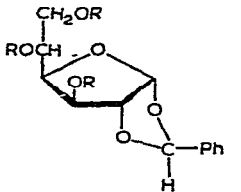
1 R = Me 2 R = H



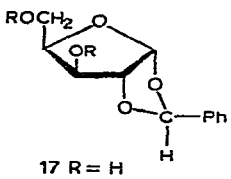
3 R = CO_2H 4 R = CO_2Na
5 R = CO_2Me 6 R = CH_2OH
7 R = CH_2OSiMe_3
8 R = CH_2OAc 9 R = H



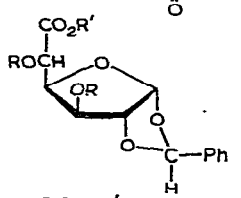
10 R = H 11 R = $SiMe_3$
12 R = Ac 13 R = C(=O)—Ph



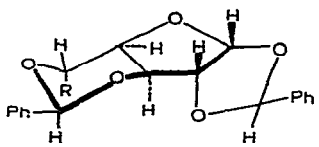
14 R = H 15 R = $SiMe_3$
16 R = Ac



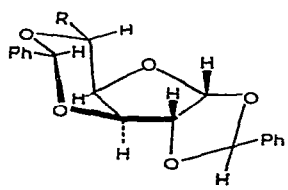
17 R = H
18 R = $SiMe_3$



19 R = R' = H
20 R = H; R' = Na
21 R = H; R' = Me
22 R = $SiMe_3$; R' = Me
23 R = Ac; R' = Me



24



25

G.l.c. of the trimethylsilyl ether **11**, prepared from analytically pure **10** from both fractions, indicated that the ratio of *exo*- to *endo*-phenyl isomers in fraction A was 57:43, whereas that in fraction B was 91:9. Specific rotations of these two mixtures of **10** in pyridine at different wavelengths are given in Table I, along with those of the dibenzylidene acid **3** and the ester **5**. From these data, the specific rotations of the pure *exo*- and *endo*-phenyl **10** were calculated for each wavelength, and are given in Table I. Derivatives of *exo*-phenyl **10** had longer retention times in g.l.c., since the *endo*-benzyl proton signal of **10** (from fraction B) in n.m.r. and the peaks with longer retention times were predominant. Hence, peaks with shorter retention times were attributed to derivatives of *endo*-phenyl **10**.

The lactone **10** (from fraction A) was converted into monobenzylidene D-glucose (**14**) by reduction with lithium aluminum hydride, which on acid hydrolysis gave D-glucose. Compound **14** was degraded to D-xylose by periodate oxidation, lithium aluminum hydride reduction, and acid hydrolysis. Although **14**, obtained from fraction B, was not converted into D-xylose to confirm the furanose ring of **10**, the alternate structure, 1,2-*O*-benzylidene-D-glucopyranurono-6,3-lactone, is precluded

by the following evidence: (*a*) The proton signal (H-5) moving downfield in **12** (τ 4.48) and **13** (τ 4.25) was a doublet (*J ca.* 4 Hz). For the pyranuronolactone structure, Dreiding models indicate nearly equal coupling of H-4 with H-3 and H-5, hence the signal moving downfield (H-4) should be a triplet and not a doublet. (*b*) The chemical shift of H-5 of **12** (τ 4.48) and **13** (τ 4.25) resembles that (τ 4.38) reported[17] for H-5 of 1,2-*O*-isopropylidene-5-*O*-*p*-tolylsulfonyl-α-D-glucofuranurono-6,3-lactone. The magnitude of the paramagnetic shift of the H-4 signal in the alternate structure should be less than that observed for H-5 in **12** and **13**, since C-4 does not have an α-carbonyl as does C-5. The influence of the lactone carbonyl group on the paramagnetic shift of H-5 can be seen by comparing the reported[17] chemical shifts of H-5 in the 5-*O*-*p*-toluenesulfonates of both 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone (τ 4.38) and 3,6-anhydro-1,2-*O*-isopropylidene-α-D-glucofuranose (τ 4.98).

TABLE I

SPECIFIC ROTATIONS (DEGREES) OF COMPOUNDS **3**, **5**, AND **10** IN PYRIDINE[a]

Wavelength (nm)	***10*** *91% exo- +9% endo-phenyl*	***10*** *57% exo- +43% endo-phenyl*	***10*** *exo-phenyl*	***10*** *endo-phenyl*	***3***	***5***
589	+46.9	+23.7	+53	−16	+48.6	+53.5
578	+48.4	+24.0	+55	−17	+50.2	+55.1
546	+54.6	+27.4	+62	−21	+56.4	+62.1
436	+91.8	+44.8	+104	−36	+90.4	+101
365	+148	+70.1	+169	−65	+136	+152

[a]For concentrations, see Experimental section.

The mixture of **10** and **19** in fraction A was converted into the methyl ester **21** *via* the sodium salt **20** by treatment with methyl iodide in *N*,*N*-dimethylformamide. G.l.c. of the trimethylsilyl ether **22** and of the acetate **23** indicated that the methyl ester **21** (75%) was accompanied by the lactone **10** (25%). The i.r. spectrum of **23** had two carbonyl peaks, one at 1810 cm^{-1} and the other at 1750 cm^{-1}. The 1750 cm^{-1} absorption was assigned to the acetyl carbonyl. The intensity of carbonyl absorption at 1810 cm^{-1} was much greater than that expected from 25% contamination with the lactone acetate **12**, thus suggesting that the methyl ester carbonyl was absorbing at 1810 cm^{-1}, a frequency higher than normally expected. It is likely that this rise in absorption frequency is due to the electronegativity of the α-acetoxy group[18] at C-5 of **23**. The n.m.r. spectrum of **23** showed two methoxyl proton singlets (τ 6.17 and 6.19), thus lending further support to the existence of two isomers.

Conformations of dibenzylidene compounds. — In view of the general preference of groups to occupy an equatorial position in six-membered rings, it was of interest to examine the n.m.r. spectra of the dibenzylidene compounds **3** and **5** to determine which of the two conformations, "O-inside" (**24**) or "H-inside" (**25**), was favored

TABLE II

CHEMICAL SHIFTS (τ-VALUES) OF DIBENZYLIDENE COMPOUNDS 3, 5, AND 8

Compound	*Solvent*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*Benzyl protons*[a]	*Other protons*	*Phenyl*
3	Acetone-d_6	3.65 (d)[b]	5.12 (d)	5.19 (d)	5.29 (q)	4.96	—	3.65 3.91	2.4 (carboxyl)	2.4
5	Chloroform-*d*	3.68 (d)	5.15 (d)	5.30[c]	5.42[c]	4.95	—	3.75 4.07	6.10 (methoxyl)	2.5
8	Chloroform-*d*	3.68 (d)	5.13 (d)	5.29 (d)	5.79 (d)	(c)	(c)	3.78 4.08	7.86 (acetoxyl)	2.5

[a]For each compound, the low-field, benzyl-proton signal is related to the 1,2-benzylic hydrogen, and the high-field signal to the 3,5-benzylic hydrogen. [b]Key: (d), doublet; (q), quartet; (c), complex band between τ 5.20 and 5.76. [c]Assignment tentative.

by these derivatives, since the former would have the C-6 substituent in an axial position, whereas the latter would have it in an equatorial position. The prediction of Mills[19] that the "O-inside" conformation would be preferred has been verified for 1,3:2,4-di-*O*-methylene- and 1,3:2,4-di-*O*-benzylidene-L-threitol[20,21]. Ferrier and Hatton[22] have shown that 1,2:3,5-di-*O*-benzylidene-α-D-xylofuranose (**9**) shows, in the n.m.r., features characteristic of an "O-inside" conformation (**24**, R=H). Conformation **24** was determined recently by Coxon[23] for various 6-*O*-substituted derivatives of **6**.

Tables II and III list the chemical shifts and the coupling constants of compounds **3**, **5**, and **8**. Arguments similar to those used for the determination of the conformation of **9** (ref. 22) and of various derivatives of **6** (ref. 23) confirmed the "O-inside" conformation (**24**) of compounds **3** and **5**.

TABLE III

COUPLING CONSTANTS (Hz) OF DIBENZYLIDENE COMPOUNDS **3**, **5**, AND **8**

Compound	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$
3	3.7	<1	*ca.* 2	*ca.*1	—
5	3.8	<1	—	<1	—
8	3.7	<1	*ca.* 2.5	—	—

Although equatorial configuration of the phenyl substituent of the *m*-dioxane ring of compounds **3** to **8** can be assigned with certainty on the basis of the work of Baggett and co-workers[9,10], evidence is not available for an unequivocal assignment of either the *endo* or the *exo* orientation to the phenyl group of the 1,2-benzylidene ring of compounds **3** to **8**. Data presented below support the *exo*-phenyl assignment of the 1,2-benzylic carbon of **3** to **8**.

Coxon[23] observed that a change in the 6-*O*-substituent of dibenzylidene-D-glucose **6** caused a greater variation in the chemical shift of the benzyl proton signal at higher field than the one at lower field. On this basis, the low-field signal was attributed to the more distant (from C-6) benzyl proton of the 1,2-benzylidene ring of various derivatives of **6**. As seen from Table II, chemical shifts for the benzyl protons of **8**, which was obtained here from **5**, differ very little from those of **5**. Hence, the low-field benzyl proton signal of **3** and **5** was assigned to the 1,2-benzylic hydrogen and the high field signal to the 3,5-benzylic hydrogen. The benzylic proton resonances at τ 3.65, 3.75, and 3.78 for **3**, **5**, and **8**, respectively, resemble the reported[22] chemical shift at τ 3.83 for the *endo*-benzyl proton of the 1,2-benzylidene ring of **9**; the corresponding *exo*-benzyl proton of **9** resonated at τ 4.10. Hence, it appears quite likely that the benzyl proton of the 1,2-benzylidene ring of **3** to **8** is *endo*. In this regard, it is interesting to note that the plots of the molecular rotation (obtained from specific rotations given in Table I) *versus* the wavelength (Fig. 1) for the dibenzylidene acid **3** and the ester **5** resemble that of the monobenzylidene lactone **10** having an *exo*-phenyl ring, whereas they are markedly different from that of **10** having an *endo*-

phenyl ring. Specific rotations of *endo*- and *exo*-phenyl **9** in chloroform at various wavelengths have been reported[22]; as in the case of *endo*-phenyl **10**, *endo*-phenyl **9**

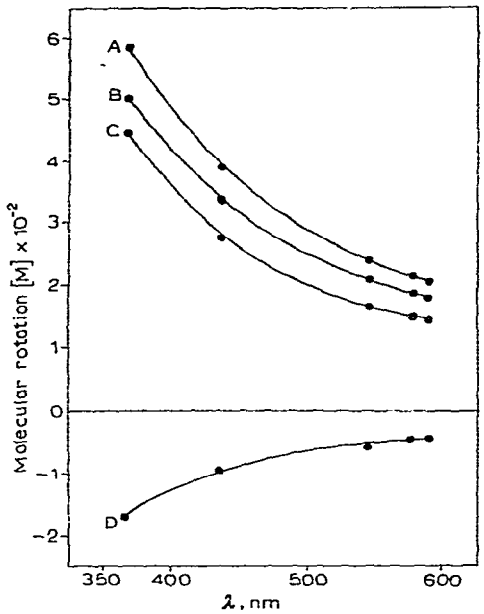


Fig. 1. Molar rotation [M] in pyridine *versus* wavelength. A, methyl 1,2:3,5-di-*O*-benzylidene-α-D-glucofuranuronate (**5**); B, 1,2:3,5-di-*O*-benzylidene-α-D-glucofuranuronic acid (**3**); C, (*1,2-exo-phenyl*)-1,2-*O*-benzylidene-α-D-glucofuranurono-6,3-lactone (**10**); D, (1,2-*endo-phenyl*)-*1,2*-*O*-benzylidene-α-D-glucofuranurono-6,3-lactone (**10**).

has a more negative rotation at 400 nm than at 589 nm. The *exo*-phenyl isomers of **9** and **10** have a more positive rotation at 400 nm than at 589 nm.

EXPERIMENTAL

Melting points were determined with a Fisher-Johns apparatus and are corrected. A Perkin–Elmer Model 257 spectrophotometer was used to determine i.r. spectra. N.m.r. spectra were obtained on a Varian A-60 spectrometer. Solvents employed for n.m.r. are indicated under appropriate compounds. Chemical shifts were determined as mid-points of multiplets and are given in τ units, downfield from the tetramethylsilane signal, as internal standard at τ 10. G.l.c. was performed on a Packard Model 7821 gas-chromatograph equipped with a flame-ionization detector and a temperature programmer. A six-foot coiled glass column packed with 3% silicone polymer, OV-1 on Gas Chrom Q, 100-120 mesh (Applied Science Labs., Inc., State College, Pa.) was used. A stream of nitrogen as carrier gas was passed through the column, at a rate of 50 ml per min. The retention times were calculated from the beginning of the solvent peak. The trimethylsilyl derivatives used for g.l.c. were prepared according to the method of Sweeley *et al.*[24]. Paper chromatography was performed on Whatman paper No. 1 with the following solvents: 14:3:3 ethyl acetate–

acetic acid–water (solvent A); 4:1:5 butyl alcohol–acetic acid–water (solvent B)[5]; 5:5:1:3 ethyl acetate–pyridine–acetic acid–water (solvent C); 2:8:1 pyridine–ethyl acetate–water (solvent D). Thin-layer chromatography (t.l.c.) was performed either on cellulose (MN-Polygram Cel 300, Brinkmann Instruments, Inc.) or on Silica Gel G. Solvents employed for t.l.c. were: 1:1 benzene–chloroform (solvent E); 98:2 chloroform–methanol (solvent F). Spots on cellulose plates and paper were made visible by spraying with silver nitrate in acetone, followed by potassium hydroxide in methanol[25], while those on silica gel plates were detected by heating the plates which had been sprayed with 10% ethanolic sulfuric acid. Thin-layer plates were scanned with a Packard Model 7201 radiochromatogram scanner to detect radioactive spots. Column chromatography was performed with silica gel, grade 950, purchased from Will Scientific Corp.; unless stated otherwise, chloroform was used as solvent. Methyl-^{14}C iodide was purchased from New England Nuclear Corp. Optical rotations were determined at room temperature in a 1-dm cell with a Perkin-Elmer Model 141 polarimeter. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn, U.S.A.

1,2:3,5-Di-O-benzylidene-α-D-glucofuranuronic acid (**3**). — A mixture of D-glucuronic acid (19.4 g, 100 mmoles), benzaldehyde (150 ml), and anhydrous zinc chloride (20 g) was stirred for 6 h at room temperature. The mixture, which contained some insoluble solid (presumably unreacted **2**), was partitioned between ether (250 ml) and water (200 ml). The organic layer was washed further with water (3 × 200 ml), and then evaporated under diminished pressure to remove ether. Benzaldehyde was removed by co-distillation with water (2 × 200 ml). The residual solid was suspended in water (200 ml), filtered off, and washed successively with water and petroleum ether. The yellow solid was recrystallized from chloroform–petroleum ether to yield 4.11 g (11%) of **3**, m.p. 189–190°. The dibenzylidene compound **3** was dissolved in a solution of sodium hydrogen carbonate (1.4 g) in water (100 ml), decolorized with Norit A, and precipitated by acidification of the solution to pH 2 with M hydrochloric acid. The product was filtered off and washed with water; yield, 3.77 g (10%), m.p. 188–190°. An analytical sample was prepared by decolorizing a solution of **3** in a small volume of acetone with Norit A, followed by precipitation with water; m.p. 195–196° (lit.[8]: m.p. 175°); $[\alpha]_D$ +48.6° (*c* 1.01, pyridine); ν_{max}^{KBr} 1738(C = O), 758 and 697 (aromatic) cm^{-1}; t.l.c. (silica gel, solvent E): streaks; n.m.r. data: see Tables II and III.

Anal. Calc. for $C_{20}H_{18}O_7$: C, 64.85; H, 4.90. Found: C, 65.09; H, 4.62.

Sodium 1,2:3,5-di-O-benzylidene-α-D-glucofuranuronate (**4**). — To a solution of **3** (3.21 g, 8.68 mmoles) in acetone (25 ml) was added a solution of sodium hydrogen carbonate (0.73 g, 8.68 mmoles) in water (25 ml). After 2 h at room temperature, the solvents were evaporated under reduced pressure to give **4** as a pale-yellow solid; yield, 3.40 g (100%); ν_{max}^{KBr} 1640 and 1605 (COO^-), 740 and 700 (aromatic) cm^{-1}.

Methyl 1,2:3,5-di-O-benzylidene-α-D-glucofuranuronate (**5**). — (*a*) *From* **4**. A solution of **4** (3.07 g, 7.85 mmoles) in *N*,*N*-dimethylformamide (15 ml) was heated for 2 h at 85° with methyl iodide (0.52 ml, 8.4 mmoles). Most of the *N*,*N*-dimethyl-

formamide was evaporated under reduced pressure at 70°, final traces being removed by co-distillation with xylene. The residual syrup was extracted with chloroform, and filtered to remove sodium iodide. The filtrate was concentrated to a dark yellow syrup which afforded 2.63 g (87%) of **5**, m.p. 113–115°, on crystallization from methanol. An additional 0.188 g (6%) of **5**, m.p. 108–110°, was obtained from the mother liquor.

A sample for microanalysis, m.p. 113–114°, was obtained from an earlier run by two recrystallizations from methanol; $[\alpha]_D$ +53.5° (*c* 1.01, pyridine); ν_{max}^{KBr} 1728 (C=O), 758 and 700 (aromatic) cm^{-1}; t.l.c. (silica gel, 3:1 benzene–chloroform): R_F 0.29; n.m.r. data: see Tables II and III.

In g.l.c. (250–275°, 5° per min), **5** had a retention time of 5.95 min. In addition, two negligible peaks (retention times, 4.95 and 5.20 min) were detected. These may be due to traces of isomers differing from **5** in the configuration of the phenyl groups.

Anal. Calc. for $C_{21}H_{20}O_7$: C, 65.61; H, 5.24. Found: C, 65.82; H, 5.33.

(*b*) *By methylation of* **3** *with diazomethane.* — A solution of **3** (0.37 g, 1 mmole) in anhydrous ether (35 ml) containing excess diazomethane was kept over the week-end at room temperature. The excess reagent was removed under a stream of nitrogen, and the ether was evaporated under reduced pressure. Crystallization of the residual syrup from methanol–petroleum ether gave 0.308 g (80%) of nearly colorless **5**, m.p. 113–114°. G.l.c. behavior was identical to that of **5** obtained by method (*a*).

1,2:3,5-Di-O-benzylidene-α-D-glucofuranose (**6**). — To a partial solution of **5** (0.384 g, 1 mmole) in anhydrous ether (20 ml) was added lithium aluminum hydride (0.380 g, 10 mmoles). The mixture was stirred under reflux for 10 h, after which time the excess hydride was decomposed by dropwise addition of a small excess of water (1 ml) to the mixture cooled in an ice-bath. The insoluble solids were removed by filtration through Filter Cel and washed with acetone and chloroform. The combined filtrates were evaporated under reduced pressure to give a nearly colorless crystalline residue (0.353 g, 99%), m.p. 160–162°. Recrystallization from methanol provided analytical **6**, m.p. 161–163°, in 80% yield, $[\alpha]_D$ +35.7° (*c* 1.06, pyridine) (m.p. and specific rotation were in close agreement with lit.[8,13,14] values); ν_{max}^{KBr} 3530 (OH), 750–700 (aromatic) cm^{-1}; the trimethylsilyl ether **7** of **6** had one major peak (retention time, 6.60 min) in g.l.c. (250–275°, 5° per min). An insignificant peak (retention time, 5.65 min) suggested the presence of other isomers of **6**.

Anal. Calc. for $C_{20}H_{20}O_6$: C, 67.41; H, 5.66. Found: C, 67.50; H, 5.80.

The 6-*O*-acetyl derivative **8**, prepared in 85% yield in the usual manner, had m.p. 145–146°, $[\alpha]_D$ +27.7° (*c* 1.02, chloroform), in good agreement with lit.[14] values; ν_{max}^{KBr} 1745 (C=O), 760 and 700 (aromatic) cm^{-1}; t.l.c. (silica gel, 3:1 benzene–chloroform): R_F 0.12; n.m.r. data: see Tables II and III; in g.l.c. (250–275°, 5° per min) the major peak appeared after 7.9 min. The retention times of three other minor peaks, attributable to the remaining three isomers of **8**, were 6.35, 6.90, and 7.50 min.

Anal. Calc. for $C_{22}H_{22}O_7$: C, 66.33; H, 5.57. Found: C, 66.47; H, 5.63.

Methyl D-glucuronate (**1**). — A mixture of **5** (1.0 g), 10% palladium-on-carbon (0.10 g), and absolute ethanol (100 ml) was shaken with hydrogen under atmospheric

pressure until the hydrogen uptake ceased (*ca.* 2 days). The catalyst was removed by filtration through Celite, and washed with ethanol. Removal of the solvent from the combined filtrates afforded syrupy **1** which reduced Benedict's solution; yield, 0.60 g (110%); ν_{max}^{Film} 3500–3200 (OH), 1780 (shoulder) and 1750–1725 (ester C=O) cm^{-1}. The i.r. spectrum of authentic[4] **1** was identical. Paper chromatography in solvents A, B, and C revealed a single spot having a mobility identical with that of authentic[4] **1**.

When **5** (1 g) was hydrogenolyzed in absolute ethanol (150 ml) with 10% palladium-on-carbon (1 g), the hydrogen uptake stopped after 1 h. Work-up as described above gave a syrup which showed, on paper chromatography in solvent B, three spots, the slowest of which was **1** (R_F 0.37). The faster moving of the other two components, R_F 0.52 and 0.62, was predominant.

Methyl-^{14}C D-*glucuronate* (**1**). — Methyl-^{14}C iodide (33.9 mg, 0.24 mmole, containing 0.5 mCi) was transferred, in a closed system, into a solution of **4** (0.145 g, 0.37 mmole) in *N*,*N*-dimethylformamide (0.6 ml). After 45 min at room temperature, the mixture was heated for 2 h at 75°. The solvent was distilled off, and the solution of the residue in chloroform (2 ml) was loaded on a column (1-cm diameter) of silica gel (10 g). The column was eluted with chloroform. Methyl-^{14}C **5** was isolated by removal of the solvent from the fractions eluted between 110–155 ml.

Labeled **5** was hydrogenated in absolute ethanol (100 ml) at atmospheric pressure over 10% palladium-on-carbon catalyst (150 mg). Work-up as described for unlabeled **1** afforded methyl-^{14}C **1**; radiochemical yield, 0.474 mCi (95%); t.l.c. (cellulose) in solvent B revealed a single radioactive spot corresponding to authentic[4] **1**.

Hydrolysis of unlabeled **1**. — Unlabeled **1** (34 mg) was heated with 0.05M sodium hydroxide (5 ml) for 2.5 h at 50°, and then freed of sodium ions by passage through a column of Dowex-50 (H^+). Concentration of the eluate to dryness yielded a syrup which was shown to consist of three components by paper chromatography in solvents A, B, and C. Similar chromatographic behavior was exhibited by D-glucuronolactone, after identical treatment. The product with the lowest mobility had the same R_F as D-glucuronic acid (**2**), whereas the fastest moving component had the same R_F as D-glucuronolactone. The third component, moving slightly ahead of **2**, was not identified, but it is likely to be D-mannuronic acid, D-fructuronic acid, or L-iduronic acid[15,16].

(1,2-endo, exo-phenyl)-*1,2*-O-*Benzylidene*-α-D-*glucofuranurono*-*6,3*-*lactones* (**10**). — To a mixture of **2** (19.4 g, 100 mmoles) and benzaldehyde (150 ml), which had been stirred for 24 h, was added anhydrous zinc chloride (20 g). After an additional 48 h of stirring, only a small amount of insoluble material remained. Ether (250 ml) was added, and the mixture was washed with water (4 × 250 ml). Ether was removed from the organic layer by evaporation under diminished pressure, and then benzaldehyde was co-distilled with water at 65°. The residual pale-yellow solid was suspended in water (100 ml), filtered off, and washed with petroleum ether to remove traces of benzaldehyde. The filtered solid was extracted with water (1350 ml, in small portions). Removal of water gave a solid (fraction A) which weighed 4.71 g (17.8%).

Fraction B (11.2 g, 42.5%) was obtained by washing the residue, remaining after extraction of fraction A with water, with ether (300 ml). T.l.c. (silica gel, solvent F): fraction A, R_F 0.33 (major) and 0.42 (major); fraction B, R_F 0.33 (major), 0.42(minor), and streaking near the origin. N.m.r. data (acetone): fraction A, τ 3.78 (doublet, $J_{1,2}$ *ca.* 4 Hz, H-1), 3.85 and 3.98 (*endo-* and *exo*-benzyl protons); fraction B, τ 3.80 (doublet, $J_{1,2}$ *ca.* 4 Hz, H-1), 3.88 (predominant) and 3.97 (small) (*endo-* and *exo*-benzyl protons), 4.07 (small, 3,5-benzylic proton of **3**).

Two recrystallizations of fraction A and one recrystallization of fraction B from butanone–petroleum ether gave the respective analytical samples of **10**; m.p. 125–127° (fraction A), 140–145° (fraction B); $[\alpha]_D$ +23.7° (fraction A, *c* 0.93, pyridine), +46.9° (fraction B, *c* 1.01, pyridine) (for specific rotations of the pure *endo-* and *exo*-phenyl isomers of **10**, see discussion); ν_{max}^{KBr} fraction A, 3470 and 3330 (OH), 1796 and 1780 (C=O), 768 and 702 (aromatic) cm^{-1}; fraction B, 3470 and 3320 (OH), 1810, 1792, 1778, and 1748 (C=O), 763 and 700 (aromatic) cm^{-1}. G.l.c. (200–250°, 6° per min) of trimethylsilyl ether **11** of **10** (retention times): fraction A, 5.2 min (*endo*-phenyl, 43%), 6.5 min (*exo*-phenyl, 57%); fraction B, 5.2 min (*endo*-phenyl, 9%), 6.5 min (*exo*-phenyl, 91%).

Anal. Calc. for $C_{13}H_{12}O_6$: C, 59.06; H, 4.59. Found (fraction A): C, 59.23; H, 4.62. Found (fraction B): C, 59.25; H, 4.50.

5-O-Acetyl-(1,2-endo, exo-phenyl)-*1,2-O-benzylidene-*α-D-*glucofuranurono-6,3-lactones* (**12**). — Fractions A and B were acetylated in the usual manner with pyridine and acetic anhydride; yield of syrupy **12**: from fraction A, 103% after chromatography on a column of silica gel (chloroform); from fraction B, 96%; ν_{max}^{Film} (both fractions): 1805 (lactone C=O), 1750 (Ac C=O), 1238–1220 (acetate C–O–C), 762 and 698 (aromatic) cm^{-1}; n.m.r. data (chloroform-*d*): fraction A, τ 3.84 (doublet, $J_{1,2}$ *ca.* 4 Hz, H-1), 3.98 (benzyl protons), 4.48 and 4.52 (two doublets, $J_{4,5}$ *ca.* 4 Hz, H-5 of *endo-* and *exo*-phenyl **12**), 7.78 and 7.81 (OAc proton singlets of *endo-* and *exo*-phenyl **12**); fraction B, τ 3.83 (H-1), 3.98 (benzyl proton), 4.48 (H-5), and 7.77 (OAc proton singlet). T.l.c. (silica gel, solvent E): fraction A, R_F 0.19 (major), 0.37 (trace); fraction B, R_F 0.17 (major), 0.34 (trace). G.l.c. (225–250°, 5° per min) (retention times): fraction A, 3.5 and 4.4 min (peak heights nearly equal); fraction B, 3.5 min (small) and 4.4 min (predominant).

5-O-Benzoyl-(1,2-endo, exo-phenyl)-*1,2-O-benzylidene-*α-D-*glucofuranurono-6,3-lactones* (**13**). — Fractions A and B were benzoylated in the usual manner with pyridine and benzoyl chloride to give the benzoates **13**; 60% (syrup) from fraction A, 72% (amorphous) from fraction B; ν_{max}^{Film} fraction A, 1810 (lactone C=O), 1725 Bz C=O), 1270 (benzoate C–O–C), 760, 712, and 700 (aromatic) cm^{-1}; fraction B, 1810 and 1790 (shoulder) (lactone C=O), 1725 (Bz C=O), 1275 (benzoate C–O–C), 760, 712, and 697 (aromatic) cm^{-1}. N.m.r. data (chloroform-*d*): fraction A, τ 3.82 (doublet, $J_{1,2}$ *ca.* 4 Hz, H-1), 3.97 (benzyl protons), and 4.25 (H-5); fraction B, τ 3.80 (H-1), 3.94 (benzyl proton), and 4.25 (doublet, $J_{4,5}$ *ca.* 4 Hz, H-5). T.l.c. (silica gel, solvent E): fraction A, R_F 0.20; fraction B, R_F 0.19 (major) and 0.05 (trace).

Methyl 3,5-di-O-acetyl-(1,2-endo, exo-phenyl)*-1,2-O-benzylidene-α-*D*-gluco-furanuronates* (**23**). — The lactone **10** (from fraction A, 0.141 g) was converted into the sodium salt **20** by use of the procedure described for the preparation of **4**; yield of pale-yellow solid, 0.153 g (100%); ν_{max}^{KBr} 1780 (small, lactone C=O of unreacted **10**), 1600 (COO^-), 760 and 695 cm^{-1} (aromatic).

Methylation of **20** was accomplished with methyl iodide in *N,N*-dimethylformamide as described for **5**. G.l.c. (200–250°, 6° per min) of the trimethylsilyl-ether **22** prepared from the crude mixture gave two predominant peaks (retention times, 6.0 and 7.7 min) corresponding to two isomers of **21**, in addition to two minor peaks due to **11**. Integration of the areas under the peaks suggested that **22** and **11** were present in *ca.* 3:1 ratio.

The crude mixture containing **21** was acetylated, in the usual manner, to give (after chromatography on a silica gel column) syrupy **23** (0.135 g, 71%); ν_{max}^{Film} 1810 (Me ester C=O), 1750 (Ac C=O), 1235–1220 (acetate C–O–C), 763 and 700 (aromatic) cm^{-1}; n.m.r. data (chloroform-*d*): τ 6.17 and 6.19 (OMe proton singlets of *endo-* and *exo-*phenyl **23**), 7.76 and 7.78 (OAc proton singlets of the two isomers of **12**), 7.88 and 7.93 (OAc proton singlets of the two isomers of **23**); t.l.c. (silica gel, solvent E): R_F 0.15 (major) and 0.34 (trace); g.l.c. (225–250°, 5° per min) showed two major peaks (retention times, 4.4 and 5.9 min) for the *endo-* and *exo-*phenyl isomers of **23** and a minor peak for **12** at 3.5 min. The second peak of **12**, if present, was superimposed on the first peak of **23** at 4.4 min. The ratio of **23** to **12** was *ca.* 3:1.

D-*Glucose and* D-*xylose from* **10**. — Monobenzylidene lactone (**10**, from fraction A) was reduced with lithium aluminum hydride in tetrahydrofuran as described for **6** to give syrupy **14** (85%) which crystallized on standing; ν_{max}^{KBr} 3400 (OH), 760 and 695 cm^{-1} (aromatic); g.l.c. (200–275°, 6° per min) of the trimethylsilyl ether **15** of **14** gave two major peaks (retention times, 6.0 and 7.4 min). The trimethylsilyl ether **15**, similarly prepared from fraction B, displayed two peaks having identical retention times (second peak predominant), in addition to a minor peak for **7**. The acetate **16** from fraction B also gave a minor peak for **8** in addition to the two peaks (retention times, 5.9 and 7.5 min, second peak predominant) of **16** (g.l.c., 225–275°, 5° per min).

Hydrolysis of **14** (from fraction A) with 0.05M sulfuric acid (5 h, 65°) followed by neutralization with barium carbonate gave a product, which was characterized as glucose by paper chromatography (solvent D) and paper electrophoresis[26] (Whatman paper No. 1, 1300 volts, 90 min, 0.1M sodium hydroxide).

To a solution of **14** (159 mg, from fraction A) in a mixture of water (20 ml) and tetrahydrofuran (10 ml) was added sodium metaperiodate (136 mg). After 4.5 h, the solution was evaporated to dryness, and the residue was extracted with tetrahydrofuran. The pale-yellow syrup, obtained after removal of the solvent, was dried by evaporation with benzene, and then reduced with lithium aluminum hydride in tetrahydrofuran, as described for **6**; yield of **17** (partially dry, yellow syrup), 181 mg; in g.l.c. (200–250°, 6° per min) the trimethylsilyl ether **18** of **17** gave two major peaks (retention times, 2.1 and 2.8 min).

Hydrolysis of **17** with 0.05M sulfuric acid gave a product which was characterized

as xylose by paper chromatography and paper electrophoresis, as described above for glucose.

Methylation with diazomethane. — Fractions A and B were methylated with excess diazomethane in anhydrous ether, as described for **5**, and the products were examined by g.l.c. (200–275°, 5° per min) after conversion to trimethylsilyl derivatives; fraction A: two major peaks of **11**, two minor peaks of **22**; fraction B: two peaks of **11** (that having a longer retention time being predominant), two minor peaks of **22**, and a minor peak due to **5**.

This indicated the presence of trace amounts of **19** in both fractions and of a small amount of **3** in fraction B.

ACKNOWLEDGMENTS

This work was supported by a grant (GM-12422) from the National Institute of General Medical Sciences, National Institutes of Health, United States Public Health Service. The author thanks Prof. F. A. Loewus for his interest and for his suggestions in the preparation of this manuscript, Prof. A. J. Solo for his helpful suggestions in the interpretation of some of the n.m.r. spectra, and Miss Anne Golebiewski for able technical assistance.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

HYDROGENOLYSIS OF CYCLIC AND ACYCLIC ORTHOESTERS OF CARBOHYDRATES WITH LITHIUM ALUMINUM HYDRIDE–ALUMINUM TRICHLORIDE*

S. S. BHATTACHARJEE** AND P. A. J. GORIN

National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan (Canada)

(Received May 20th, 1969; in revised form, June 24th, 1969)

ABSTRACT

Several cyclic and acyclic carbohydrate orthoesters have been hydrogenolyzed with 1:1 lithium aluminum hydride–aluminum trichloride, and the mechanisms of the reaction have been studied. Generally, cyclic orthoesters are cleaved *via* cyclic carbonium-ion intermediates that are stereospecifically attacked by the reducing species, giving cyclic acetals in good yield. One example is the cleavage of both diastereoisomers of 1,2-*O*-(1-methoxyethylidene)-3,4,6-tri-*O*-methyl-β-D-mannopyranose to 1,2-*O*-ethylidene-3,4,6-tri-*O*-methyl-β-D-mannopyranose having an *exo* *O*-ethylidene proton. Cleavage of carbohydrate acyclic orthoesters with the reagent is less specific. Acyclic methyl orthoesters were formed simultaneously by the action of methyl orthoformate and a trace of acid on 1,2:3,5-di-*O*-methylene-α-D-glucofuranose (**20**). After removal of the contaminating formic ester of **20**, the remainder was hydrogenolyzed with the reagent to **20** and the two methylene acyclic acetals **24** and **25** in the ratios of 5:10:3 (w/w/w).

INTRODUCTION

Eliel[1–3] and Brown[4–11] and their co-workers have investigated the reductive cleavage, with lithium aluminum hydride–aluminum trichloride, of 1,3-dioxolanes, 1,3-dioxanes, 1,3-oxathiolanes, and *O*-tetrahydropyran-2-yl and *O*-tetrahydrofuran-2-yl derivatives to ethers. We have extended these studies to carbohydrate derivatives, and have shown that the reagent has utility in the synthesis of *O*-benzyl and *O*-methyl derivatives from cyclic *O*-benzylidene and *O*-methylene acetals, respectively[12,13]. It was also found that cyclic orthoesters of sugars can be cleaved to cyclic acetals, and that the mechanism of cleavage of this type of derivative was of interest[12]. 1,2-*O*-Isopropylidene-3,5,6-*O*-orthoformyl-α-D-glucofuranose was converted by $LiAlH_4$–$AlCl_3$ (1:1) into 1,2-*O*-isopropylidene-3,5-*O*-methylene-α-D-glucofuranose; and, when $LiAlD_4$–$AlCl_3$ (1:1) was used, specific labeling with deuterium at C-2

*Issued as NRCC No. 10975.
**N.R.C. Postdoctorate Fellow, 1967–1969.

took place in the 1,3-dioxane ring. On changing the ratio of $LiAlD_4$ to $AlCl_3$ to 1:3, a small proportion of 1,2-*O*-isopropylidene-5,6-*O*-methylene-α-D-glucofuranose was formed, and this was also specifically labeled with deuterium at C-2 of the 1,3-dioxolane ring. Only one stereoisomer was formed on conversion of 1,2-*O*-isopropylidene-3,5,6-*O*-orthoacetyl-α-D-glucofuranose into 3,5-*O*-ethylidene-1,2-*O*-isopropylidene-α-D-glucofuranose; but, as this is the thermodynamically stable isomer, it is not known with certainty whether it was formed directly or arose under the reaction conditions from the thermodynamically unstable isomer. Because only one of two possible diastereoisomers was formed in each of these reactions, it was decided to conduct a systematic investigation of the hydrogenolysis products of cyclic and acyclic orthoesters, from both a synthetic and a mechanistic point of view.

RESULTS AND DISCUSSION

The orthoesters hydrogenolyzed to acetals with $LiAlH_4$–$AlCl_3$ were as follows: (*a*) 5-membered cyclic orthoesters that were the diastereoisomers of 1,2-*O*-(1-methoxyethylidene)-3,4,6-tri-*O*-methyl-β-D-mannopyranose (**1** and **2**), 1,2-*O*-(methoxybenzylidene)-3,5-di-*O*-methyl-β-D-arabinofuranose (**5**), 3-*O*-methyl-1,2,5-*O*-orthobenzoyl-β-D-arabinofuranose (**8**), mixed 2,3:5,6-di-*O*-(methoxymethylene)-D-mannose isomers, and mixed methyl 2,3:5,6-di-*O*-(methoxymethylene)-α-D-mannofuranoside isomers (**15**); (*b*) compounds containing a 6-membered, cyclic orthoester: the isomeric methyl 2,3:4,6-di-*O*-(methoxymethylene)-α-D-mannopyranosides; and (*c*) acyclic orthoesters obtained by treating 1,2:3,5-di-*O*-methylene-α-D-glucofuranose (**20**) with methyl orthoformate–hydrogen chloride.

The diastereoisomer of the 1,2-(1-methoxyethylidene) acetal of 3,4,6-tri-*O*-acetyl-β-D-mannopyranose, having an *exo* methoxyl group[14], was considered as a substrate. Since treatment with lithium aluminum hydride in the initial stages of the hydrogenolysis reaction would cause deacetylation and formation of a difficultly soluble intermediate, the hydroxyl groups were protected by conversion into the 3,4,6-trimethyl ether (**1**) by successive deacetylation and *O*-methylation. Hydrogenolysis of the trimethyl ether with $LiAlH_4$–$AlCl_3$ gave one diastereoisomer of 1,2-*O*-ethylidene-3,4,6-tri-*O*-methyl-β-D-mannopyranose (**4**), as shown by proton magnetic resonance (p.m.r.) spectroscopy; this compound has a C–CH_3 signal at τ 8.51, compared with τ 8.65 for its diastereoisomer (20% of the mixture) formed on equilibration with $CDCl_3$–HCl. By analogy with the findings of Perlin[15] on the relative C–CH_3 chemical shifts of the diastereoisomers of 3,4,6-tri-*O*-acetyl-1,2-*O*-(methoxyethylidene)-β-D-mannopyranose, the hydrogenolysis product should contain an *endo* C–CH_3 group.

Hydrogenolysis of a diastereoisomeric mixture of the 1,2-*O*-(1-methoxyethylidene)-3,4,6-tri-*O*-methyl-β-D-mannopyranoses (**1** and **2**) (prepared from mixed 3,4,6-tri-*O*-acetyl-1,2-*O*-(1-methoxyethylidene)-β-D-mannopyranoses[16] gave the same 1,2-*O*-ethylidene-3,4,6-tri-*O*-methyl-β-D-mannopyranose (**4**), having an *endo* C–CH_3, as was obtained from the pure orthoacetate. This is significant, since it means that

both reactions have a common carbonium-ion intermediate (3) which is attacked by the hydrogen of the reducing species to form a 1,2-*O*-ethylidene group having an *exo*-hydrogen atom. The hydrogenolysis reaction bears a similarity to that reported by Buchanan and Edgar[17], who prepared fluoroborate salts by treatment of a mixture of the methyl β-L-arabinopyranoside 3,4-(ethyl orthoacetates) with boron trifluoride, and then reduced this mixture with lithium borohydride to the diastereoisomeric methyl 3,4-*O*-ethylidene-β-L-arabinopyranosides. The ratio of the isomers containing the *endo* and *exo* C–CH_3 groups was 9:1, and it is possible that some acetal equilibration had occurred.

A series of reactions similar to that described for the β-D-mannopyranose series was conducted with two β-D-arabinofuranose derivatives. 2,3,5-Tri-*O*-benzoyl-1,2-*O*-(methoxybenzylidene)-β-D-arabinofuranose has an *exo*-OCH_3 group, as it is prepared by the action of methanol on 2,3,5-tri-*O*-benzoyl-α-D-arabinofuranosyl bromide in the presence of 2,6-lutidine[18], a reaction that has been used by Mazurek and Perlin[14] to prepare the *exo* OCH_3 isomer of 3,4,6-tri-*O*-acetyl-1,2-*O*-(1-methoxyethylidene)-β-D-mannopyranose from 3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl bromide. The β-D-arabinofuranose derivative was converted into 1,2-*O*-(methoxybenzylidene)-3,5-di-*O*-methyl-β-D-arabinofuranose (5), which was cleaved with $LiAlH_4$–$AlCl_3$ to a single diastereoisomer of 1,2-*O*-benzylidene-3,5-di-*O*-methyl-β-D-arabinofuranose (7). The stereochemistry is uncertain on the basis of the chemical shift of its benzylic proton (τ 3.98), which is virtually the same as that of its stereoisomer

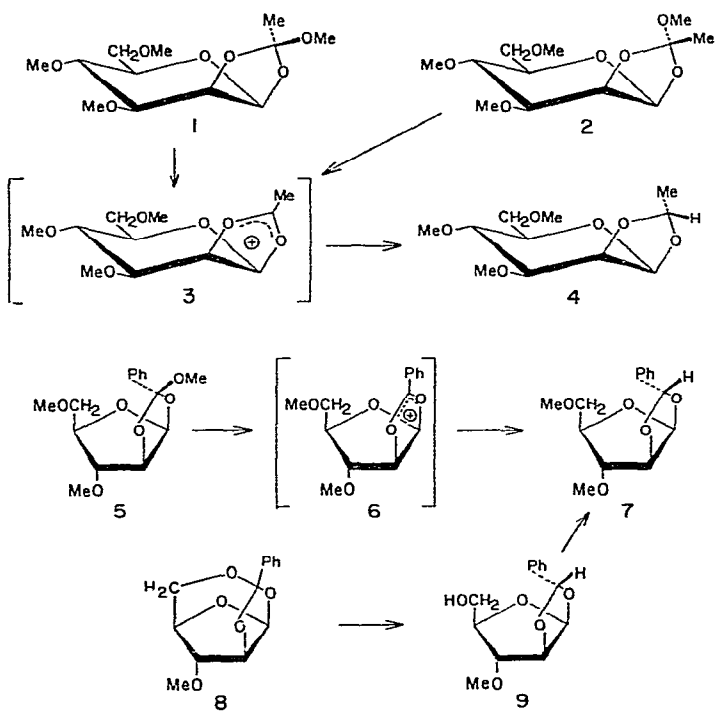
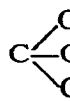


Fig. 1. Mechanism of cleavage of cyclic orthoesters with $LiAlH_4$–$AlCl_3$.

(< 35% of the mixture), formed on $CDCl_3$–HCl equilibration. However, it seems likely that the benzylidene proton is *exo*, as the intermediate carbonium-ion (**6**) would be more readily approached by the reducing agent from this side. As the 1,2-*O*-(methoxybenzylidene)-3,5-di-*O*-methyl-β-D-arabinofuranose having an *endo* OCH_3 orthoester group was not available as a hydrogenolysis substrate, a stereochemically analogous compound, namely, 3-*O*-methyl-1,2,5-orthobenzoyl-β-D-arabinofuranose (**8**) was used; this was prepared by methylation of 1,2,5-*O*-orthobenzoyl-β-D-arabinofuranose[18]. Hydrogenolysis of **8** with $LiAlH_4$–$AlCl_3$ gave 1,2-*O*-benzylidene-3-*O*-methyl-β-D-arabinofuranose (**9**), which was converted by methylation into 1,2-*O*-benzylidene-3,5-di-*O*-methyl-β-D-arabinofuranose (**7**), identical (by p.m.r. spectroscopy) with the compound prepared by hydrogenolysis of 1,2-*O*-(methoxybenzylidene)-3,5-di-*O*-methyl-β-D-arabinofuranose (**5**).

A noticeable feature of the above series of reactions is the selective, hydrogenolytic cleavage of the C-2–oxygen bonds that are external to the 1,3-dioxolane rings; this behavior is presumably due to the lower free-energies of the five-membered, cyclic carbonium-ions as compared with other possibilities. Two other examples of transformation of the 5-membered, cyclic orthoester to a 5-membered, cyclic acetal were noted. Firstly, D-mannose was treated with methyl orthoformate–HCl to give a crude orthoester mixture from which a di-*O*-(methoxymethylene)-D-mannose derivative was isolated; it was substituted in the 2,3:5,6-positions, because spin-decoupling experiments showed that it contained a free hydroxyl group on C-1. Hydrogenolysis gave 2,3:5,6-di-*O*-methylene-D-mannose (**12**) containing a trace of 2,3:5,6-di-*O*-methylene-D-mannitol (**13**) (combined yield, 40%). Secondly, methyl α-D-mannofuranoside (**14**) was dissolved in methyl orthoformate–HCl to give a syrup from which a crystalline stereoisomer of methyl 2,3:5,6-di-*O*-(methoxymethylene)-α-D-mannofuranoside (**15**) was isolated. The crude orthoester mixture was hydrogenolyzed to syrupy methyl 2,3:5,6-di-*O*-methylene-α-D-mannofuranoside (**16**) in 28% yield. (The product was identical with the compound formed by the action of sodium hydride and dichloromethane on methyl α-D-mannofuranoside in *N*,*N*-dimethylformamide.) In these two hydrogenolyses, there is a tendency for preponderance of the cyclic, carbonium-ion intermediate, as the yields are higher than 11% (the yield that would be obtained were the cleavage of the  bonds of the orthoesters random).

A lower yield of *O*-methylene acetal was obtained on hydrogenolysis of a 6-membered *O*-(methoxymethylene) ring. Methyl α-D-mannopyranoside (**10**) was converted into orthoester derivatives. Hydrogenolysis of the crude product gave methyl 2,3:4,6-di-*O*-methylene-α-D-mannopyranoside (**11**) in 17% yield. In each of these three examples, the yield of sugar orthoester is low, due to incomplete substitution of the available hydroxyl groups by action of methyl orthoformate–HCl and, probably, also, the formation of formic esters (see later).

Although cyclic methylene acetals are relatively stable to hydrogenolysis conditions, they can undergo further cleavage[4,12,13]. Prolonged hydrogenolysis of

methyl 2,3:5,6-di-*O*-methylene-α-D-mannofuranoside (**16**) gave a mixture from which syrupy methyl 6-*O*-methyl-2,3-*O*-methylene-α-D-mannofuranoside (**17**; 9% yield) and crystalline methyl 5-*O*-methyl-2,3-*O*-methylene-α-D-mannofuranoside (**18**; 4.5% yield) were isolated. A further fraction (12% yield) was treated with sodium periodate to give periodate-resistant methyl 2,6-di-*O*-methyl-α-D-mannofuranoside (**19**), identified by conversion with methanolic hydrogen chloride into the corresponding pyranoside derivative, the g.l.c. characteristics of which are known[13]. The periodate-oxidized material is probably a polyhydric alcohol containing hydroxyl groups on C-4 and C-5; this could arise *via* cleavage of the C-1 to furanose-ring-oxygen bond of one of the partially hydrogenolyzed products, such as methyl 2,6-di-*O*-methyl-α-D-mannofuranoside or methyl 6-*O*-methyl-2,3-*O*-methylene-α-D-mannofuranoside.

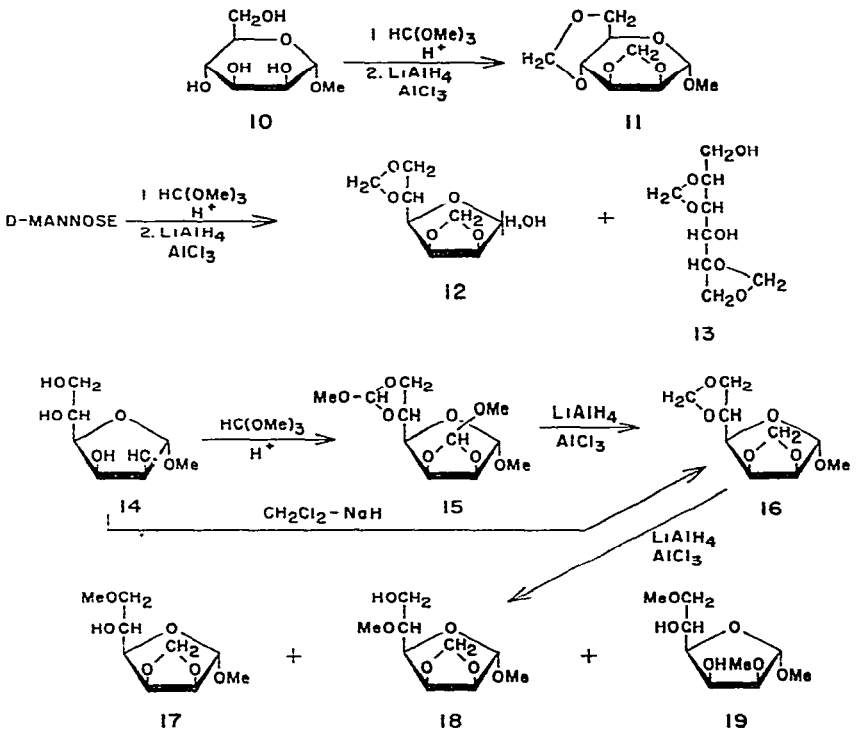


Fig. 2. Conversion of D-mannose derivatives into cyclic methylene acetals by successive treatment with methyl orthoformate–HCl and $LiAlH_4$–$AlCl_3$.

Acyclic orthoformic esters were prepared by treating 1,2:3,5-di-*O*-methylene-α-D-glucofuranose (**20**) with methyl orthoformate in the presence of a trace of acid. The product contained some formic ester, as it showed infrared absorption at 1720 cm^{-1}; it was treated with methanolic sodium methoxide, and the resulting 1,2:3,5-di-*O*-methylene-α-D-glucofuranose was removed by repeatedly washing a chloroform solution of the product with water. The resulting orthoesters were

hydrogenolyzed with $LiAlH_4$–$AlCl_3$, and the product was fractionated by column chromatography on silicic acid. 6-*O*-(Methoxymethyl)-1,2:3,5-di-*O*-methylene-α-D-glucofuranose (**24**) was eluted initially (25% yield), and was identified by its p.m.r. spectrum. The next fraction (7.5% yield) contained a crystalline compound which had a molecular weight, carbon and hydrogen analysis, and p.m.r. spectrum corresponding to 6,6'-*O*-methylenebis(1,2:3,5-di-*O*-methylene-α-D-glucofuranose) (**25**). 1,2:3,5-Di-*O*-methylene-α-D-glucofuranose (**20**) was also isolated. The ratio (w/w/w) of 6-*O*-(methoxymethyl)-1,2:3,5-di-*O*-methylene-α-D-glucofuranose (**24**), a product of high molecular weight (**25**), and 1,2:3,5-di-*O*-methylene-α-D-glucofuranose (**20**) isolated from the column was 10:3:5. Since the unresolvable mixture of orthoesters obtained by treatment of 1,2:3,5-di-*O*-methylene α-D-glucofuranose could contain **21**, **22**, and **23**, little can be deduced on the pathway of hydrogenolysis of the acyclic orthoester. For example, **21** or **22** can give rise to **24**, and **25** could be formed from either **21** or **23**. However, hydrogenolysis proceeds only to acyclic *O*-methylene derivatives, and further conversion into methyl ethers does not occur; this behavior is also a characteristic of cyclic *O*-methylene acetals[12].

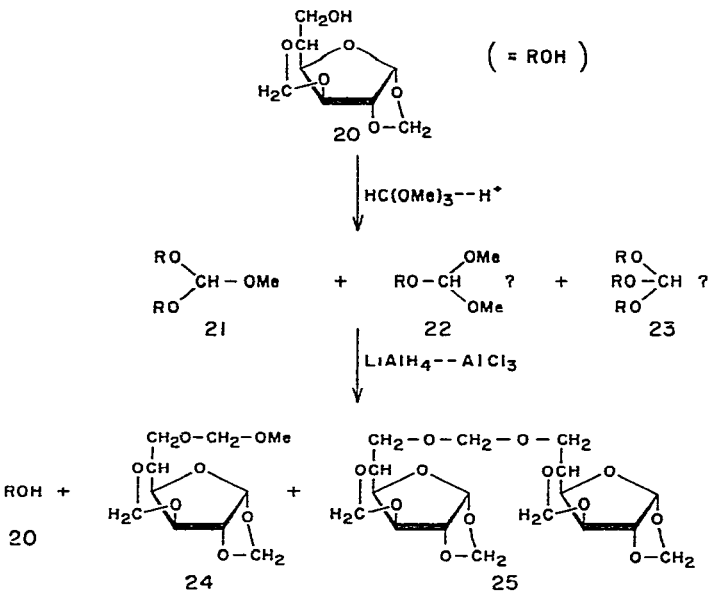
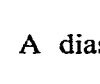
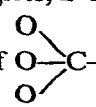


Fig. 3. Cleavage of acyclic orthoesters of 1,2:3,5-di-*O*-methylene-α-D-glucofuranose with $LiAlH_4$–$AlCl_3$.

EXPERIMENTAL

P.m.r. spectra were recorded with a Varian 100-MHz spectrometer, with tetramethylsilane as the internal standard; chemical shifts are recorded as τ values. Hydrogenolyses with lithium aluminum hydride–aluminum trichloride were conducted with a 1:1 molar ratio of the two reagents by the procedure described previously[12],

except that the reaction mixture was decomposed by means of cold, dilute, aqueous potassium carbonate solution. G.l.c. was performed with a column (0.25 in × 12 ft) of 2% neopentyl glycol succinate on Chromosorb W, at 190° and a helium pressure of 36 lb.in^{-2}. Evaporations were performed under diminished pressure. I.r. spectra were recorded with a Perkin–Elmer Model 21 spectrometer. Paper chromatography was conducted on Whatman No. 1 filter paper with 40:11:19 (v/v) butyl alcohol–ethanol–water. T.l.c. was performed on plates of Silica Gel G with detection by use of a 50% sulfuric acid spray, and charring.

Hydrogenolysis of 1,2-O-(1-methoxyethylidene)-3,4,6-tri-O-methyl-β-D-mannopyranoses. — 3,4,6-Tri-*O*-acetyl-1,2-*O*-(1-methoxyethylidene)-*β*-D-mannopyranose (200 mg), containing an *exo* OCH_3 group[14], was deacetylated with sodium methoxide in methanol, and the product was methylated with Purdie's reagents to give 1,2-*O*-(1-methoxyethylidene)-3,4,6-tri-*O*-methyl-*β*-D-mannopyranose[19] (**1**) (150 mg); $[\alpha]_D^{25}$ −1.4° (*c* 0.5, ethanol) and p.m.r. signals ($CDCl_3$) at τ 4.63 (doublet, *J* 2.5 Hz, anomeric H), τ 6.44, 6.47, 6.62, 6.70 (4 singlets, 4 OMe), τ 8.30 (singlet, CH_3 of ). A diastereoisomeric mixture[16] of tri-*O*-acetyl-*β*-D-mannopyranose 1,2-(methyl orthoacetates) consisting of 55% of the *exo* and 45% of the *endo* OCH_3 compound was similarly converted into the trimethyl ethers[19] (**1** and **2**), $[\alpha]_D^{25}$ −8° (*c* 1.0, ethanol); p m.r. signals ($CDCl_3$) at τ 4.63 (doublet, *J* 2.5 Hz, anomeric H), τ 4.86 (doublet, *J* 2.5 Hz, anomeric H), τ 6.44 (2 superimposed singlets, 2 OCH_3), τ 6.46 (2 superimposed singlets, 2 OCH_3), τ 6.57, 6.62, 6.65, 6.70 (4 singlets, 4 OCH_3), τ 8.30, 8.48 (2 singlets, 2 of ).

Each of the two preparations of trimethyl ethers was hydrogenolyzed for 45 min with 1.1 molar equivalents of $LiAlH_4$–$AlCl_3$, giving products in 65–75% yields. In each case, one stereoisomer of 1,2-*O*-ethylidene-3,4,6-tri-*O*-methyl-*β*-D-mannopyranose (**4**) was obtained, and this could be separated from other, minor components (20–25%) by chromatography on silicic acid with elution with 8:1 (v/v) chloroform–acetone. The product gave p.m.r. signals ($CDCl_3$) at τ 4.74 (quartet, *J* 5 Hz, CH of *O*-ethylidene), τ 4.84 (doublet, *J* 2.5 Hz, anomeric H), τ 6.46, 6.48, 6.63 (3 singlets, 3 OCH_3), and τ 8.51 (doublet, *J* 5 Hz, CH_3 of *O*-ethylidene). The two products had the same g.l.c. retention-time, and $[\alpha]_D^{25}$ +4° (*c* 0.5, ethanol).

Equilibration of **4** with 0.1% of hydrogen chloride in $CDCl_3$ gave a diastereoisomeric mixture. The new diastereoisomer was detected by the p.m.r. signal of its —CCH_3 group (20% of the total C—CH_3 signals; doublet at τ 8.65).

Hydrogenolysis of 1,2-O-(methoxybenzylidene)-3,5-di-O-methyl-β-D-arabinofuranose (endo *Ph group*) (**5**) *and 3-O-methyl-β-D-arabinofuranose 1,2,5-orthobenzoate* (**8**). — 2,3,5-Tri-*O*-benzoyl-*α*-D-arabinofuranosyl bromide was treated with methanol in the presence of 2,6-lutidine, and the product was debenzoylated to give 1,2-*O*-(methoxybenzylidene)-*β*-D-arabinofuranose having an *exo* OCH_3 group[18]. This

compound was methylated with Purdie's reagents, and the resulting 1,2-*O*-(methoxybenzylidene)-3,5-di-*O*-methyl-β-D-arabinofuranose (**5**) had $[\alpha]_D^{25} - 10°$ (*c* 0.5, chloroform), and p.m.r. signals (in CCl_4) at τ 2.40–2.80 (multiplet, 15 aromatic protons), τ 4.01 (doublet, *J* 4 Hz, anomeric H), τ 6.68, 6.94, 6.96 (three singlets, three OCH_3).

Hydrogenolysis of **5** (300 mg) with 1 molar equivalent of $LiAlH_4$–$AlCl_3$ for 45 min gave a syrup (210 mg) which had a p.m.r. spectrum containing one *O*-benzylidene proton (at τ 3.98) of 1,2-*O*-benzylidene-3,5-di-*O*-methyl-β-D-arabinofuranose (**7**). Slower-moving, minor contaminants, detectable by t.l.c. with 2:1 (v/v) chloroform–acetone were removed by chromatography on silicic acid with 2:1 (v/v) chloroform–acetone. The product had $[\alpha]_D^{25}$ +7° (*c* 1.0, ethanol). The original reaction-product and the purified product gave the same p.m.r. signals ($CDCl_3$); τ 1.90–2.80 (multiplet, 15 aromatic protons), τ 3.98 (singlet, *O*-benzylidene proton), τ 4.06 (doublet, *J* 4 Hz, anomeric H), τ 6.56, 6.58 (two singlets, 2 OCH_3).

Methylation of 1,2,5-*O*-orthobenzoyl-β-D-arabinofuranose[18] with Purdie's reagents gave the 3-methyl ether (**8**) which (from ether) had m.p. 126–128° and $[\alpha]_D^{25}$ −26.4° (*c* 0.5, ethanol).

Anal. Calc. for $C_{13}H_{14}O_5$: C, 62.39; H, 5.64. Found: C, 62.14; H, 5.78.

It gave p.m.r. signals ($CDCl_3$) at τ 2.3–2.75 (multiplet, 15 aromatic H), τ 3.96 (doublet, *J* 4 Hz, anomeric H), τ 6.58 (singlet, OCH_3). Hydrogenolysis of the 3-methyl ether **8** (100 mg) with 1 molar equivalent of $LiAlH_4$–$AlCl_3$, as already described, gave syrupy 1,2-*O*-benzylidene-3-*O*-methyl-β-D-arabinofuranose (**9**) (75 mg), which was homogeneous on t.l.c. with 8:1 (v/v) chloroform–acetone. The compound showed p.m.r. signals (in methyl sulfoxide-d_6) at τ 2.15–2.40 (multiplet, 15 aromatic protons), τ 3.67 (singlet, *O*-benzylidene proton), τ 3.71 (doublet, anomeric H), τ 4.70 (triplet, primary hydroxyl), and τ 6.30 (singlet, OCH_3). Methylation of **9** with Purdie's reagents gave 1,2-*O*-benzylidene-3,5-di-*O*-methyl-β-D-arabinofuranose (**7**) with a p.m.r. spectrum and $[\alpha]_D^{25}$ [+7° (*c* 0.5, ethanol)] identical with those of the known compound.

Synthesis of O-*methylene*-D-*mannose derivatives* via *cyclic orthoesters.* — *A. Methyl 2,3:4,6-di-*O-*methylene-*α-D-*mannopyranoside* (**11**). A mixture of methyl α-D-mannopyranoside (**10**) (2 g), dry *N,N*-dimethylformamide (2 ml), methyl orthoformate (3 ml), and 4 drops of 3% methanolic hydrogen chloride was shaken for 40 min. Chloroform (20 ml) and anhydrous potassium carbonate were added, and the mixture was shaken and filtered. The filtrate was evaporated to a syrup (600 mg), which was treated with 2.5 molar equivalents of $LiAlH_4$–$AlCl_3$ for 1 h. The crude reaction product (400 mg) was chromatographed on a column of silicic acid with 2:1 (v/v) chloroform–acetone as the eluant. The initial fraction (100 mg) crystallized from methanol; it had m.p. 138°, and was identical by p.m.r. spectrum, g.l.c. retention-time, and mixed m.p. with methyl 2,3:4,6-di-*O*-methylene-α-D-mannopyranoside[12] (**11**).

*B. 2,3:5,6-Di-*O-*methylene-*D-*mannose* (**12**) *and 2,3:5,6-di-*O-*methylene-*D-*mannitol* (**13**). D-Mannose (2 g) was added to dry *N,N*-dimethylformamide (2 ml), methyl orthoformate (3 ml), *p*-toluenesulfonic acid (20 mg), and Drierite (1 g), and the

mixture was shaken for 4 h (until complete dissolution of the sugar had occurred). Chloroform (25 ml) and anhydrous potassium carbonate were added, and the mixture was shaken for a few minutes, filtered, and the filtrate evaporated to dryness in the presence of a trace of potassium carbonate. The product was treated with sodium methoxide in methanol (to remove any ester groups that might have been present). Extraction of an aqueous solution of the product with ether, followed by evaporation, gave a syrup, a portion of which crystallized from chloroform–hexane (20% yield). The resulting 2,3:5,6-di-*O*-(methoxymethylene)-D-mannose had m.p. 107–109° and $[\alpha]_D^{25}$ +27.7° (*c* 1.3, ethanol).

Anal. Calc. for $C_{10}H_{16}O_8$: C, 45.45; H, 6.10. Found: C, 44.98; H, 5.78.

P.m.r. signals ($CDCl_3$): τ 4.24, 4.26 (2 singlets, 2 orthoester protons), τ 4.61 (doublet, *J* 2 Hz, anomeric H), τ 6.70, 6.73 (2 singlets, 2 OCH_3), τ 7.01 (doublet, *J* 2 Hz, secondary OH). A spin-decoupling experiment, with H-1 as the reference proton, showed that the free hydroxyl group was on C-1.

Hydrogenolysis of the crystalline derivative (100 mg) with 2.5 molar equivalents of $LiAlH_4$–$AlCl_3$ gave essentially one product (75 mg), as shown by t.l.c. After purification of the mixture by column chromatography on silicic acid with 2:1 (v/v) chloroform–acetone, the p.m.r. spectrum of the purified material (50 mg) corresponded to that for a 2,3:5,6-di-*O*-methylenemannose (such as **12**) contaminated by approximately 20% of a 2,3:5,6-di-*O*-methylenemannitol (such as **13**). It reduced Fehling solution, and, on hydrolysis with 0.5M sulfuric acid for 15 h at 100°, a mannose with a trace of a mannitol could be detected by paper chromatography.

*C. Methyl 2,3:5,6-di-*O*-methylene-α-D-mannofuranoside* (**16**). Methyl α-D-mannofuranoside (**14**) (500 mg) was shaken with methyl orthoformate (2.5 ml) containing 2–3 drops of 3% methanolic hydrogen chloride for 15 min. Benzene (5 ml) was then added, and the resulting solution was shaken for 15 min, diluted with benzene, stirred with anhydrous potassium carbonate, and filtered. The filtrate was evaporated to a syrup (600 mg) which partly (~30% of the total) crystallized from chloroform–hexane. The product (**15**) had m.p. 104–107°, $[\alpha]_D^{25}$ +81° (*c* 1.0, ethanol).

Anal. Calc. for $C_{11}H_{18}O_8$: C, 47.48; H, 6.52. Found: C, 47.78; H, 6.71.

The crude, syrupy mixture of orthoesters (500 mg) was hydrogenolyzed with 2.5 molar equivalents of $LiAlH_4$–$AlCl_3$ for 1 h, and the product was purified by column chromatography on silicic acid with 2:1 (v/v) chloroform–acetone. Methyl 2,3:5,6-di-*O*-methylene-α-D-mannofuranoside (**16**) (140 mg) was eluted initially, and it constituted ~50% of the product; it was homogeneous by t.l.c. and g.l.c.; p.m.r. signals ($CDCl_3$) at τ 4.96, τ 5.02, τ 5.08, τ 5.11, and τ 5.14 (5 singlets for 5 protons, anomeric H and 4 *O*-methylene protons), τ 6.70 (singlet, OCH_3). It had $[\alpha]_D^{25}$ +78° (*c* 1.3, chloroform), and was identical by g.l.c., p.m.r. spectroscopy, and $[\alpha]_D^{25}$ with material synthesized by the following procedure.

Methyl α-D-mannofuranoside (**14**) (1 g) was dissolved in dry *N*,*N*-dimethylformamide (10 ml), and sodium hydride (0.5 g) was slowly added, followed[20] by dichloromethane (1 ml). After the mixture had been shaken for 4 days, the same amounts of sodium hydride and dichloromethane were added, and the reaction was

continued for a further 2 days. Methanol was slowly added and, after 1 h, water was carefully added. The mixture was extracted with chloroform, and fractionation of the extract by column chromatography on silicic acid with 2:1 (v/v) chloroform–acetone afforded methyl 2,3:5,6-di-*O*-methylene-α-D-mannofuranoside (**16**) (330 mg) as the initial fraction.

*Hydrogenolysis of methyl 2,3:5,6-di-*O*-methylene-α-D-mannofuranoside* (**16**). — Methyl 2,3:5,6-di-*O*-methylene-α-D-mannofuranoside (**16**) (1 g) was treated with 3 molar equivalents of $LiAlH_4$–$AlCl_3$ for 8 days in the usual way. Column chromatography of the crude product on silicic acid with 2:1 (v/v) chloroform–acetone gave the following fractions.

Fraction A (600 mg) was identified as unreacted starting-material.

Fraction B (90 mg) had $[\alpha]_D^{25}$ +92° (*c* 0.5, ethanol) and contained methyl 6-*O*-methyl-2,3-*O*-methylene-α-D-mannofuranoside (**17**); p.m.r. signals ($CDCl_3$) at τ 5.02–5.08 (2 peaks, 3 protons, anomeric H and 2 *O*-methylene protons), τ 6.60, 6.72 (2 singlets, 2 OCH_3), and τ 7.16 (doublet, one secondary hydroxyl group). Spin-decoupling experiments established the positions of all of the ring protons, and also showed that the hydroxyl group was on C-5.

Fraction C (45 mg) was identified as methyl 5-*O*-methyl-2,3-*O*-methylene-α-D-mannofuranoside (**18**), which crystallized from chloroform–hexane and had m.p. 70–72° and $[\alpha]_D^{25}$ +98° (*c* 1.0, ethanol).

Anal. Calc. for $C_9H_{16}O_6$: C, 49.08; H, 7.32. Found: C, 49.34; H, 7.25.

It showed p.m.r. signals ($CDCl_3$) at τ 5.06–5.11 (3 peaks, 3 protons, anomeric H and two *O*-methylene protons), τ 6.52, 6.71 (2 singlets, 2 OCH_3). The p.m.r. spectrum obtained from a solution in methyl sulfoxide-d_6 at 70° showed a triplet[21] corresponding to a primary hydroxyl group.

Fraction D (120 mg) was treated overnight with $NaIO_4$, and the oxidized material was removed by column chromatography on silicic acid. The slow-moving component (50 mg) had $[\alpha]_D^{25}$ +96° (*c* 0.5, ethanol) and was methyl 2,6-di-*O*-methyl-α-D-mannofuranoside (**19**). It was homogeneous by g.l.c.; the retention times have already been reported[13]. It showed p.m.r. signals ($CDCl_3$) at τ 5.11 (doublet, *J* 2 Hz, anomeric H), τ 6.54, 6.62, 6.66 (3 singlets, 3 OCH_3). A solution of **19** in 3% methanolic hydrogen chloride was refluxed for 2 h, made neutral with silver carbonate, the suspension filtered, and the filtrate evaporated to a syrup whose p.m.r. spectrum and g.l.c. retention-time were identical with those of methyl 2,6-di-*O*-methyl-α-D-mannopyranoside[13].

*Conversion of 1,2:3,5-di-*O*-methylene-α-D-glucofuranose* (**20**) *into* O*-methylene derivatives.* — A crude mixture (1.2 g) of orthoesters was obtained by treating 1,2:3,5-di-*O*-methylene-α-D-glucofuranose (1 g) with methyl orthoformate (10 ml) containing 5 drops of 3% methanolic hydrogen chloride; following neutralization, the solution was evaporated to dryness. The crude syrup, which showed i.r. absorption at 1720 cm^{-1}, was treated with sodium methoxide in methanol, the solution was evaporated to dryness, and the product was washed with water and then dissolved in chloroform; the solution was washed with water, and evaporated to dryness.

The resulting material (**21** and **22**) (800 mg) was free from 1,2:3,5-di-*O*-methylene-α-D-glucofuranose (by t.l.c.) and its formic ester (by i.r.). The material derived from the hydrogenolysis of the orthoester mixture (400 mg) with 1 molar equivalent of $LiAlH_4$–$AlCl_3$ was resolved into three fraction by column chromatography on silicic acid with 8:1 (v/v) chloroform–acetone.

Fraction A (100 mg), a syrup, had $[\alpha]_D^{25}+42.5°$ (*c* 1.5, ethanol) and, on the basis of its p.m.r. spectrum (CCl_4), was 6-*O*-(methoxymethyl)-1,2:3,5-di-*O*-methylene-α-D-glucofuranose (**24**); signals occurred at τ 4.16 (doublet, *J* 4 Hz, anomeric H), τ 5.06–5.12 (superimposed signals, 3 protons of *O*-methylene), τ 5.34 (doublet, *J* 5 Hz, one proton of six-membered *O*-methylene group), τ 5.46 (singlet, 2 protons of 6-*O*-substituted *O*-methylene), τ 6.70 (singlet, OCH_3).

Fraction B (30 mg) crystallized from carbon tetrachloride; m.p. 100–102°, $[\alpha]_D^{25}+49.8°$ (*c* 1.0, ethanol).

Anal. Calc. for $C_{17}H_{24}O_{12}$: C, 48.57; H, 5.76; Mol. wt. 420. Found: C, 48.87; H, 5.57; Mol. wt. 431.

Its p.m.r. spectrum was consistent with structure **25**; signals ($CDCl_3$) at τ 3.98 (doublet, *J* 4 Hz, 2 anomeric H), τ 4.95 (doublet, *J* 6 Hz, 4 five-membered *O*-methylene H), τ 4.99 (doublet, *J* 6 Hz, 2 equatorial *H* of six-membered *O*-methylene group), τ 5.18 (doublet, *J* 6 Hz, 2 axial protons of six-membered *O*-methylene group), τ 5.28 (singlet, 2 protons of *O*-methylene bridge), τ 5.53–6.30 (signals for 12 ring-protons).

Fraction C (50 mg) was identified as 1,2:3,5-di-*O*-methylene-α-D-glucofuranose (**20**) by its characteristic p.m.r. spectrum.

ACKNOWLEDGMENTS

The authors thank Mr. M. Mazurek for recording the p.m.r. spectra, and Mr. W. C. Haid for recording the infrared spectra and making the microanalytical determinations.

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Carbohyd. Res., 12 (1970) 57–68

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

AMINO SUGARS: ADDITION OF ACYL NITRITES TO 3,4,6-TRI-*O*-ACETYL-D-GLUCAL

JOHANNES H. JORDAAN
Life Sciences Division, Atomic Energy Board, Pretoria (South Africa)

(Received June 24th, 1969)

ABSTRACT

Acyl nitrites, either preformed or generated *in situ*, add to 3,4,6-tri-*O*-acetyl-D-glucal in a *cis* fashion to give dimeric 2-deoxy-2-nitroso adducts having the α-D-*gluco* configuration. Adducts of acetyl, benzoyl, phenylacetyl, and propionyl nitrite were obtained. Partial, catalytic hydrogenation, or reduction with sodium hydrosulfite, afforded the corresponding azoxy derivatives. Prolonged reduction with a zinc–copper couple in acetic acid, followed by acetylation, and hydrolysis with sodium methoxide in methanol, gave 2-acetamido-2-deoxy-D-glucose.

INTRODUCTION

The stereoselective *cis*-addition of nitrosyl chloride to acetylated glycals[1,2] presents a facile route for the synthesis of 2-amino-2-deoxyglycosides[3,4]. The reported *cis*-addition of nitrosyl formate to certain olefins[4,5] prompted an investigation of the addition of acyl nitrites to tri-*O*-acetyl-D-glucal (**1**), in order to find a direct and convenient route for the synthesis of 1-*O*-acyl derivatives of 2-amino-2-deoxy-D-glucose. This report describes the addition of four acyl nitrites, generated *in situ* or preformed, to 3,4,6-tri-*O*-acetyl-D-glucal and some studies on reduction of the adducts.

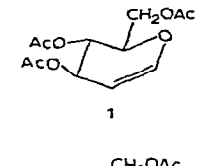
It is known that addition of nitrosyl chloride to acetate **1** gives a readily isolable, dimeric, nitroso *cis*-adduct **3**, having the α-D-*gluco* configuration[1,2]. It was shown[3] that this adduct could be readily dehydrochlorinated to give an intermediate 2-nitroso-D-glucal triacetate **2**, which rapidly added carboxylic acids or alcohols. In the latter case, 2-oximinoglycosides were obtained[4], which, in turn, could be reduced to 2-amino-2-deoxy-α-D-glycosides. Addition of a carboxylic acid to **2** produces a mixture of products, even under carefully controlled conditions; this route for synthesis of derivatives of 2-acylamino-2-deoxy-D-glucose is unattractive, because of the extreme sensitivity to moisture of the intermediate nitrosoglucal.

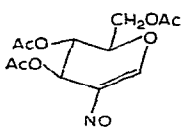
It has been shown[5] that some acyl nitrites add to olefins to give either monomeric or dimeric nitroso adducts. Evidence available indicated that the addition might proceed through a four-center transition state, leading to a *cis* product. As in the case of nitrosyl chloride addition, it seemed reasonable to expect that addition of

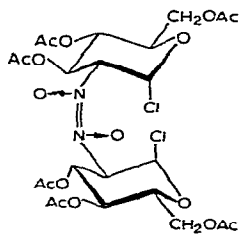
acyl nitrites to **1** might lead to *cis* adducts, unless equilibrium displacement of acylate by acylate occurred.

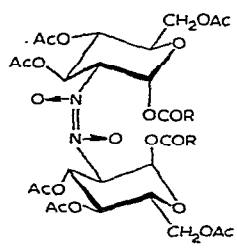
RESULTS AND DISCUSSION

Solutions of acetyl, benzoyl, phenylacetyl, and propionyl nitrites in methylene chloride were prepared by treatment of the corresponding silver salts of the carboxylic acids with nitrosyl chloride[6] at low temperature. These solutions were each treated with **1** for several days at −5°. The products **4a–d** were obtained in yields of 49–62%. Elemental analyses were in agreement with the structures shown, and ultraviolet absorption maxima at 300 nm corresponded to that of nitroso groups in other *trans*-nitroso dimers[5,7]. Assignments of the infrared spectra in the region characteristic for *trans*-nitroso dimers[5,8] were complicated by other absorption bands

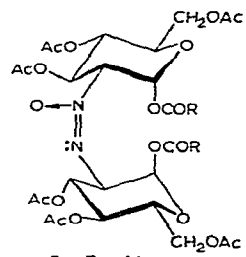








4a R = Me
b R = Et
c R = Ph
d R = $PhCH_2$



5a R = Me
b R = Ph

The mass spectra of compounds **4a–d** did not contain peaks for the molecular ions of either the monomeric or dimeric structures. In each case, the observed peak of highest mass had *m/e* 301, and an accurate mass consistent with the formula $C_{12}H_{15}NO_8$, which corresponds to the nitrosoglucal **2**. Superimposed on the spectrum of **2**, peaks were obtained which originated from the carboxylic acid that would be thermally eliminated from C-1 and C-2. Thus, apart from the peak of *m/e* 301 and its derived ions, the spectrum of **4b** contained a peak of *m/e* 74, representing propionic acid, that of **4c** showed a strong peak of *m/e* 122 (benzoic acid), and that of **4d** had an intense *m/e* 136 peak (phenylacetic acid). Since no peaks of mass above *m/e* 301 were obtained, and a probe temperature of *ca.* 190° was employed, thermal elimination of carboxylic acid from **4** is suggested.

The n.m.r. spectra of **4a–d** indicated the presence of protons on all of the ring carbon atoms (Table I). The splitting of 3.0–3.5 Hz for H-1 and H-2, and of *ca.* 11 Hz for H-2 and H-3 require the compounds to be in the α-D-*gluco* configuration; these data are in agreement with those obtained[1,2] for the corresponding product **3** from addition of nitrosyl chloride. Signals for all of the other protons were in agreement with the structures shown.

Generation[5] of the required acyl nitrites, *in situ*, in methylene chloride, or in

TABLE I

N.M.R. DATA[a] OF DIMERIC 3,4,6-TRI-*O*-ACETYL-1-*O*-ACYL-2-DEOXY-2-NITROSO-α-D-GLUCOPYRANOSES

Compound	*H-1 (d)*	*H-2 (q)*	*H-3 (q)*	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$
4a	6.48	5.43	6.02	3.5	11.0	9.1
4b	6.41	5.42	5.98	3.5	11.0	9.2
4c	6.53	5.60	6.14	3.3	10.7	9.3
4d	6.43	5.37	5.90	3.1	10.7	9.1

[a]Chemical shifts in p.p.m. and splittings in Hz.

ether, by the use of isopentyl nitrite, the relevant carboxylic acid, and 70% perchloric acid as catalyst, in the addition reactions to **1**, were only marginally successful. Since the rate of the addition reaction was found to be relatively slow, acid-catalyzed elimination of carboxylic acid from the addition products appeared to become competitive. Periodic examination of the reaction mixtures by t.l.c. showed a gradual disappearance of **1**, with concomitant appearance of the required **4** as well as several other products. Final yields varied considerably, and, after work-up of the reaction mixtures following the complete disappearance of **1**, maximal yields of 18% for **4a**, 17% for **4b**, 0.3% for **4c**, and 9.4% for **4d** were obtained.

The possibility of converting **3** into a 1-*O*-acyl derivative was investigated; treatment with silver acetate in glacial acetic acid gave a syrup that showed several spots on t.l.c. (silica gel; benzene–acetone, 85:15). The major, slow-moving component corresponded to authentic **4a** on co-chromatography in several solvent systems. Similar treatment of **3** with silver acetate in methylene chloride produced a syrup which, among several spots (t.l.c.), showed one major, fast-moving component. Isolation of this component by preparative chromatography on silica gel plates gave a chromatographically pure oil that had elemental analysis, t.l.c. behaviour, and n.m.r. and mass spectra identical with those of 3,4,6-tri-*O*-acetyl-D-glucal. It therefore seems that, after an initial electrophilic attack of silver on chlorine, the incipient positive charge on C-1 could be neutralized either by loss of nitrosonium ion from C-2 in a *cis* elimination to give **1**, or by loss of a proton from C-2 to produce **2** in a *trans* elimination. In the latter case, free acetic acid could add[2] to **2** to give **4a**. From the available data, it is uncertain whether the elimination reaction proceeds through an ionic intermediate, or concertedly.

Reduction of **4a** in refluxing ethanol–acetone with sodium hydrosulfite produced crystalline 2,2′-azoxy-bis(1,3,4,6-tetra-*O*-acetyl-2-deoxy-α-D-glucopyranose) (**5a**) in 60% yield. The product showed an i.r. peak at 1500 cm^{-1}, due to an azoxy group[10]. The mass spectrum of **5a** had a highest mass peak of *m/e* 647, which had an accurate mass corresponding to $C_{26}H_{35}N_2O_{17}$. The required molecular ion peak with *m/e* 706 was not obtained, but facile loss of 59 mass units ($CH_3COO^{\cdot}$) could be expected in peracetylated carbohydrates[9]. Similarly, sodium hydrosulfite reduction of **4b** produced crystalline 2,2′-azoxy-bis(3,4,6-tri-*O*-acetyl-1-*O*-benzoyl-2-deoxy-α-D-glucopyranose) (**5b**) in 67% yield. The product showed i.r. peaks at 1500 and 1490 cm^{-1} which could

be attributed to a phenyl group and a *trans*-azoxy group[10]. The ultraviolet spectrum showed diminished absorption in the region of 300 nm. The mass spectrum of **5b** showed a peak of highest mass at *m/e* 710, with an accurate mass corresponding to $C_{34}H_{34}N_2O_{15}$, which indicates the loss of two molecules of acetic acid from the expected molecular ion having *m/e* 830.

In symmetrically substituted azoxides, neighboring protons of the substituent on the oxygen-bearing nitrogen atom would be expected to be slightly deshielded, relative to the protons of the substituent on the other nitrogen atom[10]. As anticipated, the n.m.r. spectra of **5a** and **5b** showed two sets of signals for H-1, H-2, and H-3 of the two identical substituents in each of the azoxides. It is clear from Table II that the α-D-*gluco* configuration had been retained during reduction. All other signals in the spectra were in agreement with the structures shown. Hydrogenation of **4a** and **4b** in ethyl acetate, using 10% palladium–charcoal catalyst, gave products identical with **5a** and **5b**.

TABLE II

N.M.R. DATA[a] OF AZOXY COMPOUNDS, $R_1\overset{\overset{O}{\uparrow}}{N} = NR_2$

Compound	*H-1 (d)*	*H-2 (q)*	*H-3 (q)*	*H-4 (t)*	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$
5a, R_1	6.43	4.78	6.03	5.12	3.8	10.8	9.3
R_2	6.28	4.43	5.73	5.12	3.5	10.6	9.3
5b, R_1	6.55	4.89	6.18	5.16	3.5	10.7	9.6
R_2	6.53	4.53	5.88	5.16	3.4	10.6	9.6

[a]Chemical shifts in p.p.m. and splittings in Hz.

Reduction of **4a** with excess of a zinc–copper couple in acetic acid for 4 days, followed by treatment with acetic anhydride, produced a syrup that showed two spots on t.l.c. The major, fast-moving component was collected from preparative layer chromatograms. After *O*-deacetylation with sodium methoxide in methanol, the product proved to be identical with 2-acetamido-2-deoxy-D-glucose in co-chromatography on silica gel.

Attempts are being made to extend the addition reaction to the use of carboxylic acids having functional side-chains, and to find improved procedures for reduction of the nitroso dimers to 1-*O*-acyl derivatives of 2-amino-2-deoxy-α-D-glucose.

EXPERIMENTAL

General. — Melting points were taken in capillary tubes in a Büchi silicone bath apparatus, and are corrected. Infrared spectra were determined for KBr discs with a Perkin–Elmer 257 grating spectrophotometer. Ultraviolet spectra were determined in 2-methoxyethanol with a Beckman DK-2A ratio recording spectrophotometer. N.m.r. spectra were recorded in deuteriochloroform on a Varian A-60A

spectrometer with tetramethylsilane as internal standard. Mass spectra were obtained with an AEI MS-9 mass spectrometer. Optical rotations were determined with a Perkin–Elmer 141 polarimeter. Thin-layer chromatograms were run on pre-coated, silica gel F plates (Merck), and spots were detected with alkaline hydroxylamine followed by ferric nitrate sprays[11], unless otherwise indicated. Preparative layer chromatograms were run on pre-coated, preparative, silica gel plates (Merck). Microanalyses were performed by Dr. Franz Pascher, Bonn, Western Germany.

Silver salts were conveniently prepared by addition of excess of aqueous silver nitrate to an aqueous solution of the sodium salt of the particular acid, followed by filtration, washing with water, and drying *in vacuo*.

*1,3,4,6-Tetra-*O*-acetyl-2-deoxy-2-nitroso-α-*D*-glucopyranose dimer* (**4a**). — (*a*) To a stirred suspension of 18.26 g (110 mmoles) of dry, powdered silver acetate in 200 ml of anhydrous methylene chloride at −15°, was added a solution of 6.55 g (100 mmoles) of nitrosyl chloride in 50 ml of methylene chloride, previously cooled to −15°. The mixture was stirred for 1 h at ambient temperature and then filtered through a sintered-glass funnel in a dry-box. The salts on the filter were washed with 50 ml of methylene chloride, and the yellow filtrate was cooled to −50° and added to a solution of 27.2 g (100 mmoles) of **1** in 100 ml of methylene chloride. The mixture was kept for 4 days at −5° and then poured into 250 ml of ice–water. The blue, organic layer was washed with water (3 × 250 ml), dried over magnesium sulfate, filtered, and then evaporated to dryness *in vacuo* at a bath temperature below 40°. The blue, solid residue was triturated with 200 ml of ether and cooled to −10°, and the crystalline precipitate was collected. The product was washed with 50 ml of ether and recrystallized from acetone–hexane to give 16.7 g (62%) of colorless crystals that showed one spot on t.l.c. (benzene–acetone, 85:15) and had m.p. 144–145°, $[\alpha]_D^{20}$ +151° (*c* 1.19, chloroform); λ_{max} 300 nm (ε 9200)*; n.m.r. data (Table I): δ 1.94–2.13 (4 peaks, CH_3CO_2-); 3.95–4.30, 3-proton signal.

Anal. Calc. for $C_{14}H_{19}NO_{10}$ (monomer): C, 46.54; H, 5.30; N, 3.88. Found: C, 46.62; H, 5.30; N, 3.81.

(*b*) A solution of 5.44 g (20 mmoles) of **1** in a mixture of 4.7 g (40 mmoles) of isopentyl nitrite and 10 ml of dry tetrahydrofuran was added dropwise during 30 min to a stirred solution of 5 ml glacial acetic acid in 20 ml of dry methylene chloride containing 0.1 ml of 70% perchloric acid at −10°. The mixture was kept for 48 h at 0°, washed with water (3 × 50 ml), dried over magnesium sulfate, filtered, and spin-evaporated to dryness *in vacuo* at a bath temperature below 40°. The blue residue was triturated with 20 ml of ether. Colorless crystals separated on cooling; yield, 1.3 g (18%), after recrystallization from acetone–hexane. The product showed one spot on t.l.c. and was identical with **4a** in all aspects described in (*a*).

*3,4,6-Tri-*O*-acetyl-2-deoxy-2-nitroso-1-*O*-propionyl-α-*D*-glucopyranose dimer* (**4b**) — (*a*) A suspenson of 9.95 g (55 mmoles) of dry, powdered silver propionate in 100 ml of methylene chloride was treated with a solution of 3.23 g (50 mmoles) of

*Calc. by using the molecular weight of the dimer.

nitrosyl chloride in 25 ml of methylene chloride (to obtain a solution of propionyl nitrite), followed by treatment with 13.6 g (50 mmoles) of **1**, as described for **4a**. Two recrystallizations from ethyl acetate–hexane gave 9.7 g (52%) of colorless needles that showed one spot on t.l.c. (benzene–acetone, 85:15) and had m.p. 136–137°, $[\alpha]_D^{20}$ +140° (*c* 1.19, chloroform); λ_{max} 300 nm (ε 9000); n.m.r. data (Table I): δ 2.38 (2-proton quartet, *J* 7.3 Hz, CH_3CH_2), 1.11 (3-proton triplet, *J* 7.3 Hz, CH_3CH_2), 1.9–2.1, 3 signals for CH_3CO_2.

Anal. Calc. for $C_{15}H_{21}NO_{10}$ (monomer): C, 48.00; H, 5.64; N, 3.73. Found: C, 48.27; H, 5.37; N, 3.55.

(*b*) A solution of 6 g of propionic acid in 20 ml of methylene chloride containing 0.1 ml of 70% perchloric acid was treated with a solution of 5.44 g (20 mmoles) of **1** in a mixture of 4.7 g of isopentyl nitrite and 5 ml of dry tetrahydrofuran, as described for **4a**. Recrystallization of the product from ethyl acetate–hexane gave 1.31 g (17%) of colorless needles that were identical with **4b** described in (*a*).

*3,4,6-Tri-*O*-acetyl-1-*O*-benzoyl-2-deoxy-2-nitroso-α-*D*-glucopyranose dimer* (**4c**). — (*a*) Treatment of a suspension of 28.5 g (125 mmoles) of silver benzoate in 100 ml of dry methylene chloride with a solution of 10 g (150 mmoles) of nitrosyl chloride in 100 ml of methylene chloride (to obtain a solution of benzoyl nitrite), followed by treatment with 27.2 g (100 mmoles) of **1** in 100 ml of methylene chloride, as described for **4a**, gave a blue residue after work-up and evaporation of the solvent *in vacuo*. A solution of the residue in boiling ether was cooled to −50°, and the crystalline precipitate was collected after 1 h; yield, 23.4 g. Evaporation of the mother liquor *in vacuo*, followed by trituration with a small volume of ether, gave 2.1 g of product. Two recrystallizations from acetone–hexane gave 23.9 g (56%) of colorless crystals that showed one spot on t.l.c. (benzene–acetone, 85:15) and had m.p. 140–141°, $[\alpha]_D^{20}$ +203° (*c* 1.03, chloroform); λ_{max} 300, 288 nm (ε 9600, 8400); n.m.r. data (Table I): δ 1.9–2.2 (4 peaks, CH_3CO_2), 4.1–4.4 (3-proton signal), 7.4–7.6 and 7.9–8.2 (5 phenyl protons).

Anal. Calc. for $C_{19}H_{21}NO_{10}$ (monomer): C, 53.90; H, 5.00; N, 3.31. Found: C, 53.67; H, 4.84; N, 3.58.

(*b*) Treatment of a solution of 6.1 g (50 mmoles) of benzoic acid in 20 ml of methylene chloride containing 0.1 ml of 70% perchloric acid with a solution of 5.44 g (20 mmoles) of **1** in a mixture of 4.7 g of isopentyl nitrite and 10 ml of dry tetrahydrofuran, as described for **4a**, gave a white solid. Recrystallization from acetone–hexane gave 265 mg (0.3%) of colorless crystals that were identical with **4c** described in (*a*).

*3,4,6-Tri-*O*-acetyl-2-deoxy-2-nitroso-1-*O*-phenylacetyl-α-*D*-glucopyranose dimer* (**4d**). — (*a*) Treatment of a suspension of 14.5 g (60 mmoles) of dry, powdered silver phenylacetate in 100 ml of methylene chloride with a solution of 3.23 g (50 mmoles) of nitrosyl chloride in 100 ml of methylene chloride, followed by treatment of the filtered solution of phenylacetyl nitrite with 13.6 g (50 mmoles) of **1**, and work-up as described for **4a**, gave a crystalline product. Two recrystallizations from acetone–hexane gave 10.7 g (49%) of colorless crystals that showed one spot on t.l.c. (benzene–

acetone, 85:15) and had m.p. 135–136°, $[\alpha]_D^{20}$ +133° (*c* 1.06, chloroform); λ_{max} 300 nm (ε 9000); n.m.r. data (Table I): δ 1.9–2.1, three CH_3CO_2 signals; 3.5–4.2, multiple peaks for 5 protons; 7.26 (5-proton singlet).

Anal. Calc. for $C_{20}H_{23}NO_{10}$ (monomer): C, 54.92; H, 5.30; N, 3.20. Found: C, 54.71; H, 5.38; N, 3.26.

(*b*) A solution of 7.8 g (50 mmoles) of phenylacetic acid in 20 ml of methylene chloride, containing 0.2 ml of 70% perchloric acid, was treated with a solution of 5.44 g (20 mmoles) of **1** in a mixture of 4.7 g of isopentyl nitrite and 10 ml of dry tetrahydrofuran, as described for **4a**. Two recrystallizations of the product from acetone–hexane gave 820 mg (9.4%) of colorless crystals that were identical with **4d** in (*a*).

*2,2'-Azoxy-bis(1,3,4,6-tetra-*O*-acetyl-2-deoxy-α-*D*-glucopyranose)* (**5a**). — To a solution of 3.61 g (10 mmoles of monomer) of **4a** in a mixture of 75 ml of ethanol and 25 ml of acetone was added a solution of 1 g of sodium hydrosulfite in 10 ml of water. The mixture was stirred under reflux, and enough water was added to clarify the solution. Refluxing was continued for 6 h; the mixture was then cooled and poured, with stirring, into 600 ml of ice–water. The mixture was extracted with chloroform (5 × 25 ml), and the combined extracts were washed with water, dried over magnesium sulfate, and evaporated *in vacuo*. The residue was recrystallized twice from aqueous ethanol to give 2.13 g (60%) of colorless crystals that showed one spot on t.l.c. (benzene–ethyl acetate, 3:2; or benzene–acetone, 7:3) and had m.p. 224–225°, $[\alpha]_D^{20}$ +160° (*c* 1.18, chloroform); ν_{max} 1500 cm^{-1} (*trans* N=NO); n.m.r. data (Table II): δ 1.9–2.2 (8 CH_3CO_2 signals), 3.8–4.15 (6 protons).

Anal. Calc. for $C_{28}H_{38}N_2O_{19}$: C, 47.60; H, 5.42; N, 3.96. Found: C, 47.52; H, 5.28; N, 4.03.

*2,2'-Azoxy-bis(3,4,6-tri-*O*-acetyl-1-*O*-benzoyl-2-deoxy-α-*D*-glucopyranose)* (**5b**). — Reduction of 4.23 g (10 mmoles as monomer) of **4b** with sodium hydrosulfite, as described for **5a**, gave 2.78 g (67%) of colorless crystals, after an additional recrystallization from chloroform–light petroleum (60–80° fraction). The product showed one spot on t.l.c., as described for **5a**, and had m.p. 211–212°, $[\alpha]_D^{20}$ +209° (*c* 1.25, chloroform); λ_{max} 270, 276, 283 nm (ε 2000, 2100, 1700); ν_{max} 1515 cm^{-1} (N=NO); n.m.r. data (Table II): δ 1.85–2.15 (6 CH_3CO_2 signals), 4.05–4.37 (six protons), 7.4–8.2 (10 phenyl protons).

Anal. Calc. for $C_{38}H_{42}N_2O_{19}$: C, 54.94; H, 5.10; N, 3.37. Found: C, 55.10; H, 5.14; N, 3.41.

Reduction of **4a** *with zinc in acetic acid.* — A mixture of 5 g of zinc dust and 1.5 g of hydrated copper sulfate was stirred at room temperature in a mixture of 40 ml of glacial acetic acid and 10 ml of water. To the mixture was added a solution of 1.8 g (5 mmoles, as monomer) of **4a** in 25 ml of glacial acetic acid. Stirring was continued during 72 h, with the addition of 1-g portions of zinc dust at 12-h intervals, and the addition of 5 ml of acetic anhydride after 60 h. The mixture was filtered, diluted with 600 ml of water, and extracted with chloroform (5 × 20 ml). The extracts were washed with water, dried over sodium sulfate, and evaporated to give 1.5 g

of a colorless glass. The product showed two spots on t.l.c. (benzene–acetone, 2:1); the faster-moving spot was the major component. The mixture was fractionated on ten preparative plates of silica gel (benzene–acetone, 2:1), to give the faster-moving (700 mg) and slower-moving components (190 mg) as homogeneous (t.l.c.) glasses. Hydrolysis of each component with sodium methoxide in anhydrous methanol gave products of which only that obtained from the faster-moving component was identical with authentic 2-acetamido-2-deoxy-D-glucose (t.l.c., ethyl acetate–pyridine–acetic acid–water, 5:5:1:3).

*Reaction of 3,4,6-tri-*O*-acetyl-2-deoxy-2-nitroso-α-*D*-glucopyranosyl chloride dimer with silver acetate.* — (*a*) *In benzene.* To a stirred solution of 3.3 g (10 mmoles as monomer) of $3^{1,2}$ in 25 ml of glacial acetic acid was added 2.5 g (15 mmoles) of dry, powdered silver acetate, and stirring was continued for 24 h at room temperature. The mixture was filtered, the residue washed with 10 ml of glacial acetic acid, and the filtrate diluted with 250 ml of water. The solution was extracted with chloroform (5 × 20 ml), and the combined extracts were washed well with water. The solution was dried over magnesium sulfate, filtered, and evaporated *in vacuo*, to leave 3 g of a syrupy product that showed several spots on t.l.c. (benzene–acetone, 85:15), with one major, slower-moving component. Co-chromatography of the product with authentic **4a** on silica gel, using either benzene–acetone (85:15), chloroform–acetone (9:1), or benzene–methanol (95:5), showed that the major component was identical with **4a**.

(*b*) *In dichloromethane.* Treatment of 3.3 g (10 mmoles) of **3** with silver acetate in methylene chloride, as described above, followed by filtration, washing with water, and drying over magnesium sulfate, gave 3.5 g of a syrup on evaporation *in vacuo*. The product showed several spots on t.l.c. (benzene–acetone, 85:15), but one major, fast-moving spot predominated. A 1-g portion of the product was fractionated on ten preparative plates of silica gel (benzene–acetone, 85:15), to give the faster-moving component (540 mg) as an oil, which was indistinguishable from 3,4,6-tri-*O*-acetyl-D-glucal (**1**) by t.l.c. (benzene–methanol, 95:5; or benzene–acetone, 85:15), elemental analysis, and i.r., n.m.r., and mass spectra.

ACKNOWLEDGMENT

The South African Council for Scientific and Industrial Research is thanked for the n.m.r. and mass spectra.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

STUDIES ON STARCH-DEGRADING ENZYMES

PART XII*. THE INITIAL STAGES OF THE ACTION ON AMYLOSE OF THE ALPHA-AMYLASES FROM *B. subtilis*, HUMAN SALIVA, MALTED RYE, AND PORCINE PANCREAS

W. BANKS, C. T. GREENWOOD, AND K. M. KHAN
Department of Chemistry, The University, Edinburgh EH9 3JJ (Great Britain)

Received June 27th, 1969)

ABSTRACT

The action pattern on amylose of the alpha-amylases from *B. subtilis*, human saliva, malted rye, and porcine pancreas has been studied. In the initial stages, the reaction has been followed by measuring the weight-average degree-of-polymerization ($\overline{DP_w}$) by viscometry, and also the number-average value ($\overline{DP_n}$) by enzymic assay. The mode of enzymic hydrolysis was established by following changes in the value of the ratio $\overline{DP_w}/\overline{DP_n}$, and also by chromatographic examination of the products of low molecular weight. We have confirmed that the porcine, pancreatic alpha-amylase is unique amongst those enzymes examined, in showing evidence of multiple attack at certain pH-values, and have shown that the presence of glycerol (40%, v/v) changes the mode of action in the initial stages to an essentially random form. The significance of this finding is discussed.

INTRODUCTION

It is now known that the mode of action of alpha-amylases isolated from various sources is not the same, but the exact details of these differences are not yet completely established[1]. Elsewhere[2–5], we have suggested that the initial stages of the alpha-amylolysis of amylose involves the random degradation of the α-D-(1→4) linkages in the polysaccharide. These conclusions were based on the change in degree-of-polymerization (as measured by the viscosity number) with time of enzymic hydrolysis.

Viscosity experiments possess the inherent disadvantage, however, that they would be unaffected by the presence of low concentrations of such small molecules as maltose or maltotriose, as has been pointed out by Robyt and French[6]. From studies of the change in absorbance of the residual amylose–iodine complex and reducing-power measurements, these authors suggested that the alpha-amylases from human saliva, porcine pancreas, and *Aspergillus oryzae* degraded amylose by varying degrees of multiple attack. Robyt and French[6] obtained a measure of this degree of multiple attack by comparing the reducing value of the residual polisaccharide-

* Part XI: *Stärke*, 21 (1969) 177.

fraction with that of the corresponding oligosaccharide-fraction at various stages of digestion. Here, we present another approach to the problem of investigating the initial stages of the alpha-amylolysis of amylose which avoids the disadvantage of the viscosity technique.

If an alpha-amylase initially degrades amylose by multiple attack[6], the resulting oligosaccharides of low molecular weight will have a profound influence on the number-average degree-of-polymerization, ($\overline{DP_n}$) of the total system[6], whereas their effect on the weight-average value ($\overline{DP_w}$) will be insignificant. Multiple attack will, in fact, cause the ratio of $\overline{DP_w}/\overline{DP_n}$ to increase with time of enzymic hydrolysis. On the other hand, if linear amylose, which has the most probable, or exponential, distribution[7], is used as the substrate, random attack will be reflected by constancy in the ratio $\overline{DP_w}/\overline{DP_n}$, the numerical value being *ca.* 2.

In this work, we have investigated changes in the value of this ratio by determining $\overline{DP_w}$ from viscosity measurements[8], and measuring $\overline{DP_n}$ enzymically[9]. This approach has been used to investigate the early stages of the action on amylose of the alpha-amylases from *B. subtilis*, human saliva, malted rye, and porcine pancreas.

EXPERIMENTAL

Substrate. — Samples of linear amylose were obtained by leaching potato-starch granules[10].

Enzymes. — Crystalline alpha-amylases from porcine pancreas and *B. subtilis* were obtained from the Sigma Chemical Company, London. The specific activities were 4.4×10^4 and 1.8×10^4 iodine–dextrin colour units[5,11] per mg of protein per ml of digest, respectively. Human salivary alpha-amylase was prepared by the method of Fischer and Stein[12] (specific activity = 2.8×10^4 units), and the alpha-amylase from malted rye was prepared by the method of Greenwood and Milne[5] (specific activity = 1.2×10^4 units).

Control experiments showed that all of the enzymes were free from maltose-splitting activity.

Digestion conditions. — Digests were set up at pH 4.8 (0.02M acetate buffer), 7 and 8.5 (0.02M phosphate buffer), and 10.5 (0.02M sodium hydroxide–glycine buffer), and incubated at 37° under toluene. For convenience in the measuring of $\overline{DP_n}$, high concentrations of amylose (20 mg per ml) were required; digests were therefore made 40% v/v with respect to glycerol, to stabilize the polysaccharide[9], unless otherwise stated.

Analytical techniques. — The reducing power of the digests was measured by the alkaline ferricyanide method[13], and results were expressed as the apparent percentage of maltose.

Absorbance measurements of the amylose–iodine complex[14] were carried out on a Hilger Spekker (filter 608).

Quantitative estimations of the oligosaccharides produced at different stages of hydrolysis in the digests were made after precipitating the residual polysaccharide

with ethanol[6]. Salts were removed by shaking the supernatant liquors with resin (Amberlite IRA-400, saturated with CO_2). The oligosaccharides were then concentrated on a rotary evaporator before quantitative separation by multiple, ascending, paper (3 MM) chromatography with 70% aqueous propyl alcohol as solvent[15] at 20°. Standard sugars were run as controls. After elution, the oligosaccharides were estimated by enzymic degradation with amyloglucosidase[13], and then the amount of liberated D-glucose was determined by using D-glucose oxidase, as described below, after appropriate dilutions. Control experiments with standard sugars showed that recovery of oligosaccharide was > 95%.

For determinations of $\overline{DP_w}$ and $\overline{DP_n}$, samples of the digest were removed at appropriate intervals, and the enzyme was destroyed by heating for 10 min at 98°. Digests for $\overline{DP_n}$ were then set up as described[9], whilst for $\overline{DP_w}$-values, the amylose product was precipitated as the complex with butyl alcohol, before drying and dissolving in 0.15M potassium hydroxide[8].

Determination of $\overline{DP_w}$ by viscosity measurements. — The technique for measuring the limiting-viscosity number, $[\eta]$, has been detailed[16]. Measurements were made in 0.15M potassium hydroxide. Weight-average molecular weights were found from the relation[8]:

$$[\eta] = 8.36 \times 10^3 \times \overline{M}_w^{0.77},$$

and hence the corresponding value of $\overline{DP_w}$ was calculated.

Determination of $\overline{DP_n}$ by an enzymic technique. — The enzymic technique used to measure the number-average molecular weight, and hence $\overline{DP_n}$, was that described earlier[9], except for the assay of D-glucose by D-glucose oxidase*.

Assay for D-glucose. — Tris–glycerol buffer was prepared as previously described[9]. In 100 ml of this buffer were dissolved[21] D-glucose oxidase (30 mg), peroxidase (3 mg), and *o*-dianisidine hydrochloride (15 mg). The enzyme–chromogen mixture was stored at 4° in a brown bottle.

For the assay, a 1.0-ml sample was thoroughly mixed with 1.5 ml of enzyme–chromogen solution, and incubated for 2 h at 35°. The digests were then cooled in ice–water, and acidified with 12M sulphuric acid (1 ml). The absorbance (A) at a wavelength of 5400 Å was then measured in a spectrophotometer (Unicam SP.600).

The resultant calibration relation for D-glucose in pure water was: D-glucose content in μg = $38.4 \times A$; in the presence of 40% aqueous glycerol, the relation was: D-glucose content in μg = $43.6 \times A$.

RESULTS AND DISCUSSION

The essential differences between random degradation and multiple attack have been discussed by Robyt and French[6]. These authors stressed the profound effect

*In our original paper[9], we included a description of the technique given to us by Professor W. J. Whelan, which we had erroneously assumed to have been taken from the literature[17–20]. We now learn that we gave a method described by Dr. Catley[21], but not at that time published. We apologise to Dr. Catley for this oversight.

that the presence of small oligomers (arising from multiple attack) would have on the number-average of the system.

The best method of establishing the extent of multiple attack is to measure simultaneously $\overline{DP_w}$ and $\overline{DP_n}$. As a measure of the former, Robyt and French[6] used the absorbance of the amylose–iodine complex, but the range of degradation over which the absorbance is proportional to the $\overline{DP_w}$ of the amylose is quite limited[22]. Similarly, the accuracy of reducing-power measurements to determine $\overline{DP_n}$ for a range of molecular sizes is questionable; Robyt and French[6] had to extrapolate from a control sugar of $\overline{DP_n} = 4$ to values of $\overline{DP_n} \sim 1000$ for amylose. Our present technique is inherently more accurate, provided any small sugars produced by multiple attack are statistically divided between odd and even numbers of residues. This point is dealt with in more detail later.

Experiments in the presence of 40% of glycerol (v/v)

Value of the ratio, $\overline{DP_w}/\overline{DP_n}$. — In our first experiments, the enzymic digests contained 40% by volume of glycerol for convenience in dealing directly with high concentrations of amylose in the $\overline{DP_n}$-determination. Results obtained under these conditions for the four enzymes are shown in Table I. It can be seen that, in the

TABLE I

ACTION OF VARIOUS ALPHA-AMYLASES ON AMYLOSE IN PRESENCE OF 40% OF GLYCEROL: VALUE OF $R = \overline{DP_w}/\overline{DP_n}$

B. subtilis				*Human saliva*			
Sample	$\overline{DP_w}$	$\overline{DP_n}$	R	*Sample*	$\overline{DP_w}$	$\overline{DP_n}$	R
1	1980	810	2.5	1	1810	890	2.0
2	1780	635	2.8	2	1160	650	1.9
3	1450	590	2.5	3	1160	585	2.0
4	1210	480	2.5	4	1040	490	2.1
5	1040	425	2.5	5	940	480	2.0
Malted rye				*Porcine pancreas*			
Sample	$\overline{DP_w}$	$\overline{DP_n}$	R	*Sample*	$\overline{DP_w}$	$\overline{DP_n}$	R
1	1720	775	2.2	1	1720	775	2.2
2	950	367	2.6	2	1040	400	2.6
3	510	230	2.2	3	660	295	2.2
4	385	190	2.0	4	500	240	2.1
5	350	160	2.2	5	530	220	2.4

initial stages of enzymic action, there is no change in the ratio $\overline{DP_w}/\overline{DP_n}$, within experimental error. Multiple attack would cause $\overline{DP_n}$ to change more rapidly than $\overline{DP_w}$, and so the ratio between the two would increase. The constancy of the experi-

mental ratio implies that multiple attack is not occurring for any of the four enzymes under our experimental conditions.

These results are in contrast to those of Robyt and French[6], who found evidence for a degree of multiple attack for the three enzymes that they examined, particularly so for porcine, pancreatic alpha-amylase.

The effectiveness of our method of determining $\overline{DP_n}$ depends on there being no preferential build-up in the digest of oligosaccharides having an even number of D-glucose residues. The digests were therefore analysed chromatographically for soluble sugars at the early stages of hydrolysis. Traces of these were found (Table II)

TABLE II

ACTION PATTERN OF HUMAN SALIVARY ALPHA-AMYLASE: YIELDS OF MALTODEXTRINS PRODUCED FROM AMYLOSE AS FUNCTION OF DECREASE IN ABSORBANCE AT pH 4.8

Sample	*Decrease in absorbance* (%)	*Nmoles of maltodextrin*[a] *per ml*					
		G_1	G_2	G_3	G_4	G_5	G_6
1	26	3	32	30	24	10	15
2	45	3	62	68	39	16	30
3	64	6	146	195	130	52	70

[a] G_1 = D-glucose; G_2 = maltose; etc.

for the action pattern of human salivary alpha-amylase, but there was no evidence for any pronounced build-up of even-numbered sugars. It should be noticed that there were only traces of D-glucose, indicating the absence of any maltase-splitting activity, and also no appreciable build-up of maltose and maltotriose, indicating the absence of multiple attack.

When the results of the viscosity experiments were treated as previously[2-5], the resultant graphs of $\overline{DP_w^{-1}}$ *versus* time were linear, indicating again the occurrence of a random, hydrolytic scission of α-D-(1→4) bonds in the earlier stages of reaction (see Fig. 1).

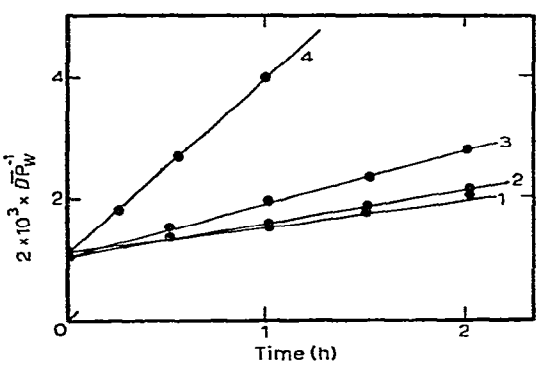

Fig. 1. Graph of $\overline{DP_w^{-1}}$ against time (h) for the action on amylose of the alpha-amylase from 1. *B. subtilis*, 2. human saliva, 3. malted rye, 4. porcine pancreas.

Experiments in the absence of 40% of glycerol

These experiments differed from the earlier ones[6], however, as the digests contained 40% of glycerol. In order to investigate whether the presence of this reagent was influencing the action pattern of the enzyme, digests without this reagent were set up.

Value of the ratio $\overline{DP_w}/\overline{DP_n}$. — Experiments were made with the alpha-amylases from *B. subtilis* and porcine pancreas at pH 4.8. Table III shows that the value of

TABLE III

ACTION OF *B. subtilis* AND PORCINE PANCREAS ALPHA-AMYLASES ON AMYLOSE IN ABSENCE OF 40% OF GLYCEROL: VALUE OF $R = \overline{DP_w}/\overline{DP_n}$

B. subtilis				*Porcine pancreas*			
Sample	$\overline{DP_w}$	$\overline{DP_n}$	R	*Sample*	$\overline{DP_w}$	$\overline{DP_n}$	R
1	1820	810	2.2	1	2110	850	2.5
2	1540	685	2.2	2	1760	565	3.1
3	1280	595	2.2	3	1540	575	3.0
4	1110	505	2.2	4	1430	410	3.5
5	940	450	2.1	5	1230	300	4.1

the ratio $\overline{DP_w}/\overline{DP_n}$ was unchanged for the *B. subtilis* enzyme, but that there was a profound alteration in the value of this ratio with time of digestion for the porcine, pancreatic enzyme. This result implies that, under these experimental conditions, the enzyme was producing an excess of small sugars, in agreement with the results of Robyt and French[6].

Absorbance–reducing-power curves. — Robyt and French[6] showed that, at the adverse pH of 10.5, porcine, pancreatic alpha-amylase appeared to have no multichain action, and that this was reflected by characteristic changes in absorbance–reducing-power curves. We compare in Fig. 2, therefore, the behaviour of porcine, pancreatic alpha-amylase at pH 4.8 without glycerol (curve 1) with its behaviour in the presence of glycerol (curve 2) and also at pH 10.5 (curve 3). It can be seen that the action pattern of the enzyme at pH 4.8 in the presence of glycerol and at pH 10.5 are the same. From the values of the ratio $\overline{DP_w}/\overline{DP_n}$ under these conditions (Table III) and from Robyt and French's conclusions[6], we suggest that curves 2 and 3 in Fig. 2 are characteristic for random degradation, *i.e.*, under these conditions, there is a rapid fall in absorbance with a corresponding relatively small increase in reducing power. The fact that the same shape of graph is obtained for the action of *B. subtilis* alpha-amylase at pH 4.8 without glycerol (curve 4), together with the results in Table III, confirms this suggestion.

In contrast, curve 1 (Fig. 2) for a digest of porcine, pancreatic alpha-amylase at pH 4.8 without glycerol shows a much more rapid increase in reducing power for much smaller changes in absorbance, and this type of curve must be characteristic for enzymic action that results in a build-up of small sugars in the digest.

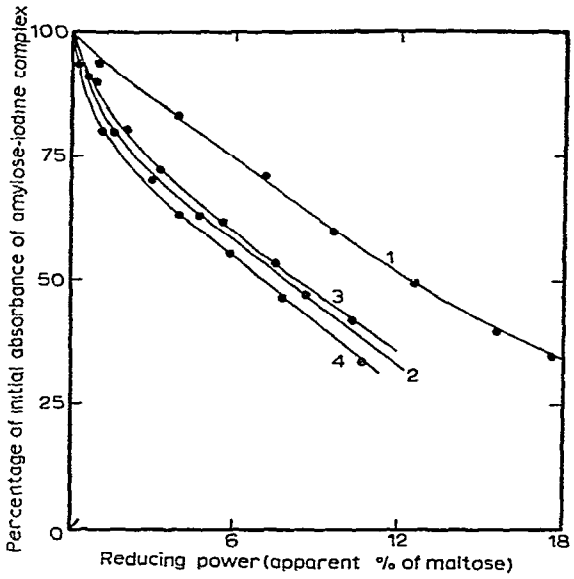

Fig. 2. Graph of the change in absorbance of the amylose–iodine complex as a function of the apparent reducing-power for the action on amylose of 1. porcine, pancreatic alpha-amylase at pH 4.8 without glycerol; 2. porcine, pancreatic alpha-amylase at pH 4.8 in presence of 40% aqueous glycerol; 3. porcine, pancreatic alpha-amylase at pH 10.5; 4. *B. subtilis* alpha amylase at pH 4.8 without glycerol.

It was found that the enzymes from human saliva and malted rye also gave absorbance–reducing-power curves which indicated that random degradation still occurred in the absence of glycerol.

Quantitative estimations of oligosaccharide products

Porcine, pancreatic alpha-amylase appears to be the only enzyme of those examined which has any pronounced degree of multiple attack on amylose. However, at an adverse pH of 10.5, the enzyme reverts to an essentially random attack, in agreement with the results of Robyt and French[6]. Additionally, we have now shown that the presence of 40% of glycerol, at optimal values of pH, is analogous to the use of high pH.

The difference in action pattern of the porcine amylase was further demonstrated when the sugars produced in the early stages of the digestion were determined quantitatively after paper-chromatographic separation. Digests corresponding to the two extremes of action pattern, *i.e.*, pH 4.8 without glycerol, and pH 10.5, were analysed with the results shown in Table IV. It can be seen that at pH 10.5 the amounts of oligosaccharides produced are comparable to those in Table II. However, at pH 4.8 and without glycerol, vastly increased amounts of all sugars (with the exception of D-glucose) are formed. Particularly noticeable are the relatively large amounts of maltose and maltotriose.

Even under optimal conditions and allowing the reaction to proceed to the

achroic point, the other alpha-amylases do not produce such excessive amounts of the small sugars. Table V shows typical results for estimations of sugar composition at pH 5.5, 7.0, and 8.5 using *B. subtilis* alpha-amylase. The same trends as in Table II are apparent.

TABLE IV

ACTION PATTERN OF PORCINE, PANCREATIC ALPHA-AMYLASE: YIELDS OF MALTODEXTRINS PRODUCED FROM AMYLOSE AS A FUNCTION OF pH AND DECREASE IN ABSORBANCE

Sample	*pH of digest*	*Decrease in absorbance* (%)	*Nmoles of maltodextrin*[a] *per ml*					
			G_1	G_2	G_3	G_4	G_5	G_6
1	4.8	27	2	290	127	72	38	29
2	4.8	50	4	620	268	142	86	70
3	4.8	71	6	910	300	222	135	66
1a	10.5	28	4	15	16	8	9	6
2a	10.5	47	4	47	30	15	14	11
3a	10.5	68	6	75	48	33	25	21

[a]G_1 = D-glucose; G_2 = maltose; etc.

TABLE V

ACTION PATTERN OF *B. subtilis* ALPHA-AMYLASE: YIELDS OF MALTODEXTRINS PRODUCED FROM AMYLOSE AT THE ACHROIC LIMIT AS A FUNCTION OF pH

Digest	*pH*	*Digestion stage*	*Percentage by weight*[a]						
			G_1	G_2	G_3	G_4	G_5	G_6	$>G_6$
1	4.8	Achroic	0.2	6	19	6	5	18	46
	4.8	Post-achroic	0.8	11	26	8	10	29	16
2	4.8[b]	Achroic	0.5	5	20	8.5	6	31	30
	4.8[b]	Post-achroic	1.5	8	23	8.5	9	29	21
3	7.0	Achroic	0.1	2	6	2	1	8	81
	7.0	Post-Achroic	0.3	3	9	2	2	10	74
4	8.5	Achroic	0	1.8	3	1.5	1.3	4	88
	8.5	Post-achroic	0.2	2.4	7	2	1.5	7	80

[a]G_1 = D-glucose; G_2 = maltose, etc. [b]Concentration of enzyme 5× greater than in digest 1.

CONCLUSIONS

The initial stages of the action pattern on amylose of the alpha-amylases from *B. subtilis*, human saliva, and malted rye do not involve multiple attack, as measured by changes in the ratio of $\overline{DP_w}/\overline{DP_n}$. This conclusion holds for digests at pH 4.8 with, and without, the presence of 40% of glycerol.

In contrast, the action of the enzyme from porcine pancreas is different. Although there is no evidence of multiple attack at pH 4.8 in the presence of 40%

of glycerol, there is a preferential build-up of small sugars in its absence. This difference in action pattern is reflected by changes in the absorbance–reducing-power curves, in agreement with Robyt and French[6]. The action pattern at the adverse pH of 10.5 is the same as that at pH 4.8 in the presence of 40% of glycerol. Reasons for this difference in action pattern are difficult to establish. Aqueous glycerol (40%) is a viscous medium in which it might be expected that the rate of breakdown of the enzyme–substrate complex would be slower than in a purely aqueous system: multi-chain attack would, therefore, be expected to be favoured in this medium. The experimental evidence suggests, in fact, that the converse effect occurs. Unfavourable ionization of the catalytic groups, as suggested by Robyt and French[6], is unlikely to be occurring in aqueous glycerol (40%). This problem is being investigated further, but we tentatively suggest that the porcine, pancreatic alpha-amylase may be a mixture of two multiple forms[23] having slightly different action-patterns, one of which is apparently suppressed in the presence of aqueous glycerol (40%).

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

THE SPECIFIC SUBSTANCE FROM *Diplococcus pneumoniae* TYPE 31

NIRMOLENDU ROY*, WILLIAM R. CARROLL, AND C. P. J. GLAUDEMANS

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service, Department of Health, Education, and Welfare, Bethesda, Md. 20014 (U. S. A.)

(Received June 28th, 1969)

ABSTRACT

The capsular polysaccharide (or specific substance, S) of *Diplococcus pneumoniae* Type 31 (S.31) contains L-rhamnose, D-galactose, and a glucuronic acid in the approximate molar ratios of 2:2:1. The glucuronic acid is linked to a D-galactose moiety in the polymer. The polysaccharide is very susceptible to hydrolytic cleavage, indicating that one or more of the sugar components may occur in its furanose form. The molecular weight, obtained from sedimentation and diffusion studies, was 120,000. The sedimentation behavior is that which would be expected for a linear polymer.

INTRODUCTION

In 1949, Laros[1] showed that, when subcultured in the presence of rabbit serum cross-reacting to pneumococcus Type 31, *Diplococcus pneumoniae* Type 61 loses its cross-reactivity, but maintains its type specificity. Mills[2] showed that this modified strain of pneumococcus 61 (called M61) had acquired cross-reactivity to anti pneumococcus Type 13 rabbit serum, and that it would revert to the parent Type 61 when subcultured in the latter serum. Types 61, 31, and M61 must, therefore, have common structural features; this should also hold for Types M61, 13, and 61. The present paper describes some work on polysaccharide S.31, one of the antigens of this group.

RESULTS AND DISCUSSION

The capsular polysaccharide of pneumococcus Type 31 was found to be readily hydrolyzed, even in 45% aqueous formic acid. Thin-layer chromatography on cellulose powder† showed three components, one moving as rhamnose, one as galactose, and one at about the same rate as cellobiose. The last component had a much retarded movement in a basic eluant (10:5:4 ethyl acetate–pyridine–water), in which its mobility was almost zero (the same as for a sample of synthetic gentio-

*Chemical Foundation Fellow, 1967–1968.
†Avicel plates, Analtech., Inc., 100 S. Justison St., Wilmington, Delaware 19801, U. S. A.

biouronic acid[3]), suggesting that the compound was an acidic disaccharide. A large amount of S.31 was hydrolyzed, and the hydrolyzate was neutralized, and separated into its components by paper chromatography, to yield L-rhamnose, D-galactose, and the acidic disaccharide. The last component, having $[\alpha]_D^{20}$ +15°, was obtained in 40% yield, and its composition was determined as follows. The compound had an equivalent weight of 405; a (hexosyluronic acid)hexose ($C_{12}H_{20}O_{12}$) has an equivalent weight of 356. It therefore seemed that the material might be a di- (not a tri-) saccharide. Hydrolysis gave only a galactose as the neutral sugar constituent. Reduction of the carboxyl group of the acetate of the saccharide with diborane, followed by deacetylation and hydrolysis, gave a glucose and a galactose in about equimolar amounts (see Fig. 1). When the reducing group of the aldobiouronic acid was first reduced with sodium borohydride, the product acetylated, and the carboxyl group of the resulting acetate reduced, the product yielded, after deacetylation and hydrolysis, a glucose and galactitol. Hence, the disaccharide must be a (glucosyluronic acid)-galactose.

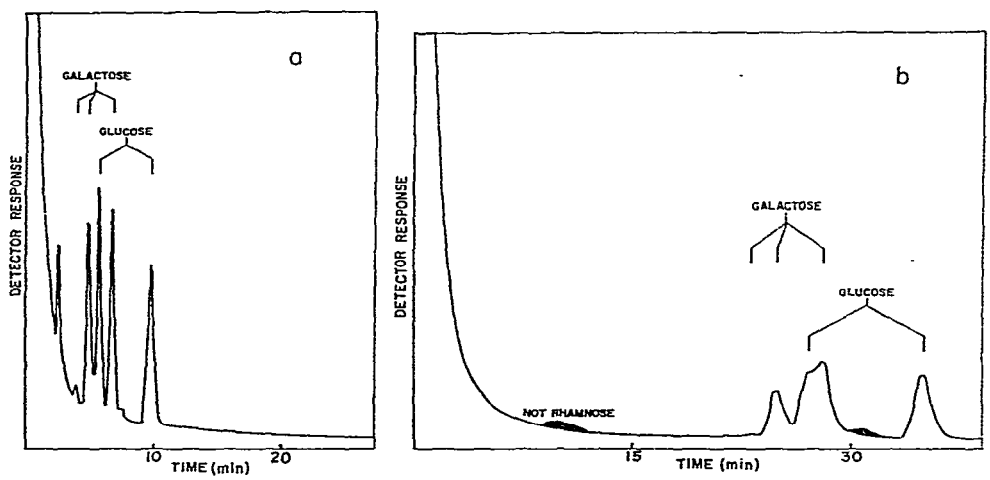

Fig. 1. Gas–liquid chromatograms of the per(trimethylsilyl) derivatives of carboxyl-reduced and hydrolyzed aldobiouronic acid from polysaccharide S.31. (a. Column *1*, 190°; b. Column *2*, 150–180° at 1°/min.)

Polysaccharide S.31 has an approximate acetyl content of 12%. When it is deacetylated, much of the interaction between the antigen and its homologous antibody is lost. When S.31 is acetylated, the acetate reduced with diborane, and the product deacetylated, a carboxyl-reduced polysaccharide is obtained that is free from acetyl groups. This neutral compound (CR-S.31) shows an even more diminished interaction with homologous antibody*. The neutral polysaccharide CR-S.31 was hydrolyzed, and the products were converted into their per(trimethylsilyl)[4] derivatives.

*This observation and some quantitative studies of inhibition will be described in a future communication.

Examination by g.l.c. showed the presence of a rhamnose, a galactose, and a glucose in the ratios of 2.25:2.06:1.00 (see Fig. 2).

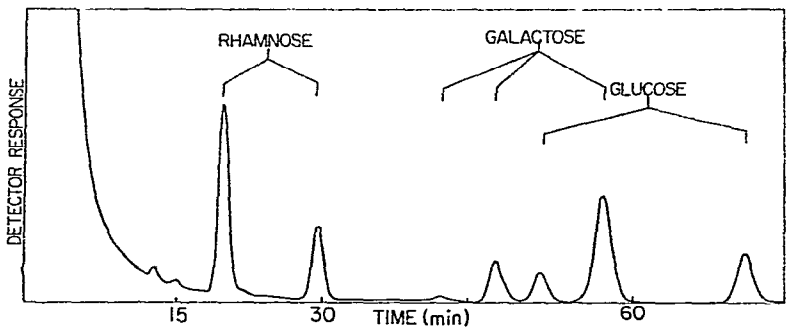

Fig. 2. Gas–liquid chromatogram (column *1*) of the per(trimethylsilyl) derivatives of the materials in the hydrolyzate of carboxyl-reduced polysaccharide S.31.

Native S.31 was hydrolyzed with acid, the product was acetylated, the acetate was reduced with diborane and deacetylated, and the product was hydrolyzed (to cleave the linkage in the reduced aldobiouronic acid). The component sugars were then converted into the related alditols by reduction with sodium borohydride, the mixture was acetylated, and the product was examined by g.l.c. A rhamnitol, galactitol, and a glucitol were found, in the ratios of 2.8:2.5:1.0. It seems likely that this procedure would entail some destruction of glucuronic acid during the hydrolysis; this would lead to slightly higher proportions of rhamnose and galactose than those originally present.

The sedimentation and diffusion behavior of polysaccharide S.31 is similar to that reported by Record and Stacey[5] for the type-specific polysaccharides from pneumococcus Types I, II, and III, namely, a marked increase of the sedimentation constant and a *decrease* of the diffusion coefficient with dilution. During sedimentation at concentrations of 0.5% or higher, the boundaries were hypersharp, as would be expected for linear chains. At all concentrations, only one symmetrical, sedimentation boundary was present. Plots of $1/S$ *versus* the concentration were linear, and permitted evaluation of S^0 and k in the equation $S^0 = S(1+kc)$ as given in Table I. The diffusion boundaries of S.31 are asymmetric, and analysis of the Rayleigh fringe-

TABLE I

SEDIMENTATION AND DIFFUSION PARAMETERS FOR POLYSACCHARIDE S.31

Solution	$S^0_{25w}, X\ 10^{13}$ (*sec.*)	k (*dl/g*)	D^0_{25w}, ($X10^7$) (cm^2sec^{-1})	k_1 (*dl/g*)	M
A	4.40	1.49	1.94	0.80	120,000
B	3.65	0.72	2.53	0.55	77,000
C	3.55	0.64	3.60	0.42	53,000

patterns for concentration dependence was performed as suggested by Creeth[6], the extensive calculations required being performed with a computer. The fringe-deviation diagram for a fresh solution (solution *A*) is shown in Fig. 3. In the equation

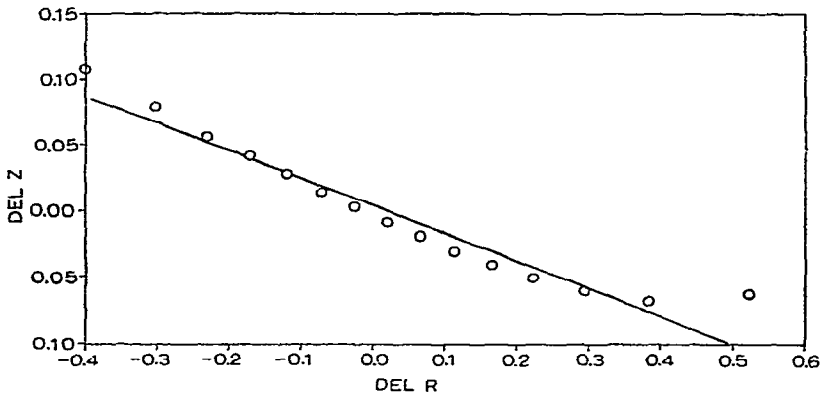

Fig. 3. Fringe-deviation plot for diffusion of solution *A*. DEL Z is a normalized measure of the difference between the actual fringe distances and those expected for an ideal system. DEL R is related to the distance from the center of the boundary (see ref. 6).

$k_1 = (\text{Del } Z/\text{Del } R)\cdot(2/\Delta C)$, the slope of the deviation plot defines k_1, the coefficient of the concentration term in the expression

$$D^0 = D_{\bar{c}}(1 - k_1 \tfrac{\Delta C}{2}),$$

where D^0 is the diffusion coefficient at zero concentration, $D_{\bar{c}}$ is the average diffusion coefficient across the boundary of average concentration $\bar{c}$, and ΔC is the difference between the concentrations of solute in the upper and lower solutions in the diffusion experiment. For solution *A*, these values are shown on the first line of Table I.

As diffusion proceeded beyond twelve hours with solution *A*, the average diffusion coefficient increased somewhat, suggesting a progressive breakdown of the polymer as a function of time. Consequently, two further solutions were prepared after incubation at room temperature for another ~3 and ~8 days (solutions *B* and *C*, respectively). The results, given in Table I, confirmed the hypothesis of progressive hydrolysis, the diffusion coefficients showing marked increases, and the sedimentation constants, appreciable decreases. In addition, the deviation plot for solution *C* (see Fig. 4) is severely curved, indicating considerable heterogeneity; this curvature obviously makes the evaluation of k_1 uncertain.

From the sedimentation and diffusion values at infinite dilution and the partial specific volume, 0.537 (given by Record and Stacey[5] for a similar polysaccharide), the molecular weights were calculated for the three solutions (see Table I). As the fringe-deviation plot for the fresh solution (*A*) showed little indication of heterogeneity, it was concluded that the original preparation had a molecular weight of 120,000, and that incubation at room temperature in aqueous buffer at pH 6.0 caused a slow, random hydrolysis that produced mixtures of materials having average

molecular weights that were progressively lower. The molecular weight of the original sample is somewhat lower than that found by Record and Stacey for S.I and S.III, and considerably lower than that found[5] for S.II.

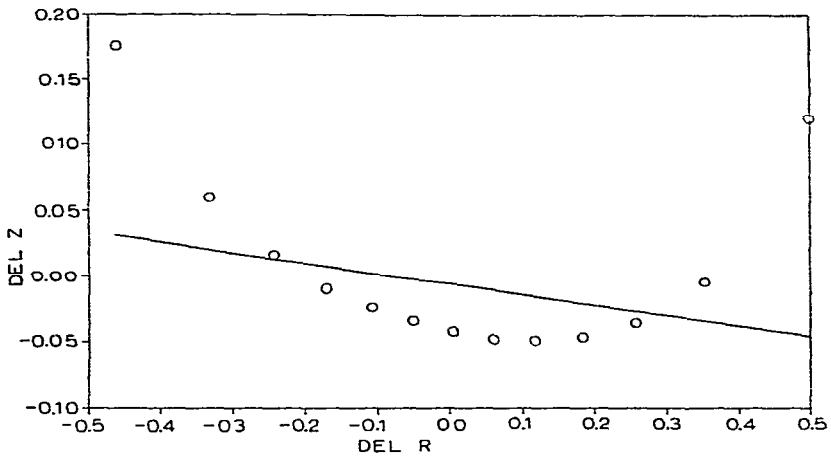

Fig. 4. Fringe-deviation plot for diffusion of solution *C*.

EXPERIMENTAL

General. — G.l.c. was performed with a Hewlett–Packard F & M Scientific Model 5750 chromatograph, with use of four different columns: (*1*) 10% NPGS (Succinate) on Chromosorb W (6 ft. × 0.25 in.) programmed from 150 to 180° at 1°/min; (*2*) 3% SE52 on Gas-Chrom A (6 ft. × 0.25 in.), 150–225° at 2°/min; (*3*) 3% ECNSS-M on Gas-Chrom Q (either 10 ft. × 0.25 in. or 6 ft. × 0.125 in.) at 180°; and (*4*) 15% DEGS (Succinate) on Chromosorb W (6.4 ft.), 150–180° at 1° /min. Descending paper-chromatography was conducted with solvent *X*, 9:2:2 ethyl acetate–acetic acid–water; *Y*, 8:2:1 ethyl acetate–pyridine–water; or *Z*, 10:5:4 ethyl acetate–pyridine–water.

Isolation and properties of S.31. — The capsular polysaccharide was prepared, in several batches, as previously described[7]; it was checked for the presence of C-substance[7] by agar double-diffusion by the method of Ouchterlony[8], but, under conditions that would have revealed 0.1% in the sample*, none was found. The capsular material, which moved as a single peak in chromatography on DEAE-Sephadex and G-200 Sephadex[7], showed a (weak) second line in Ouchterlony

*Previously, we reported that C-substance and S.31 can be resolved by chromatography on DEAE-cellulose[7]. We have now found that, when crude S.31 from lysed cells is eluted from such a column with 0.05M sodium acetate (pH 5.7), a major band of C-substance is, indeed, eluted prior to S.31, but that there is also a minor band, overlapping with S.31, which shows C-substance activity when tested with anti-C serum. We then checked a sample of S.31 kindly presented to us by Dr. Rachel Brown; it showed a single peak on chromatography on DEAE-Sephadex with 0.05M sodium acetate (pH 6.0) and gradient elution with sodium chloride, but each fraction containing the capsular material showed weak C-activity also. Hence, under these conditions, formation of a single peak is, alone, an inadequate criterion for the complete absence of C-substance.

double-diffusion[8] *versus* some rabbit homologous antisera. This line, which was not due to C/anti-C interaction, had the same immunoelectrophoretic mobility as the main bulk, and may be attributable to molecular-weight heterogeneity of the antigen, the antibody, or both.

After precipitation with alcohol, the polysaccharide was used without further purification; it had $[\alpha]_D^{20}$ −17° (water), and contained 0.23% of N. An analytical sample purified by chromatography[7] had $[\alpha]_D^{20}$ −19°, and contained N, 0.68; P, 0.0; S, 0.18; and acetyl, 11.68%. A solution (0.05%) in water showed no absorption at 260 nm.

Acid hydrolysis of S.31. — A solution of the polysaccharide (10.5 mg) in 0.5M sulfuric acid (1 ml) was divided into four parts; these were hydrolyzed in sealed ampoules for different periods of time at 100°. The acid was neutralized with barium carbonate, the insoluble salts were filtered off, the filtrate was treated with Amberlite IR-120 cation-exchange resin, and filtered, and the filtrate was examined by paper chromatography (solvents *X*, *Y*, and *Z*). Three spots were detected; these respectively traveled at the same rate as rhamnose, galactose, and that expected for an acidic disaccharide. Higher oligosaccharides could be detected in traces only. Examination by g.l.c. showed only a galactose and a rhamnose, the proportions of galactose increasing with the length of the hydrolysis. The hydrolyzate gave a positive naphtho-resorcinol test for uronic acid. Hydrolysis of S.31 with aqueous 45% formic acid for 8 h at 100° afforded the same components.

Isolation of the acidic disaccharide from S.31. — The polysaccharide (90 mg) was hydrolyzed with aqueous 0.5M sulfuric acid for 2.5 h at 95–100°. Neutralization with barium carbonate, filtration, treatment of the filtrate with Amberlite IR-120 cation-exchange resin, and evaporation of the filtrate yielded a syrup. This was resolved by preparative paper-chromatography (solvent *X*) into L-rhamnose, $[\alpha]_D^{20}$ +7.7° (*c* 1.6, water); D-galactose, $[\alpha]_D^{20}$ +71° (*c* 0.6, water); and the aldobiouronic acid (35.8 mg), $[\alpha]_D^{20}$ +15° (*c* 0.4, water). Calc. for $C_{12}H_{20}O_{12}$: equivalent wt., 356. Found: 405.

The disaccharide (1 mg) was hydrolyzed with aqueous 0.5M sulfuric acid to give a galactose as the only neutral product (g.l.c., column *1*). Another portion (2 mg) was acetylated with pyridine (0.2 ml) and acetic anhydride (0.05 ml) for two days, and the reagents were removed by azeotropy with toluene and then ethyl alcohol. The acetate was thoroughly dried, suspended in dry tetrahydrofuran, and treated with a solution of diborane in the same solvent. After 2.5 h, the excess of diborane was cautiously decomposed by addition of methanol. As soon as effervescence had ceased, the mixture was evaporated *in vacuo*, and the borate was removed by repeated addition and evaporation of methanol. The product was suspended in methanol, and deacetylated by the addition of methanolic sodium methoxide (final concentration, 0.05M). Sodium ions were removed with Amberlite IR-120 cation-exchange resin, the suspension was filtered, and the filtrate was evaporated to a syrup. The material was then hydrolyzed with 0.5M sulfuric acid, and the hydrolyzate passed through a column of Amberlite IR-45 anion-exchange resin, yielding a neutral

eluate which was evaporated to a syrup, and the latter dried. The per(trimethylsilyl) derivative[4] was prepared in the usual way; examination of the resulting mixture by g.l.c. (columns *1* and *2*) showed the presence of a galactose and a glucose in approximately equal amounts (see Fig. 1). A small peak, moving unlike rhamnose and accounting for ~6% of the total, was also present.

The components, as their alditol acetates, were also characterized by g.l.c., with column *3*. The material in the hydrolyzate was reduced with sodium borohydride, and the product was acetylated with acetic anhydride in pyridine.

In another experiment, the acidic disaccharide (1.8 mg) was reduced (at the reducing end) with sodium borohydride (0.5M, 2 ml) for 3 h at room temperature, and then acetylated and the carboxyl group reduced with diborane; after removal of the excess of borate, the product was hydrolyzed as already described. Part of the neutral product was acetylated, and the acetate examined by g.l.c. with column *3*; part was converted into the per(trimethylsilyl) derivatives[4], and these were examined by g.l.c. with columns *1* and *2*. A glucose pentaacetate and galactitol hexaacetate, but no glucitol hexaacetate, were observed. A number of peaks of highly volatile compounds were observed on the solvent shoulder, but these could not be identified.

Preparation of carboxyl-reduced S.31. — The polysaccharide S.31 (14 mg) was suspended in formamide (0.25 ml), pyridine (1.25 ml) followed by acetic anhydride (0.25 ml) were added, and the mixture was kept for one day at room temperature. The solution was evaporated to dryness *in vacuo* at 40°, and the product was suspended in dry tetrahydrofuran (5 ml). An excess of diborane in the same solvent was added, and the mixture was stirred for 16 h at 25°. Methanol was then cautiously added to decompose the excess of diborane, and the methyl borate was removed in the usual way. The product was deacetylated with sodium methoxide, sodium ions were removed with Amberlite IR-120 cation-exchange resin, the suspension was filtered, and the filtrate was evaporated to dryness, giving carboxyl-reduced S.31 (CR-S.31; 9 mg).

Total hydrolysis of carboxyl-reduced S.31. — The neutral polysaccharide CR-S.31 was heated in 0.5M sulfuric acid in a sealed tube for 15 h at 95–100°. The acid was neutralized with barium carbonate, the suspension was filtered, and the filtrate was treated with Amberlite IR-120 cation-exchange resin, and evaporated to dryness. Conversion of the constituent sugars in the mixture into their per(trimethylsilyl) derivatives[4], and examination by g.l.c. (column *1*) showed a rhamnose, a galactose, and a glucose in the ratios of 2.25:2.06:1.00 (see Fig. 2).

Alditol acetates from S.31. — The material from acid hydrolysis of 9 mg of S.31 was acetylated with acetic anhydride in pyridine, the carboxyl group of the product was reduced with diborane in tetrahydrofuran, and the product was hydrolyzed with aqueous 0.5M sulfuric acid. The acid was removed as previously described, and the neutral residue was reduced with sodium borohydride in water. The product was acetylated, and the resulting alditol acetates were examined by g.l.c. (column *3*). From the peak heights, the proportions of rhamnitol, galactitol, and a glucitol were estimated to be 2.8:2.5:1.0.

Preparation of solutions for ultracentrifuge measurements. — Polysaccharide S.31 (~164 mg) was dissolved in ~30 ml of 0.1M sodium acetate buffered to pH 6.0. This solution, and all subsequent solutions, contained 0.01% of merthiolate as a preservative, and all of the solutions were dialyzed, with continuous rocking, against buffer for 16 h at 25°. The first solution (solution *A*, or "fresh" solution) was assayed by the phenol–sulfuric acid method[9] (native S.31 was used for providing the standard curve), and found to contain 0.606% of polysaccharide. Two more solutions were prepared from solution *A*, and their concentrations were determined from the number of fringes in the diffusion experiment. Thus, solution *B* contained 0.622% of S.31 in 0.1M sodium acetate and 0.4M sodium chloride at pH 6.0, and solution *C* contained 0.536% of S.31 (in the same solvent as for solution *B*). At the time of measurement, solution *A* had been kept for 4 days at room temperature; solution *B*, for a total of 7 days; and solution *C*, for a total of 15 days. Between experiments, the solutions were kept at −15°.

Sedimentation and diffusion measurements. — The diffusion behavior was studied in an Aminco Model B diffusion apparatus at 25.48°, by use of Rayleigh-interference optics and the techniques described by Longsworth[10]. Determination-of velocity of sedimentation were made at 25.0° on the solutions prepared for ths diffusion experiments, and on dilutions of these, by use of a Spinco Model E ultrae centrifuge.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

LE 3-*O*-MÉTHYL-L-RHAMNOSE, SUCRE ISOLÉ DU MYCOSIDE G DE *Mycobacterium marinum*

CLAUDE VILLÉ* ET MIREILLE GASTAMBIDE-ODIER
Faculté des Sciences de Reims, Laboratoire de Biochimie Microbienne, B. P. 347, 51 Reims (France)
(Reçu le 3 mars 1969; sous forme révisée le 1er juillet 1969)

ABSTRACT

The structure of the monosaccharide isolated from mycoside G, a specific glycolipid of *Mycobacterium marinum*, is established as follows: demethylation experiments show that the monosaccharide is a methyl ether of L-rhamnose. Spectra (mass and n.m.r.) of the derived, acetylated methyl glycoside suggest a 6-deoxy-3-*O*-methylpyranoside structure, which is confirmed by periodate oxidation. The L-configuration of the sugar is deduced from the optical rotation, which is identical with that of 3-*O*-methyl-L-rhamnose (acofriose). The circular dichroism curve (negative Cotton effects) of the acetylated mycoside establishes the existence of an α-L-glycosidic link between the sugar and the aglycon. Mycoside G is therefore a 3-*O*-methyl-α-L-rhamnopyranoside of a *p*-substituted phenol.

SOMMAIRE

La structure du monosaccharide isolé du mycoside G, un glycolipide spécifique de *Mycobacterium marinum*, a été établie comme suit : par déméthylation, il a été démontré que ce sucre est un dérivé *O*-méthylé du rhamnose. Les spectres de r.m.n. et de masse du méthyl glycoside acétylé sont en faveur d'un 6-désoxy-3-*O*-méthyl-pyranoside. La position 3 de la fonction méthoxyle est confirmée par oxydation periodique. La configuration L est déduite du pouvoir rotatoire qui est identique à celui du 3-*O*-méthyl-L-rhamnose (acofriose). La courbe de dichroïsme circulaire du mycoside G acétylé présente des effets Cotton négatifs, le sucre est donc fixé à l'aglycone par une liaison α-L glycosidique. En conséquence, le mycoside G est un 3-*O*-méthyl-α-L-rhamnopyranoside de *p*-phénol.

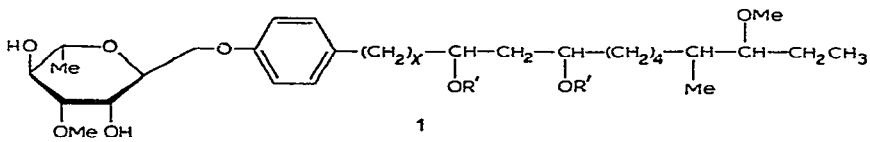
INTRODUCTION

Le mycoside G est un glycolipide spécifique, isolé de *Mycobacterium marinum* (*Mycobacterium balneï*)[1]. La structure chimique de l'aglycone de ce mycoside a été déterminée antérieurement[2]. Le présent travail est consacré à l'étude du résidu glycosidique.

Les propriétés du mycoside G et de son dérivé acétylé indiquent que ce résidu est un α-L-rhamnoside monométhylé dont l'hydroxyle en position 2 est libre. Il a été isolé sous forme de méthyl α,β-L-glycosides acétylés qui ont été séparés et analysés

*Ce travail fait partie de la Thèse de 3e cycle de C. Villé.

par r.m.n. et spectrométrie de masse, puis desacétylés et déméthylés pour identifier le L-rhamnose dont ils dérivent. La configuration du résidu glycosidique a été élucidée par comparaison avec le 3-*O*-méthyl-L-rhamnose et une oxydation periodique a confirmé la position 3 de la fonction méthoxyle. La formule du mycoside G est donc la suivante (**1**) :



1

R′ = Acyl (acides gras normaux et ramifiés)
x = 18, 19, 20, 21, 22

A notre connaissance, c'est la première fois que le 3-*O*-méthyl-L-rhamnose est isolé d'un produit naturel bactérien. Il a déjà été trouvé dans une plante par Reichstein et coll.[3].

RÉSULTATS ET DISCUSSION

Les spectres de r.m.n. du mycoside G (δ 3,51, méthoxyle, 3 protons) et du mycoside G acétylé (δ 3,38, méthoxyle, 3 protons) indiquent que le sucre est porteur d'une seule fonction méthoxyle. La chaîne glycosidique serait monosaccharidique en raison des rapports suivants (spectre r.m.n. du mycoside G acétylé) : 2 groupements méthoxyles, dont un dû à l'aglycone, pour 2 groupements acétyles, pour 4 protons

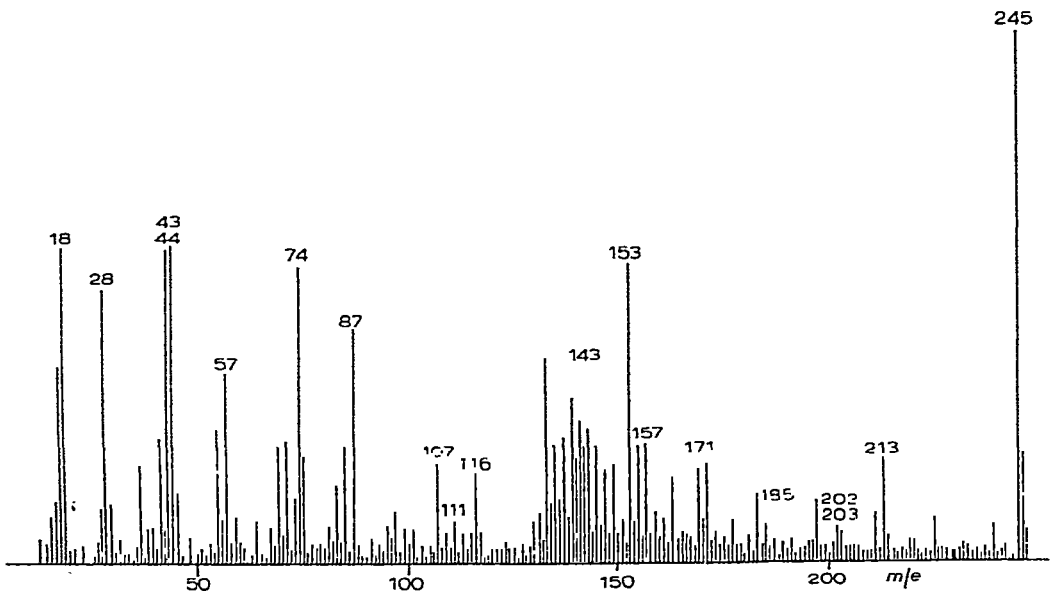


Fig. 1. Spectre de masse du mycoside G acétylé.

aromatiques. La constante de couplage $J_{1,2}$ 2 Hz du proton anomérique du mycoside G exclut[4-6] une conformation *trans*-diaxiale des protons fixés sur les carbones C-1 et C-2.

Le spectre de masse du mycoside G acétylé (Fig. 1) ne présente pas de pics moléculaires. Les pics au-delà de *m/e* 245 ne sont guère analysables, mais les pics *m/e* 18 à *m/e* 245 sont tout à fait caractéristiques de la chaîne glycosidique terminale (voir Schéma I). Le pic intense à *m/e* 245 correspond à l'ion oxonium[7-11], produit par fragmentation selon la voie B de Heyns[12]. La masse de cet ion est compatible avec la présence d'un groupement désoxy et d'une fonction méthoxyle. La perte de méthanol (*m/e* 245−32, *m/e* 185−32, *m/e* 143−32) au cours de la fragmentation, ainsi que l'existence de pics à *m/e* 116 et *m/e* 74 sont également en faveur[13] de la présence d'une fonction méthoxyle. L'ion *m/e* 245 se fragmente selon un schéma établi par Kochetkov et Chizhov[7], Heyns[12,14] et Biemann et coll.[13,15] et caractérisé par la nette prédominance des ions de la série B de Heyns[7,12,14]. Il s'agit de perte de méthanol (−32), de cétène (−42) et d'acide acétique (−60). L'ion *m/e* 111 provient de la perte de méthanol à partir de l'ion *m/e* 143 ou de la perte de cétène à partir de l'ion *m/e* 153. Les ions suivants, *m/e* 116 et *m/e* 74, se formeraient par coupure selon les voies A, et C′ de Heyns[12]. Le pic à *m/e* 107, OC_6H_4-CH_2 +H, provient du noyau aromatique de l'aglycone[7-9,11].

La courbe de dichroïsme circulaire (Fig. 2) présente des minima d'absorption nets à 222, 273 et 281 nm qui correspondent à la position des bandes dûes au noyau

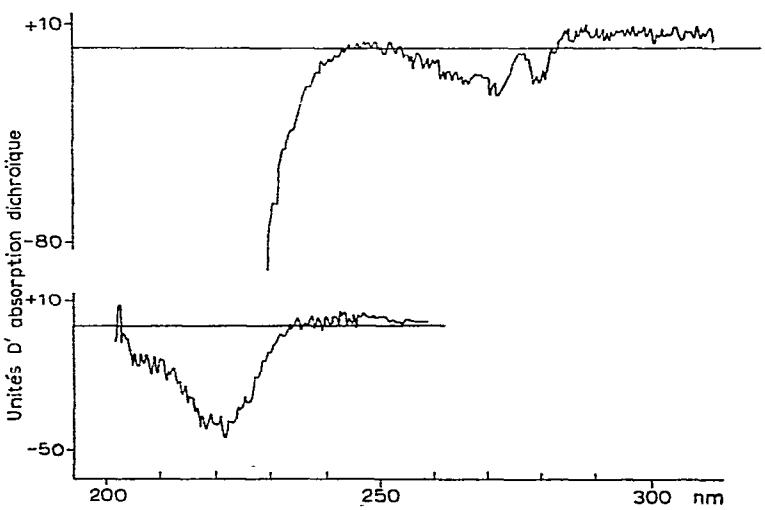


Fig. 2. Courbe de dichroïsme circulaire du mycoside G acétylé.

aromatique dans l'u.v.[16-20]. Selon Sticzay *et al.*[21], les phényl α-glycosides de configuration D présentent des effets Cotton positifs entre 260 et 280 nm, alors que les phényl β-glycosides de configuration D présentent des effets Cotton négatifs dans la même région. L'asymétrie induite par le groupe acétal du sucre dans le noyau phéno-

lique est donc de même signe que celle du groupe acétal. Les courbes de dispersion rotatoire optique ou de dichroïsme circulaire des phényl glycosides donnent ainsi des résultats conformes aux règles de Hudson[22] qui prévoient un pouvoir rotatoire positif pour les α-D et β-L-glycosides et un pouvoir rotatoire négatif pour les β-D- et α-L-glycosides[23]. Nous démontrons ultérieurement que le sucre du mycoside G est de configuration L. Il en résulte que ce dernier est fixé à l'aglycone par une liaison α-glycosidique.

Le mycoside G a été déméthylé[24,25] et hydrolysé et le rhamnose obtenu a été identifié par chromatographie en couche mince (c.c.m.) et chromatographie en phase gazeuse (c.p.g.). Le sucre non déméthylé, isolé au cours de la même réaction, réagit positivement au chlorure de triphényltétrazolium, ce qui indique que l'hydroxyle en position 2 est libre[26].

Le spectre de r.m.n. du méthyl 2,4-di-*O*-acétyl-3-*O*-méthyl-α-L-rhamnopyranoside présente un doublet centré à 1,2 δ, $J_{5,Me}$ 6 Hz, qui est caractéristique d'un groupement[27,28] désoxy en position 6. Deux groupements méthoxyles et deux groupements acétyles sont également identifiables, ce qui est conforme aux données du spectre r.m.n. du mycoside G acétylé.

Le spectre de masse du méthyl 2,4-di-*O*-acétyl-3-*O*-méthyl-α-L-rhamnopyranoside (Fig. 3) est caractéristique de la structure proposée (Schéma I) : le pic à *m/e* 245

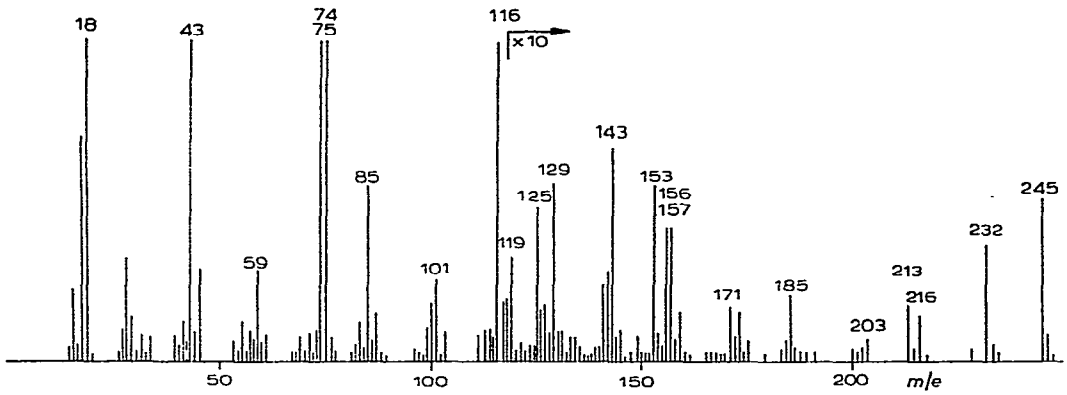


Fig. 3. Spectre de masse du méthyl 2,4-di-*O*-acétyl-3-*O*-méthyl-α-L-rhamnopyranoside.

(PM–31) est dû à l'ion oxonium[7,12–15] dont la masse est compatible avec la présence d'un groupement désoxy et d'une fonction méthoxyle. Cet ion est identique à celui de la Fig. 1 et se fragmente selon le schéma B classique[12,14]. L'ion moléculaire se fragmente également selon les voies A, C et C′ (Schéma I). La preuve de la nature pyranique du cycle est donnée par la présence du pic à *m/e* 232 et l'absence des pics *m/e* 87 et 189 qui sont propres aux isomères furaniques[7,12,13,29]. La présence du groupement méthoxyle est indiquée[13] par le pic à *m/e* 116 qui est particulièrement intense. Cette fonction est en position 3 en raison de la présence des pics *m/e* 75, 101 et 119[12,14]. Une telle fragmentation serait impossible avec un dérivé possédant un groupe méthoxyle en C-4. Le groupement méthoxyle ne peut pas être en C-2, car le

pic à *m/e* 88 $MeOCH^{+}{-}CHOMe$ qui a été trouvé pour les méthyl 3,4-di-*O*-acetyl-2-*O* méthyl et méthyl 3-*O*-acétyl-2,4-di-*O*-méthyl-rhamnosides[29] ainsi que pour les méthyl 2-*O*-méthyl-glucosides[14] est absent. Les résultats de la coloration au chlorure de triphényltétrazolium sont donc confirmés. L'oxydation periodique apportera une preuve supplémentaire indiquant que la fonction méthoxyle est fixée sur C-3.

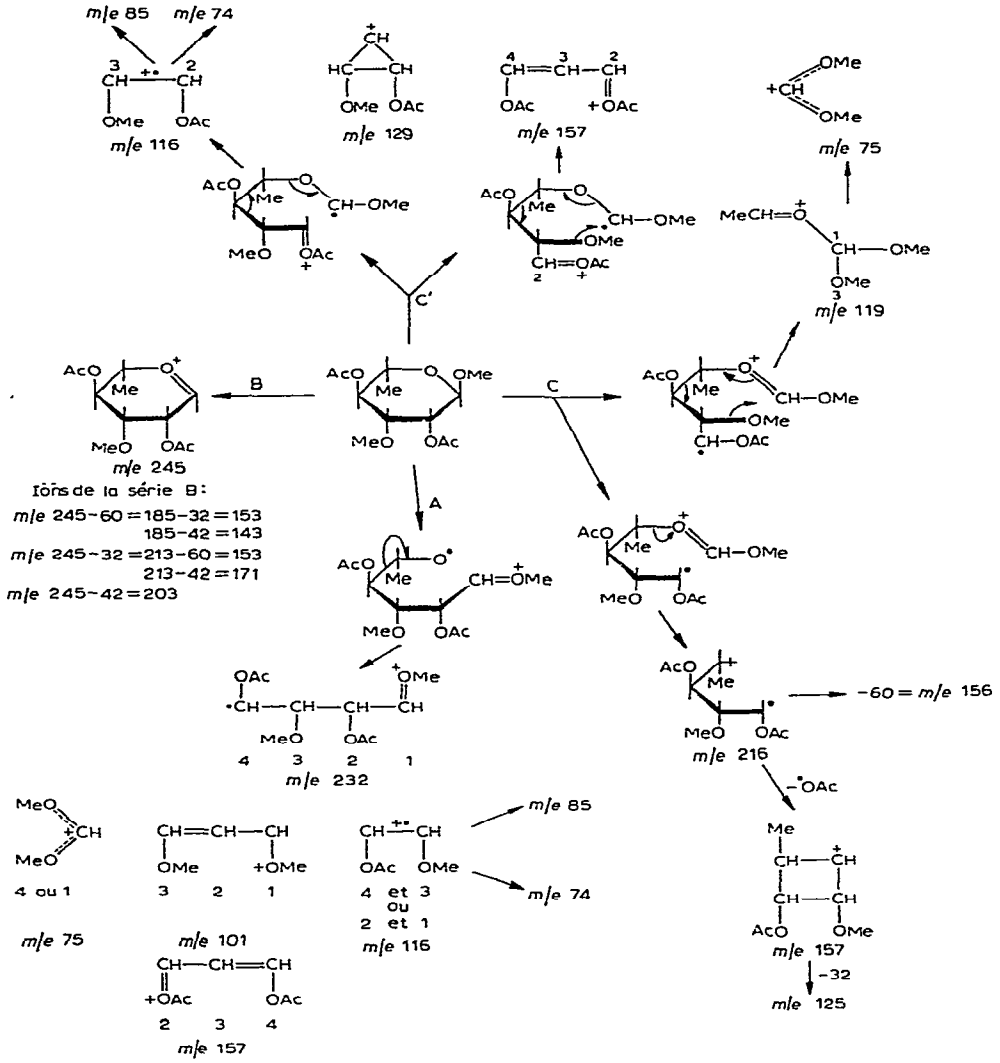


Schéma I. Fragmentation du méthyl 2,4-di-*O*-acétyl-3-*O*-méthyl-α-L-rhamnopyranoside.

Le spectre de masse de l'isomère β est pratiquement identique au spectre de l'isomère α. La seule différence est la présence d'un pic net à *m/e* 217 (PM 276−59) et l'absence de pics significatifs à *m/e* 216 et 213. Kochetkov et Chizhov[7], ainsi que Biemann[15] ont déjà signalé la grande similitude des spectres de masse des anomères α et β d'un même dérivé de sucre.

Les données suivantes confirment la nature monosaccharidique du mycoside G ainsi que l'absence de substituant en position 2 du rhamnose : les R_F des méthyl 3-*O*-méthyl-α- et β-L-rhamnopyranosides obtenus par desacétylation des méthyl 2,4-di-*O*-acétyl-3-*O*-méthyl-α- et β-L-rhamnopyranosides sont identiques à ceux des sucres obtenus par méthanolyse du mycoside G et aucun autre sucre n'a été détecté.

TABLEAU I

C.C.M.[a] ET C.P.G.[b] DES PRODUITS OBTENUS PAR DÉMÉTHYLATION DES MÉTHYL *O*-MÉTHYL-L-RHAMNOPYRANOSIDES

Composés	R_F[a]	*Temps de rétention (sec) des dérivés pertriméthylsilylés*[b]
L-Rhamnose crist.	0,25	119
L-Rhamnose après dissolution dans l'eau		115, 162
3-*O*-Méthyl-L-rhamnose	0,38	
Méthyl 3-*O*-méthyl-α-L-rhamnopyranoside		
Produit de déméthylation et hydrolyse	0,25	122, 169
Produit d'hydrolyse	0,42	83
Méthyl 3-*O*-méthyl-β-L-rhamnopyranoside		
Produit de déméthylation et hydrolyse	0,25	122, 169
Produit d'hydrolyse	0,40	83

[a]Sur gel de silice dans benzène–éthanol (3:1). [b]Sur colonne de 10% de QF I sur Aeropak 30.

Le 3-*O*-méthyl-L-rhamnopyranose résultant de l'hydrolyse des méthyl glycosides réagit au chlorure de triphényltétrazolium et les temps de rétention des anomères du L-rhamnose provenant de la déméthylation et de l'hydrolyse des méthyl glycosides sont identiques à ceux du L-rhamnose authentique (Tableau I).

Le pouvoir rotatoire du 3-*O*-méthyl-L-rhamnose est positif, $[\alpha]_D^{20}$ +34° (à l'équilibre) et correspond pratiquement à celui de l'acofriose (3-*O*-méthyl-L-rhamnose), $[\alpha]_D^{25}$ +39,1 ±2° (à l'équilibre), isolé par Muhr *et al.*[3]*.

Après oxydation au métaperiodate, il a été démontré par c.c.m. que le cycle des anomères du méthyl 3-*O*-méthyl-L-rhamnopyranoside reste intact tandis que le méthyl L-rhamnoside est complètement détruit. Sachant que les anomères du méthyl 3-*O*-méthyl-L-rhamnoside ont une structure pyranique (spectrométrie de masse), ce résultat ne s'explique que si la fonction méthoxyle est en 3, ce qui confirme les données de la spectrométrie de masse.

La formule **1** pour le mycoside G est donc démontrée. Les recherches en cours indiquent qu'il existe des variantes de mycoside G dont les sucres diffèrent du 3-*O*-méthyl-L-rhamnopyranose. Elles sont pondéralement peu importantes et leur structure est à l'étude.

*Les résultats préliminaires communiqués à la réunion commune des Sociétés de chimie biologique allemande, suisse et française, Fribourg en Brisgau, janvier 1969, *Z. Physiol. Chem.*, 350 (1969) 4, sont à corriger en fonction de ces données.

PARTIE EXPÉRIMENTALE

Général. — Les spectres de r.m.n. ont été enregistrés à l'aide d'un spectromètre Varian A-60, en microcellule. Les déplacements chimiques sont exprimés en δ (p.p.m.) et comptés à partir de la raie du tétraméthylsilane prise comme zéro de référence. Les spectres de masse ont été enregistrés sur un appareil MS 9 (AEI) à la température de 300° pour le mycoside G acétylé et 180° pour les sucres acétylés. La courbe de dichroïsme circulaire a été enregistrée sur un dichrographe Roussel-Jouan, modèle CD 185, dans le cyclohexane dist., sensibilité 1×10^{-5} unité d'absorption dichroïque par mm de déviation, traversée optique 1 mm, de 200 à 250 nm, et 1 cm au-delà de 250 nm, la constante de temps est de 2 sec. Les pouvoirs rotatoires ont été mesurés à l'aide d'un Quick Polarimètre Roussel-Jouan, les spectres u.v. ont été enregistrés sur un appareil Leres, Spila, et les spectres i.r. sur un appareil Perkin-Elmer, Infracord. Les chromatographies en couche mince (c.c.m.) ont été effectuées sur gel de silice (Merck), selon Stahl, 15 g de gel pour 35 ml d'eau dist. et épaisseur des plaques 0,25 mm. La révélation a été effectuée par la 2,4-dinitrophénylhydrazine pour les variantes cétoniques du mycoside G, par le chlorure de triphényltétrazolium pour les sucres (quantité minimum de sucre 25 μg) et par l'acide sulfurique pour tous les constituants. La chromatographie en phase gazeuse (c.p.g.) a été effectuée sur les dérivés pertriméthylsilylés avec un appareil F. et M., modèle 609, muni d'un détecteur à ionisation de flamme, colonne spiralée acier inoxydable ordinaire, diamètre 0,6 mm, remplie à 10% de QF I sur Aeropak 30 80/100, longueur 1,80 m; gaz vecteur, azote; débits : N 60 ml par min, H 60 ml par min, air 600 ml par min, température du four 150°, température de l'injecteur 269 ou 335° suivant les analyses, température du détecteur 297 ou 377° suivant les analyses. Pour les dérivés acétylés, les conditions d'analyses sont identiques à celles décrites ci-dessus, à part les points suivants : analyse sur une deuxième colonne apolaire, spiralée, acier inoxydable ordinaire, diamètre 0,6 mm, remplie à 3% de SE 30 sur Aeropak 100/120, longueur 2,10 m, température du four 175°. Pour les analyses sur 10% de QF I sur Aeropak, la température du four est de 190 et 160°.

Isolement et propriétés du mycoside G. — La souche de *Mycobacterium marinum* (*balneï*), 695-Runyon, a été cultivée pendant 32 jours sur milieu de Sauton[30]. L'extrait alcool–éther de cette bactérie a été fractionné selon la méthode d'Anderson[31], puis les graisses ont été purifiées par chromatographies successives sur colonnes de trisilicate de magnésium–Célite (2:1) et sur colonnes sèches d'alumine II-III[32,33]. Une fraction pure (520 mg) a été éluée de la dernière colonne d'alumine par des mélanges d'éther–méthanol (98:2 et 95:5). Le rendement en mycoside G par rapport à l'extrait alcool–éther de départ est d'environ 1,4%. L'analyse par c.c.m. (chloroforme–méthanol 98:2) donne une seule tache, R_F 0,51. Le mycoside G contient des traces de constituants cétoniques[2]. Ses propriétés physiques sont les suivantes : p.f. 26,5–27,5°, $[\alpha]_D^{20}$ $-18 \pm 0{,}5°$ (*c* 2, chloroforme); $\lambda_{max}^{cyclohexane}$ 222, 275 et 282 nm; λ_{max}^{NaCl} 1618, 1597 et 1515 (aromatiques), 3500 (OH), 1740 (ester), 2930, 1460 (Me et méthylène), 1380 (Me), 720 cm^{-1} (méthylène); r.m.n. (δ, *c* 17, deutériochloroforme) :

0,84 (multiplet, Me), 1,27 (CH_2), 3,29 (3 protons, singlet, OMe aglycone), 3,51 (3 protons, singlet, OMe sucre), 4,84 (2 protons, multiplet, R^1—$R^2CH(CH_2R^3)$—OR^4, aglycone), 5,48 (1 proton, doublet, $J_{1,2}$ 2 Hz, probablement H-1), 6,98 (4 protons, doublet, noyau arom.).

Mycoside G acétylé. — Le mycoside G acétylé a été préparé à partir d'un échantillon riche en mycoside G authentique élué de colonnes sèches d'alumine II-III par un mélange d'éther–méthanol (80:20). Le mycoside (302 mg) a été acétylé en présence de pyridine (2 ml) et d'anhydride acétique (3 ml) pendant 16 h à la température ord. Par chromatographies successives sur colonnes sèches d'alumine II-III, une fraction pure (39,4 mg) a été éluée par un mélange de benzène–éther (10:1). L'analyse par c.c.m. (chloroforme) donne une seule tache, R_F 0,65, R_F du mycoside G 0,08; r.m.n. (δ, *c* 25, deutériochloroforme) : 2,06+2,14 (6 protons, 2 singlets, 2 OAc sucre), 3,3 (3 protons, 1 singlet, OMe aglycone), 3,38 (3 protons, 1 singlet, OMe sucre), 6,98 (4 protons, noyau arom.).

Déméthylation du mycoside G. — Le mycoside G (10 mg) a été déméthylé[24,25] et hydrolysé. Les sucres solubles dans le méthanol ont été analysés par c.c.m. (benzène–éthanol 3:1) : R_F du sucre non déméthylé 0,4; sucre déméthylé 0,25; rhamnose 0,26; fucose 0,21. Ces sucres réagissent au chlorure de triphényltétrazolium. La présence de rhamnose a été contrôlée par c.p.g. des dérivés pertriméthylsilylés : les temps de rétention pour les deux pics principaux sont de 115 (dérivé non déméthylé) et 173 sec. Ce dernier correspond exactement à celui du dérivé pertriméthylsilylé du L-rhamnose (173 sec).

Méthanolyse du mycoside G et purification des méthyl α,β-L-glycosides sous forme de dérivés acétylés. — Le mycoside G (250 mg) a été hydrolysé par chauffage à reflux dans 15,5 ml de méthanol chlorhydrique M. A des temps différents, le mélange réactionnel a été extrait à l'eau et à l'éther et la réaction a été poursuivie sur la phase éthérée (aglycone + mycoside inattaqué) après évaporation du solvant et une nouvelle adjonction de méthanol chlorhydrique M. Au bout de 39 h d'hydrolyse, il n'y avait plus trace de mycoside inattaqué (contrôles par c.c.m.). Au cours de l'hydrolyse, la phase aqueuse est toujours identique à elle-même. Le sucre principal a un R_F de 0,59. Un anomère est présent en quantité mineure (R_F 0,47). La quantité de sucres bruts et d'aglycone, $[\alpha]_D^{20}$ +3° (*c* 9,5 chloroforme), est respectivement de 34 et de 213 mg. Le mélange brut de méthyl α,β-L-glycosides (25 mg) provenant d'une méthanolyse de 4 h est passé sur colonne sèche d'alumine II-III[32,33]. Les méthyl α,β-L-glycosides sont élués par un mélange d'éther–méthanol (80:20). Ils sont acétylés pendant 16 h à la température ord. en présence de pyridine (0,1 ml) et d'anhydride acétique (0,15 ml). Après neutralisation par l'hydrogénocarbonate de sodium aq. et extraction à l'éther, on obtient 19,1 mg de sucres acétylés bruts qui sont purifiés par chromatographie sur colonnes sèches d'alumine II-III. Le méthyl 2,4-di-*O*-acétyl-3-*O*-méthyl-α-L-rhamnopyranoside est élué par le benzène (6,7 mg), p.f. 108–109°, $[\alpha]_D^{20}$ −25,5 ±0,5° (*c* 1,6, chloroforme); r.m.n. (δ, *c* 17,5, deutériochloroforme) : 1,2 (3 protons + 1 proton dû à une impureté, doublet, $J_{5,Me}$ 6 Hz, groupement méthyle en C-6), 2,08 et 2,13 (6 protons, 2 singlets, 2 groupements acétyles), 3,32 et 3,37 (6 protons, 2 sin-

glets, 2 groupements méthoxyles), 3,33–3,34 (2 protons, multiplet), 4,63 (1 proton, doublet), 4,96 (1 proton, triplet), 5,29 (1 proton quadruplet). Le méthyl 2,4-di-*O*-acétyl-3-*O*-méthyl-β-L-rhamnopyranoside est élué par un mélange de benzène–éther (10:1) (2,3 mg), p.f. 90–91°. Le Tableau I indique le temps de rétention de ces anomères en c.p.g. par rapport aux dérivés analogues isolés du mycoside A[29].

TABLEAU II

CHROMATOGRAPHIE EN PHASE GAZEUSE DES MÉTHYL DI-*O*-ACÉTYL-*O*-MÉTHYL-RHAMNOSIDES

Composés	*Colonne QF-1* (*sec*)	*Colonne SE-30* (*sec*)
Isolés de Mycoside G		
3-*O*-Méthyl-α-L-pyranoside	102	118
3-*O*-Méthyl-β-L-pyranoside	159	143
Isolés de Mycoside A[29]		
3-*O*-Méthylfuranoside	102	122–123
2-*O*-Méthylfuranoside	159	136

Le temps de rétention de l'anomère α correspond à celui du méthyl 2,4-di-*O*-acétyl-3-*O*-méthyl-rhamnofuranoside isolé du mycoside A. Toutefois les résultats ultérieurs montrent que cet anomère a un cycle pyranique et que l'anomère β n'est pas identique au méthyl 3,4-di-*O*-acétyl-2-*O*-méthyl-rhamnofuranoside.

*Desacétylation et déméthylation des méthyl 2,4-di-*O*-acétyl-3-*O*-méthyl-α- et β-L-rhamnopyranosides.* — Le méthyl 2,4-di-α-*O*-acétyl-3-*O*-méthyl-α-L-rhamnopyranoside (14 mg) est mélangé avec 14 ml de carbonate de potassium aqueux à 2% et gardé 3 jours à la température ord. Le mélange est neutralisé par passage sur résine Amberlite MB-3 anal., filtré et la solution est évaporée sous vide à 40°. Le méthyl 3-*O*-méthyl-α-L-rhamnopyranoside obtenu (9,9 mg) se présente sous forme d'un liquide incolore, $[\alpha]_D^{20}$ −49 ±0,5° (*c* 0,8, chloroforme), R_F 0,58. L'anomère β est également desacétylé et on obtient le méthyl 3-*O*-méthyl-β-L-rhamnopyranoside, R_F 0,45. La comparaison avec les pouvoirs rotatoires négatifs du méthyl-α-L-rhamnoside et de son dérivé triacétylé et les pouvoirs rotatoires positifs du méthyl β-L-rhamnoside et de son dérivé triacétylé[34] permet de conclure que l'anomère le plus abondant qui a été isolé du mycoside G est l'anomère α. Le R_F élevé du méthyl 3-*O*-méthyl-α-L-rhamnopyranoside et le temps de rétention court du méthyl 2,4-di-*O*-acétyl-3-*O*-méthyl-α-L-rhamnopyranoside par rapport aux R_F et temps de rétention des anomères β correspondants sont en faveur de cette conclusion. Les résultats de la c.c.m. et de la c.p.g. des produits obtenus après déméthylation sont donnés dans le Tableau II.

Hydrolyse des méthyl glycosides et isolement du 3-O-méthyl-L-rhamnose. — La solution de méthyl 3-*O*-méthyl-α,β-L-rhamnopyranosides est neutralisée par passage sur résine Amberlite MB-3 anal., évaporée sous vide à 40° et chromatographiée sur Florisil. Le méthyl 3-*O*-méthyl-α-L-rhamnopyranoside, $[\alpha]_D^{20}$ −49 ±0,5° (*c* 0,8,

chloroforme), est élué par un mélange d'éther–méthanol (9:1), puis hydrolysé dans l'acide chlorhydrique M pendant 24 h. On neutralise à nouveau et le 3-*O*-méthyl-L-rhamnose est élué d'une colonne de Florisil dans l'éther–méthanol (8:2). Une solution aqueuse de ce sucre est filtrée et évaporée sous vide à 44°, $[\alpha]_D^{20}$ +34° (après 24 h, *c* 0,7, eau).

Oxydation periodique des méthyl 3-O-méthyl-α- et β-L-rhamnopyranosides. — Le méthyl 3-*O*-méthyl-α- ou β-L-rhamnopyranoside (0,4 mg) est dissous dans l'acide chlorhydrique 0,1M (0,15 ml) et additionné d'une solution de métaperiodate de sodium (0,03 ml; 40 mg par ml d'acide chlorhydrique 0.1M)[35–39]. L'oxydation dure 50–60 h à la température ambiante et à l'obscurité. Un échantillon de méthyl L-rhamnoside témoin est traité de manière identique. Les produits de réaction sont analysés par c.c.m.

REMERCIEMENTS

Nous remercions vivement M. le Professeur E. Lederer de l'intérêt qu'il a porté à ce travail, Monsieur le Professeur T. Reichstein et Madame E. Percival qui nous ont fourni des échantillons authentiques de sucres, ainsi que M. le Professeur D. W. Smith et Monsieur H. K. Kim qui nous ont fourni l'extrait alcool–éther de *Mycobacterium marinum*.

Le spectre de dichroïsme circulaire a été enregistré au Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette et M. M. Legrand a eu l'amabilité d'examiner et de discuter nos résultats. Les spectres de masse ont été enregistrés grâce à la collaboration de Messieurs K.A. Gwilliam (compagnie A.E.I.), Malliard et Carter (École de Pharmacie, Londres, Prof. Whalley) et Monsieur Y. Bruant (Chimie générale, Reims) a enregistré les spectres de résonance magnétique nucléaire; nous les remercions vivement de leur bienveillante collaboration. Nous remercions également le Commissariat à l'Énergie Atomique, Département de Biologie, Saclay, pour une subvention ayant facilité l'achat de solvants deutérés.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

AMINATION OF AMYLOSE AT THE C-2 POSITION[1]

M. L. WOLFROM* AND P. Y. WANG
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210 (U. S. A.)
(Received July 14th, 1969)

ABSTRACT

6-*O*-Tritylamylose was oxidized with methyl sulfoxide and acetic anhydride, and the product oximated; the oxime was reduced and detritylated, and the product dialyzed. The resultant, colorless solid contained, on C-2, an amino group (D.S. 0.8) which was almost entirely oriented as in the D-*gluco* configuration.

INTRODUCTION

The oxidizing system of methyl sulfoxide and a suitable acid anhydride[2-6] has been widely applied in carbohydrate chemistry. A variety of rare or new monosaccharide derivatives has since been prepared in good yields[5-9]. This mild oxidation method suggests yet another promising approach among the known procedures[10-12] for the amination of the secondary hydroxyl groups in amylose.

RESULTS AND DISCUSSION

Commercial amylose was first converted into its 6-trityl ether[13,14] and this was oxidized with a solution of acetic anhydride in methyl sulfoxide for 20 h at room temperature. The oxidized 6-*O*-tritylamylose was recovered by precipitation with methanol, and was immediately oximated with hydroxylamine hydrochloride in pyridine, to avoid possible degradation of the polysaccharide through a mechanism suggested by Theander[15]. The oximated product was obtained as a light-purple powder having a degree of substitution (D.S.) of 0.8. Reduction of the oxime groups was effected in tetrahydrofuran solution with lithium aluminum hydride for 3 h at reflux, followed by stirring for 24 h at room temperature. The reduced product was precipitated with cold, dilute hydrochloric acid, dissolved in bis(2-methoxyethyl) ether, and treated with sodium borohydride (to eliminate any keto groups regenerated from unreduced oxime after the previous reduction) and then with acid (to eliminate the hydride decomposition products). The aminated 6-*O*-tritylamylose was obtained as a completely white, odorless powder. Detritylation in methanol containing a small proportion of hydrochloric acid, followed by addition of ether to facilitate filtration,

*Deceased, June 20th, 1969.

gave a white, slightly hygroscopic powder in almost quantitative yield; over 60% of this material was non-dialyzable. The aminated amylose was readily soluble in water, dilute acid or base, methyl sulfoxide, and *N*,*N*-dimethylformamide. The product showed a characteristic ninhydrin test for amino sugars, and formed a water-insoluble, Schiff base with anisaldehyde[16].

The acid hydrolyzate of the aminated amylose contained only two major components, with paper-chromatographic mobilities indistinguishable from those of D-glucose and 2-amino-2-deoxy-D-glucose hydrochloride. The latter component was isolated from the mixture by means of a column of ion-exchange resin, followed by preparative paper-chromatography, to give a crystalline material whose physical characteristics were identical with those of an authentic sample of 2-amino-2-deoxy-D-glucose hydrochloride. The isolation of a single amino sugar in substantial quantity indicated that the oxidation of 6-*O*-tritylamylose with a solution of acetic anhydride in methyl sulfoxide occurs predominantly at C-2. An analogous conclusion has been reported by Bredereck[17] who, however, found much D-mannose in the acid hydrolyzate of a similarly oxidized 6-*O*-tritylcellulose after reduction with sodium borohydride. Retention of configuration in the reduction of the oximated 6-*O*-tritylamylose is probably attributable to the use of an excess of lithium aluminum hydride and of immediate refluxing. Keto or oxime groups in monosaccharide derivatives can be reduced preferentially so as to favor inversion of the original configuration. However, the course of reduction is determined by the type, or rate of addition, of the reducing agent[18], the stereochemistry of the adjacent groups[7,8,19], and the anomeric configuration of the glycoside[20].

The maximum extent of amination attainable by the present method corresponded to a D.S. of 0.8 (as estimated by the nitrogen content of the oximated product). Further treatment of the previously oxidized 6-*O*-tritylamylose with methyl sulfoxide and acetic anhydride did not give a product having a higher degree of oxidation. However, when methyl 4,6-*O*-benzylidene-α-D-glucopyranoside was oxidized with the same reagent for 16 h, g.l.c. showed no trace of the starting material, and t.l.c. indicated the presence of seven components. This mixture, derived from the oxidation of the model compound, was then treated with sodium borohydride and the product debenzylidenated. Analysis of the resulting product by g.l.c. showed the presence of only one component; its retention time was identical with that of methyl α-D-glucopyranoside. Therefore, the products obtained from the oxidation of a monosaccharide derivative were quite different from that from a polysaccharide. The extended-coil shape of amylose probable in methyl sulfoxide solution[21] thus appeared to control the limit and the specificity of this oxidation reaction.

The formation of by-products containing (methylthio)methyl ether groups does not seem to occur appreciably when this oxidation method is employed to convert secondary hydroxyl groups in monosaccharide derivatives into the corresponding keto function[6-8], although there are examples in which this undesired side-reaction can give over 20% of the by-product[9]. Only traces of sulfur were found in the aminated amylose prepared by the present method.

In addition to its simplicity and high yield, the present method affords an amylose derivative containing, almost entirely, 2-amino-2-deoxy-D-glucose as the modified unit; the method is distinctly superior to other procedures[10-12] reported by this laboratory for the amination of secondary hydroxyl groups in amylose.

EXPERIMENTAL

General methods. — Descending paper-chromatography was effected on Whatman No. 1 or 3 MM paper by using 9:2:2 (v/v) ethyl acetate–acetic acid–water (Solvent A) and 5:1:5:3 ethyl acetate–acetic acid–pyridine–water (Solvent B). Zones were located by the silver nitrate–sodium hydroxide procedure, or with 0.2% ninhydrin in 1-butanol. R_{GN} indicates mobility relative to that of 2-amino-2-deoxy-D-glucose hydrochloride. T.l.c. was accomplished on glass plates coated with Silica Gel G, activated at 110°. The plates were developed with 9:1 benzene–acetone (Solvent C) and sprayed with sulfuric acid. R_F refers to mobilities relative to the solvent front (ascending technique). G.l.c. was performed on a Beckman GC-5 chromatograph equipped with a thermal-conductivity detector and a column (6 ft. × 0.125 in. I.D.) of copper tubing packed with 15% SE-52 silicone rubber on Chromosorb W (120 mesh). The temperature of the column was programmed at 4° per min from 160 to 210°, with a flow rate of helium of 100 ml/min. All evaporations were effected at 40° under diminished pressure. Analyses were made by W. N. Rond.

Oxidation of 6-O-tritylamylose. — 6-*O*-Tritylamylose[13] (40 g) was dissolved in purified[22] methyl sulfoxide (300 ml), with magnetic stirring at room temperature. Acetic anhydride (200 ml) was then added to the viscous solution, and stirring was continued for 16 to 20 h, to give a thin, light-brown solution. The oxidized product was precipitated by pouring into methanol (5 l), filtered off, washed exhaustively with methanol, and pressed to remove excess of methanol. (In a separate preparation, the yield of the oxidized product, after drying under diminished pressure, was 90%.)

Oximation of the oxidized 6-O-tritylamylose. — The oxidized product, still wet with methanol, was dissolved in pyridine (400 ml) containing hydroxylamine hydrochloride (35 g), and the solution was kept for 1 h at 60°. The solution was cooled, poured into ice–water (5 l) with vigorous stirring, and the precipitate washed exhaustively. The precipitate was filtered off, and dried in a vacuum oven at 45° to give a purple solid; yield 35 g, $[\alpha]_D^{25}$ +50° (*c* 0.3, *p*-dioxane).

Anal. Calc. for $[C_6H_5O_2(HOH)_{1.2}OC(C_6H_5)_3\ (NOH)_{0.8}]_n$: N, 2.70. Found: N, 2.76.

Repeated treatment of the oxidized 6-*O*-tritylamylose (6 g, vacuum-dried) with a solution of acetic anhydride (30 ml) in methyl sulfoxide (45 ml) gave a product (5.8 g) having a similar nitrogen content (2.71%) after oximation; yield 5.7 g.

Reduction of the oxime. — The oximated 6-*O*-tritylamylose (20 g) was dissolved in purified tetrahydrofuran (1 l) to give a cherry-red solution; powdered lithium aluminum hydride (25 g) was added, whereupon the color disappeared. The slurry

was magnetically stirred, immediately heated to boiling under reflux, and refluxed for 3 h; thereafter, the reduction was continued for 24 h at room temperature. Hydrochloric acid (M) at 0° was slowly added to the cooled solution (0°), to decompose the excess of hydride and to bring the pH to 7.0. The resultant slurry was poured into M hydrochloric acid (6 l) at 0°, with vigorous stirring. The precipitate was filtered off, washed with M acid, followed by distilled water until neutral, and air-dried, to give 18 g of a light-yellow powder. The product was dissolved in bis(2-methoxyethyl) ether (300 ml), the suspension was filtered to remove a small amount of insoluble material, and the filtrate was treated with sodium borohydride (5 g). Precipitation with 0.2 M hydrochloric acid (3 l) at 0°, followed by exhaustive washing with distilled water, and drying, gave a white powder; yield 18 g, $[\alpha]_D^{21}$ +45° (*c* 1.0, *p*-dioxane).

Anal. Calc. for $[C_6H_5O_2(HOH)_{1.4}\ OC(C_6H_5)_3\ (NH_3Cl)_{0.6}]_n$: N, 1.89. Found: N, 1.88.

Detritylation. — The aminated 6-*O*-tritylamylose (8 g) was suspended in methanol (250 ml) containing concentrated hydrochloric acid (2.5 ml), and the suspension was stirred magnetically for 1 h. Ether (500 ml) was added to facilitate the filtration of the detritylated product (yield, 3 g). The aminated amylose was dissolved in water, residual hydrochloric acid was neutralized with sodium hydrogen carbonate, and the solution was dialyzed in a cellophane tube against running tap-water for 24 h. The undialyzed portion was filtered through a Millipore filter, and the filtrate was freeze-dried to give a white material; yield 1.8 g, $[\alpha]_D^{21}$ +156° (*c* 0.5, water).

Anal. Calc. for $[C_6H_7O_2(OH)_{2.4}\ (NH_3Cl)_{0.6}]_n$: C, 39.22; H, 5.94; N, 4.58. Found: C, 39.17; H, 5.79; N, 4.51.

Acid hydrolysis of the aminated amylose. — The aminated amylose (0.6 g) was hydrolyzed[23] with 1.5 M hydrochloric acid (60 ml) in sealed tubes for 3 h at 100°. The hydrolyzate was evaporated to dryness, the residue was dissolved in water, and the remaining hydrochloric acid was neutralized with Amberlite IRA-410 (CO_3^{2-}) ion-exchange resin. The aqueous solution was decolorized with carbon, and evaporated to a syrup (0.25 g). A small drop of this syrup was diluted with distilled water, and chromatographed on paper (solvent A), against standards. Spraying with alkali–silver nitrate revealed the presence of two major components having mobilities identical with those of D-glucose and 2-amino-2-deoxy-D-glucose. Small proportions of ninhydrin-positive materials having R_{GN} 0.35 and 0.66 were also present. Irrigation of another chromatogram with solvent B gave identical resolution of the same four components.

A solution of the syrup in distilled water (1.5 ml) was percolated through a column (1 × 10 cm) of Amberlite IR-120 (H^+) ion-exchange resin. After thorough washing of the column with distilled water (300 ml), the amino sugars were desorbed from the resin with 0.05M hydrochloric acid (50 ml), and the acid eluate was evaporated. Residual acid was removed by evaporation with 1-propanol, to give a mixture of crystals and syrup (0.16 g). Preparative paper-chromatography (solvent B) separated a fraction having a mobility identical with that of 2-amino-2-deoxy-D-glucose from slower-moving components (R_{GN} 0.35 and 0.66). Rechromatography with

solvents A and B showed that the faster-moving fraction was homogeneous, yet no crystals formed in several attempted crystallizations from aqueous ethanol. The solution was evaporated to dryness, and the resulting syrup was dried under diminished pressure (oil pump) to give a glassy mass; yield 130 mg. Concentrated hydrochloric acid (4 drops) was added to dissolve the material, and acetone was added to incipient turbidity. Needle-shaped crystals developed on refrigeration overnight. Recrystallization from aqueous ethanol gave fine needles; yield 114 mg, $[\alpha]_D^{21}$ +71° (water; equilibrium); the i.r. spectrum and X-ray powder diffraction pattern were indistinguishable from those of an authentic sample of 2-amino-2-deoxy-D-glucose hydrochloride.

Oxidation of the model compound. — Methyl 4,6-*O*-benzylidene-α-D-glucopyranoside[24] (5.0 g) was oxidized with 100 ml of 3:2 (v/v) methyl sulfoxide–acetic anhydride in the same way as for 6-*O*-tritylamylose. The oxidized glycosides were recovered as a yellow, glassy mass by exhaustive freeze-drying; yield 5.05 g. T.l.c. with solvent C resolved the oxidized material into seven components: R_F 0.22, 0.29, 0.44, 0.56, 0.62, 0.69, and 0.75. G.l.c. of the oxidized glycosides as their per(trimethylsilyl) ethers[25] indicated complete absence of the starting material. Attempted separation of the components by column chromatography on silica gel was unsuccessful.

Reduction of the oxidized model compound. — The glassy, oxidized product (1.0 g) was dissolved in methanol (100 ml), sodium borohydride (0.3 g) was added, and the mixture was stirred for 1 h. The excess of hydride was decomposed with solid carbon dioxide, the solution was evaporated to dryness, and the residue was extracted with chloroform, to separate the product from the inorganic salts. Evaporation of the solution gave a white powder; yield 0.95 g. G.l.c. of the per(trimethylsilyl) ether of the debenzylidenated[24] product showed that the reduced material contained a single compound having a retention time identical with that of methyl α-D-glucopyranoside.

ACKNOWLEDGMENTS

This work was supported by funds from the State of Ohio, administered by the Office of Research, The Ohio State University. The technical assistance of Mrs. Jane Winer is acknowledged.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

SYNTHESIS AND O.R.D./C.D. SPECTRA OF THE ANOMERS OF 2-AMINO-5-ETHOXYPHENYL D-GLUCOPYRANOSIDURONIC ACID AND SOME DERIVATIVES THEREOF

J. KISS AND F. BURKHARDT
Chemical and Physical Research Department, F. Hoffmann-La Roche & Co., Ltd., Basel (Switzerland)
(Received May 19th, 1969; in revised form, July 7th, 1969)

ABSTRACT

Metabolites of phenetidine and phenacetin, namely, 2-amino-5-ethoxyphenyl β-D-glucopyranosiduronic acid and 2-acetamido-5-ethoxyphenyl β-D-glucopyranosiduronic acid were synthesized from 5-ethoxy-2-nitrophenol and the D-glucosyluronic bromide, by the method of Koenigs and Knorr. The β anomers of the D-glucopyranosiduronic acid were obtained; these were identical with the natural metabolites.

For proof of the anomeric configuration, the corresponding α-anomers of the aryl D-glucosides and D-glucosiduronic acids were also synthesized: condensation of the above aglucon with the Brigl anhydride led to the α-D-glucopyranoside, which was then oxidized to the corresponding α-D-glucopyranosiduronic acid.

The o.r.d. and c.d. spectra of these aryl α- and β-D-glucopyranosides and -D-glucopyranosiduronic acids were recorded, and preliminary indications as to a relationship between the sign of the Cotton effect in the region of 274–280 nm and the anomeric configuration were obtained.

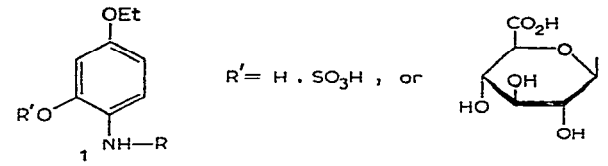
Furthermore, some data from the n.m.r. spectra of the pure anomers of these D-glucosides and D-glucopyranosiduronic acids are given.

INTRODUCTION

During the metabolic breakdown of phenetidine and phenacetin, water-soluble anionic metabolites are produced. Thus far, the following classes of such compounds have been isolated: (1) phenols, (2) phenol sulfates, (3) phenyl D-glucosiduronic acids, and (4) conjugates with cysteine[1]. A summary of the main metabolic pathway for D-glucosiduronic acids was recently published[2]. Enzymic cleavage of the ethoxyl group leads to *p*-amino(or *p*-acetamido)-phenol, which combines with sulfuric acid, giving the corresponding phenol sulfates, or with D-glucuronic acid, resulting in the D-glucosiduronic acid[3]. These compounds were found to be the main metabolites.

From the urine of cats, dogs, and man, Conney *et al.*[4] isolated a further metabolite, namely, 2-acetamido-5-ethoxyphenol. Recently, Ullrich *et al.*[5] have, in addition, reported the existence, as metabolites, of 3-acetamido-6-ethoxyphenol and its conjugate with sulfuric acid. The proportion of each of these metabolites was determined and discussed.

Two further special D-glucosiduronic acid metabolites were isolated by Dreiding *et al.*[6] from the urine of a patient sensitive to acetophenetidine, namely, 2-amino-5-ethoxyphenyl D-glucosiduronic acid (**1a**, R = H, R′ = D-glucosyluronic acid) and 2-acetamido-5-ethoxyphenyl D-glucosiduronic acid (**1b**, R = Ac, R′ = D-glucosyluronic acid).



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(a R=H, b R=Ac)

These products were characterized analytically and by physical methods (u.v., i.r., and n.m.r. spectra). The structure of the aglucons (2-amino-5-ethoxyphenol and 2-acetamido-5-ethoxyphenol) was proved by synthesis. Furthermore, Dreiding *et al.*[6] prepared some readily crystallized derivatives of these D-glucosiduronic acids, *e.g.*, methyl [5-ethoxy-2-(trifluoroacetamido)phenyl 2,3,4-tri-*O*-acetyl-D-glucopyranosid]-uronate (**2i**).

We have synthesized the above-mentioned D-glucosiduronic acids in order to check their anomeric configuration and biological activity. For comparative studies, we have synthesized both anomers of the D-glucopyranosides and D-glucopyranosiduronic acids by two independent methods, resulting in the pure anomers.

RESULTS AND DISCUSSION

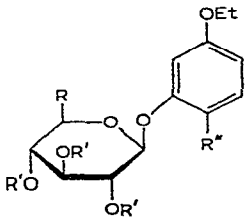
Synthesis of the β-D-glucopyranosides and β-D-glucopyranosiduronic acids. — By the classical method of Koenigs and Knorr[7], tetra-*O*-acetyl-α-D-glucopyranosyl bromide was condensed with 5-ethoxy-2-nitrophenol* in pure quinoline, affording the corresponding phenyl β-D-glucopyranoside derivative (**2a**) in a yield of > 90%. After cleavage of the acetyl groups, the primary hydroxyl group of the resulting β-D-glucopyranoside **2b** was catalytically oxidized with oxygen in the presence of platinum to the corresponding β-D-glucopyranosiduronic acid **2d** by the method of Heyns and Paulsen[8].

The purification of **2d** can be accomplished by recrystallization, or by preparation of its benzyl ester (**2f**) by treating the free acid with α-diazotoluene[9]. Hydrogenation of the nitro group and hydrogenolysis of the benzyl group gave the pure, free D-glucosiduronic acid **2j**, identical with one of the metabolites of phenetidine isolated by Dreiding *et al.*[6].

The *N*-acetyl derivative, the second metabolite, was obtained synthetically in the following the way: the β-D-glucopyranoside **2a** was reduced to the corresponding amine, and the latter was acetylated. The *O*-acetyl groups on the D-glucopyranosyl group were removed, and the resulting free D-glucopyranoside (**2c**) was oxidized

*B. Hegedüs has developed for 5-ethoxy-2-nitrophenol a rational synthesis that will be described elsewhere.

catalytically to the corresponding D-glucosiduronic acid (**2e**). The latter proved to be identical with the second "anomalous" metabolite of phenacetin isolated by Dreiding *et al.*[6].



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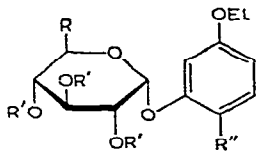


3

2 and 3	R	R'	R''
a	CH_2OAc	Ac	NO_2
b	CH_2OH	H	NO_2
c	CH_2OH	H	NHAc
d	CO_2H	H	NO_2
e	CO_2H	H	NHAc
f	CO_2CH_2Ph	H	NO_2
g	CO_2Me	Ac	NO_2
h	CO_2Me	Ac	NHAc
i	CO_2Me	Ac	$NHCOCF_3$
j	CO_2H	H	NH_2
k	$CONH_2$	H	NHAc
l	CH_2OAc	Ac	NHAc

In addition, we also obtained these uronic derivatives by condensation of methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-*β*-D-glucopyranuronate[10] with 5-ethoxy-2-nitrophenol by the method of Koenigs and Knorr[4]. The D-glucosiduronate **2g** obtained was reduced to the corresponding aniline derivative, which gave the *N*-acetyl derivative (**2h**) on acetylation, and the *N*-(trifluoroacetyl) derivative (**2i**) with trifluoroacetic anhydride. The latter compound proved to be identical with a product obtained by Dreiding *et al.*[6] from the metabolite of phenacetin and phenetidine by esterification and acylation.

Besides these compounds, some other D-glucopyranosiduronic acid derivatives were also prepared: the acetylated methyl ester (**2h**) was obtained from the acid **2d** by (*a*) methylation of the carboxyl group with diazomethane, (*b*) reduction of the nitro group to an amine group, and (*c*) acetylation of the free hydroxyl and amino groups. Ammonolysis of **2h** led to amide **2k**.

Synthesis of the α-D-glucopyranosides and α-D-glucopyranosiduronic acids. — According to the literature[12], and also our own experiments*, by reaction with the Brigl anhydride[13], phenols give mainly the α-anomers of the phenyl D-glucopyranosides. We have used this method for the synthesis of the α-D-glucopyranosides of the 2-substituted 5-ethoxyphenols. The corresponding phenyl α-D-glucopyranosiduronates were obtained therefrom by catalytic oxidation with oxygen in the presence of platinum by the method of Heyns and Paulsen[8].

*In connection with the synthetic analogs of Novobiocin, which were studied in 1958–1960, we had

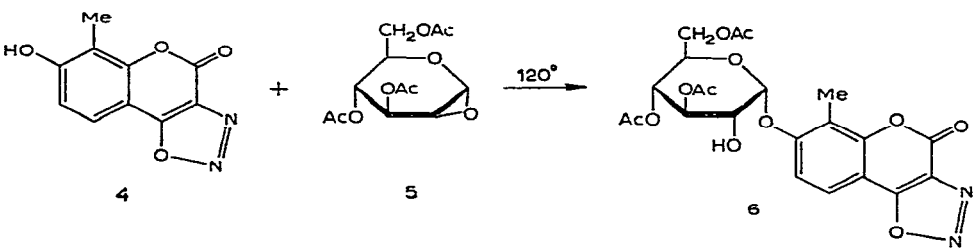
The condensation of 5-ethoxy-2-nitrophenol with the Brigl anhydride was conducted at 120° in boiling toluene. Subsequent reduction of the nitro group, and acetylation of the resulting aminohydroxy compound, gave compound **3l**. Cleavage of the *O*-acetyl groups resulted in the free α-D-glucopyranoside, which was oxidized to the corresponding α-D-glucosiduronic acid (**3e**). The methyl ester of compound **3e** was prepared with diazomethane, and this was acetylated to give the D-glucosiduronate (**3h**), which proved to be the pure α-anomer.

N.m.r. spectra. — As is well known, measurement of the proton–proton coupling-constant $J_{1,2}$ has proved to be an ideal method for determining the anomeric configuration of glycosides[11] in solution. We have, however, found that it is not possible to deduce, from their n.m.r. spectra, the configuration of the described aryl D-glucopyranosides and D-glucopyranosiduronates obtained by synthesis by the method of Koenigs and Knorr, because the chemical-shift differences in $CDCl_3$ solution were rather small at 60, and even at 100 MHz. The problem seems to be similar to the determination of the anomeric configuration of glycopyranosyl and glycopyranosyluronic bromides and chlorides[10]: in these cases, the determination of the stereochemical position of the β-anomeric proton (of the α-halides) can be carried out normally, in contrast to that for the α-anomeric proton (of the β-halides)[10].

Independent of these problems, we have recently found that the determination of the spin–spin coupling-constant of 3,4,6-tri-*O*-acetyl-2-*O*-(trichloroacetyl)-β-D-hexopyranosyl chloride cannot be carried out in $CDCl_3$ solution, but is rather easily performed in benzene-d_6 at 100 MHz. On this basis, we used benzene-d_6 as the solvent for the D-glucopyranosides and D-glucopyranosiduronates prepared by the Koenigs–Knorr method, but without any success.

Therefore, we synthesized the *other* anomer of the D-glucopyranosides and D-glucopyranosiduronates by a stereospecific synthesis, as already mentioned, to obtain evidence for the anomeric configuration by the indirect way of exclusion. The group of the aryl α-D-glucopyranosides and α-D-glucopyranosiduronates gave no difficulties, and determination of the stereochemical position of the anomeric protons was achieved (in $CDCl_3$ or in benzene-d_6 solution) at 60 or 100 MHz. The peaks are sufficiently resolved to permit configurational and conformational deductions. Thus, for 2-acetamido-3-ethoxyphenyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside (**3l**), the

found that such hydroxycoumarin derivatives as 7-hydroxy-8-methyl-3,4-(oxadiazolo)coumarin react with the Brigl anhydride[13] to give the α-D-glucopyranosides (see Experimental section).



following proton–proton coupling-constants were found: $J_{1,2} = 3.5$ Hz, $J_{2,3} = 10$ Hz, and $J_{3,4} = 9.5$ Hz. For the corresponding methyl uronate (**3h**), $J_{1,2} = 3.2$ Hz.

These aryl D-glucopyranosides and D-glucopyranosiduronates proved to be the pure α anomers; therefore, the other group of D-glucopyranosides (which are identical with the "anomalous" metabolites) must have the β-anomeric configuration. Thus, the "anomalous" metabolites of phenacetin and phenetidine have the same (*i.e.*, the β) conjugation as the "normal" metabolites*.

After conclusion of these experiments, we were able to use a 220-MHz n.m.r. spectrometer for studying the β-anomers of the aryl D-glucopyranosides and D-glucopyranosiduronates. In benzene-d_6 or in $CDCl_3$ solution, it was found possible to establish the anomeric configuration by determining the H-1–H-2 coupling-constant, $J_{1,2}$; the values were: for **2i**, 7.5 Hz; **2h**, 7.0 Hz; **2a**, 7.7 Hz; and **2l**, 7.0 Hz. These results are in agreement with the β-D anomeric configuration if the compounds assume the *CI*(D) conformation.

O.r.d. and c.d. spectra. — In connection with the stereochemistry of the anomeric carbon atom in the synthetic α- and β-D-glucopyranosides and -D-glucopyranosiduronic acids, we have also studied the i.r., o.r.d., and c.d. spectra. Comparative studies of some of the aryl α- and β-D-glucopyranosides and -D-glucopyranosiduronic acids are briefly described.

In Figs. 1–3, the o.r.d., c.d., and u.v. spectra of some derivatives of 2-amino-5-ethoxyphenyl α- and β-D-glucopyranosides and -D-glucopyranosiduronic acids are given; the difference between the spectra of each pair of anomers is distinct.

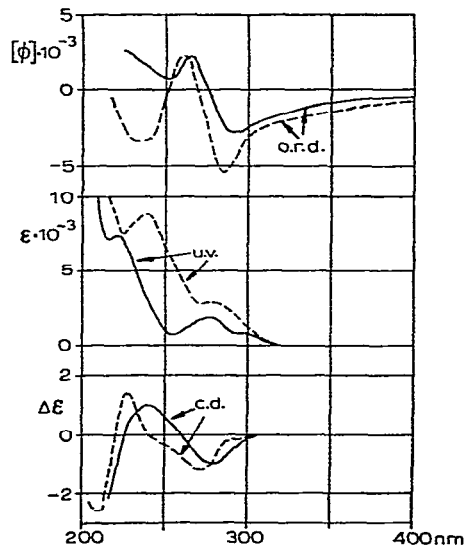


Fig. 1. O.r.d., c.d., and u.v. spectra of 2-amino-5-ethoxyphenyl β-D-glucopyranosiduronic acid (**2j**) (— — — —) and 2-acetamido-5-ethoxyphenyl β-D-glucopyranosiduronic acid (**2e**) (————).

*Determination of the anomeric configuration of the "normal" metabolic D-glucosiduronic acids was also performed by the method of configurative correlation between the aryl and alkyl D-glucopyranosiduronates. The results of these experiments will be published later.

The c.d. curves show at least three absorption bands in the region of 200–300 nm; generally, the c.d. extrema do not agree exactly with the u.v. absorption maxima. On the other hand, it is not possible to read, from the o.r.d. curves, the number, magnitude, and sign of all of the Cotton effects.

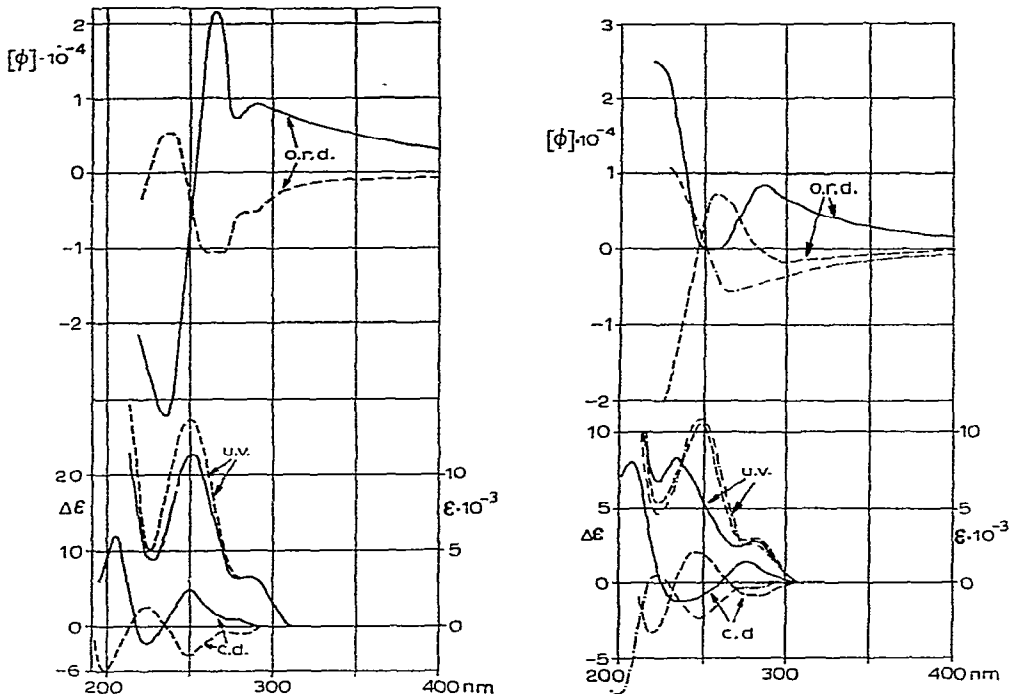


Fig. 2. O.r.d., c.d., and u.v. spectra of 2-acetamido-5-ethoxyphenyl β-D-glucopyranoside (**2c**) (— — — —) and 2-acetamido-5-ethoxyphenyl α-D-glucopyranoside (**3c**) (————).

Fig. 3. O.r.d., c.d., and u.v. spectra of methyl (2-acetamido-5-ethoxyphenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid)uronate (**2h**) (— — — —), methyl (2-acetamido-5-ethoxyphenyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosid)uronate (**3h**) (————), and 2-acetamido-5-ethoxyphenyl β-D-glucopyranosiduronamide (**2k**) (—.—.—.—.).

On the basis of the present results for 2-acetamido-5-ethoxyphenyl D-glucopyranosides and D-glucopyranosiduronic acids, the following rule is established: the c.d. curves (and, also, to a minor extent, the o.r.d. curves) are approximately mirror images of one another if the anomeric π-electron sextet in the α- or β-position is linked through an oxygen bridge to the anomeric carbon atom. The sign of the Cotton effect in the region of 274–280 nm is positive for the aryl α-D-glucopyranosides, and negative for the β anomers thereof. The sign seems to be independent of the other substituents on the pyranoside ring, but was found to be influenced by the substituents on the aromatic moiety; the latter question will be discussed in the near future.

EXPERIMENTAL

Melting points were determined in a Büchi (Flawil, Switzerland) capillary, melting-point apparatus, and are uncorrected. Microanalyses were performed in our microanalytical laboratory (Dr. A. Dirscherl). The i.r. spectra were recorded with a Beckman i.r. spectrophotometer, Model IR-9, with KBr optics (Dr. L. Chopard) for potassium bromide pellets. The u.v. spectra were recorded with a Cary Recording Spectrophotometer, Model 14. For determination of the n.m.r. spectra, Varian A-60, A-100, and 220 spectrometers were used (Dr. G. Englert). The X-ray diagrams were recorded on a Philips Diffractometer (Dr. W. Oberhänsli). The o.r.d. spectra were recorded on an automatic spectropolarimeter, constructed in our physical laboratories, that provided measurements down to 215 nm. The c.d. spectra were recorded on a Roussel–Jouan Dichrographe c.d. 185. The concentration of the solutions was 0.1%, if not otherwise indicated. The optical path-length of the solutions was so chosen as to give sufficient transmission of light. The temperature of measurements was +25°. The exact values of the extrema are listed in Table I.

For recrystallization of most of the compounds obtained, a mixture of ethyl acetate and petroleum ether (b.p. 40–45°), solvent *A*, was used in the following way: the compound was dissolved in warm ethyl acetate (5–10 ml per g), and petroleum ether was added slowly, with stirring, until the resulting turbidity disappeared. The crystallization then proceeded mostly at room temperature.

5-Ethoxy-2-nitrophenyl 2,3,4,6-tetra-O-acetyl-β-D-glucoside (**2a**). — 5-Ethoxy-2-nitrophenol (20 g) and tetra-*O*-acetyl-α-D-glucopyranosyl bromide (62 g) were dissolved in 350 ml of freshly distilled quinoline. To this solution was added a micronized mixture of dry silver oxide (20 g) and anhydrous calcium sulfate ("Siccon"-Fluka, 40 g). The flask was wrapped in aluminum foil (to protect the contents from light), and was shaken for 18–20 h at 22–25°. After dilution with 600 ml of dichloromethane, the dark-brown mixture was filtered, and the inorganic residues were washed with fresh dichloromethane (2 × 200 ml). The dichloromethane solutions were combined, cooled to 5–8°, and washed successively with cold (5–8°) 3M hydrochloric acid (3 × 500 ml), sodium hydrogen carbonate solution (5%, 300 ml), and distilled water. The red solution was dried (anhydrous sodium sulfate), and evaporated *in vacuo* at 40°; the oily residue crystallized immediately. For purification, the product was dissolved in benzene, and chromatographed on Silica Gel or on Florisil (1 kg), by using benzene and benzene–ether for elution. The pure D-glucoside was eluted with 10–40% ether–benzene, giving yellowish white crystals, which were recrystallized from solvent *A*; yield: 51 g (91%), m.p. 145–146°, $[\alpha]_D^{25}$ +27.7° (*c* 1.09, ethyl acetate).

Anal. Calc. for $C_{22}H_{27}NO_{13}$: C, 51.46; H, 5.31; N, 2.73. Found: C, 51.72; H, 5.43; N, 3.03.

5-Ethoxy-2-nitrophenyl β-D-glucopyranoside (**2b**). — 5-Ethoxy-2-nitrophenyl β-D-glucopyranoside tetraacetate (4 g) was dissolved in methanol (30 ml) at 30°, the solution was cooled to room temperature, and dry ammonia gas was introduced for 10 min. After the exothermic reaction had ceased, the solution was cooled in a

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TABLE I

NUMERICAL VALUES OF O.R.D., C.D., AND U.V. DATA AT WAVELENGTHS OF EXTREMA; FOR O.R.D., AT THE SODIUM D LINE, ALSO

Cpd.	*O.r.d.*				*C.d.*				*U.v.*			
	Solvent	*c* (%)	λ (*nm*)[a]	[α] (*degrees*)	*Solvent*	*c* (%)	λ (*nm*)[a]	Δε (l.cm^{-1}.mol^{-1})	*Solvent*	*c* (%)	λ (*nm*)[a]	ε (l.cm^{-1}.mol^{-1})
2j	H_2O	0.1	Na_D	−83 ±5	H_2O	0.02	298 (sh)	−0.14 ±0.04	H_2O	0.005	280	2,920
						0.02	274	−1.23 ±0.04		0.005	239	8,830
		0.1	262	+600 ±500		0.02	245 (sh)	−0.12 ±0.04				
		0.1	235	−900 ±500		0.1	224	+1.1 ±0.4				
2e	MeOH	0.1	Na_D	−62 ±5		0.004	280 (sh)	−0.73 ±0.2		0.002	284	3,480
		0.1	286 (sh)	−1,400 ±500	MeOH	0.004	248	−4.0 ±0.2	EtOH	0.002	250	13,800
		0.1	265	−3,000 ±5,000		0.1	220	+2.9 ±0.3	+ 4% of			
		0.1	236	+5,000 ±5,000					H_2O			
3c	H_2O	0.1	Na_D	−67 ±5	H_2O	0.2	278	−0.88 ±0.04	H_2O	0.006	294 (sh)	970
		0.1	292	−800 ±500		0.04	239	+1.1 ±0.3		0.006	275	1,900
		0.1	264	+700 ±500						0.006	220	7,500
		0.1	250	+200 ±500								
2h	MeOH	0.1	Na_D	−23 ±5		0.1	350	+0.025 ±0.005		0.005	282	2,960
		0.1	300	−300 ±100	MeOH	0.1	280	−1.1 ±0.3		0.005	249	10,600
		0.1	257	+1,500 ±1,000		0.004	245	+1.7 ±0.3	EtOH			
						0.004	220	−2.8 ±0.3	+ 4% of			

						0.1	225	+0.5 ±0.2						
						0.1	200	−7.4 ±1						
3c	MeOH	0.1	Na_D	+169 ±5		0.004	275 (sh)	+1.0 ±0.2			0.005	285	3,300	
		0.1	286	+2,600 ±500	MeOH	0.004	250	+4.9 ±0.2	EtOH		0.005	251	13,100	
		0.1	270	+2,100 ±500		0.1	220	−2.8 ±0.7	+ 4% of H_2O					
		0.1	263	+6,000 ±5,000		0.1	204	+12 ±2						
		0.1	232	−9,000 ±5,000										
3h	MeOH	0.1	Na_D	+120 ±5	MeOH	0.004	276	+1.23 ±0.25	EtOH		0.005	281	2,800	
		0.1	288	+1,600 ±500		0.004	235	−1.25 ±0.25	+4% of H_2O		0.005	233	8,140	
		0.1	264	−100 ±500		0.1	211	+8.5 ±2						
		0.02	225	+5,000 ±2,500										

[a]Sh = shoulder.

bath at −15°, and saturated with ammonia gas (30 min). After the mixture had been kept for 20 h at room temperature, the solution was evaporated, and the residue was recrystallized from isopropyl alcohol–isopropyl ether; m.p. 141–142°, $[\alpha]_D^{25}$ −95.5° (*c* 0.3, ethanol).

Anal. Calc. for $C_{14}H_{19}NO_9$: C, 48.69; H, 5.55; N, 4.06. Found: C, 48.64; H, 5.61; N, 4.16.

5-Ethoxy-2-nitrophenyl β-D-glucopyranosiduronic acid (**2d**) *and benzyl (5-ethoxy-2-nitrophenyl β-D-glucopyranosid)uronate* (**2f**). — In a 4-necked, 500-ml flask (provided with a Vibro mixer, gas-inlet tube, reflux condenser, and thermometer), 5-ethoxy-2-nitrophenyl β-D-glucopyranoside (**2b**) (3 g) was dissolved in 200 ml of water; platinum (4 g) and sodium hydrogen carbonate (0.9 g) were introduced, and, with mechanical stirring, oxygen was bubbled into the solution during 15 h at 80°. The catalyst was filtered off by suction, and the clear filtrate was concentrated *in vacuo* at 40–45° to 80 ml. The free uronic acid was obtained by treating this solution with Amberlite IRC-120 (H^+) ion-exchange resin, and the aqueous, acidic solution was concentrated *in vacuo* at 25–30° (bath temp.) to ~50 ml, and kept in a refrigerator; part of the free acid crystallized (400 mg); m.p. 123° (dec.), $[\alpha]_D^{25}$ −125° (*c* 0.198, water).

Anal. Calc. for $C_{14}H_{17}NO_{10}$: C, 46.80; H, 4.76. Found: C, 46.75; H, 5.05.

The mother liquor was diluted with methanol (100 ml) and ethyl ether (50 ml), and α-diazotoluene* in petroleum ether (b.p. 40–45°) was added. When the evolution of nitrogen had ceased, the mixture was evaporated at 40°, and the residue (2.2 g) was chromatographed on Florisil (50 g). The benzyl D-glucosiduronic acid was eluted with 1:1 and 1:3 (v/v) benzene–ethyl acetate. The crystals obtained were recrystallized from solvent *A*; yield 400 mg, m.p. 152–154°.

Anal. Calc. for $C_{21}H_{23}NO_{10}$: C, 56.12; H, 5.16; N, 3.12 Found: C, 56.09; H, 5.13; N, 3.25.

2-Amino-5-ethoxyphenyl β-D-glucosiduronic acid (**2j**). — *A. By catalytic hydrogenation of 5-ethoxy-2-nitrophenyl β-D-glucopyranosiduronic acid* (**2d**). Compound **2d** (250 mg) was hydrogenated with 200 mg of 5% palladium-on-carbon in water (50 ml). After the theoretical volume of hydrogen had been absorbed, the mixture was filtered, and the filtrate was lyophilized. A snow-white, microcrystalline substance was obtained, which turned brownish red on standing in air; m.p. 225–230° (dec.), $[\alpha]_D^{25}$ −82 ±5° (*c* 0.1, water).

Anal. Calc. for $C_{14}H_{19}NO_8 \cdot 0.5H_2O$: C, 49.80; H, 5.96. Found: C, 49.40; H, 6.08.

B. By hydrogenolysis of benzyl (5-ethoxy-2-nitrophenyl β-D-glucopyranosid)uronate (**2f**). Compound **2j** can also be obtained from the benzyl derivative **2f**. Hydrogenation of the nitro group and hydrogenolysis of the benzyl ester (250 mg)

*α-Diazotoluene was obtained by oxidation of benzaldehyde hydrazone with HgO in petroleum ether by the method of Staudinger[9].

were conducted in 5:2 (v/v) water–*p*-dioxane (70 ml) in the presence of palladium black (100 mg) and Norit (200 mg).

2-Acetamido-5-ethoxyphenyl 2,3,4,6-tetra-O-acetyl-β-D-glucoside (**2l**). — 5-Ethoxy-2-nitrophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucoside (**2a**; 4 g) was dissolved in ethyl acetate (80 ml), and hydrogenated with 0.4 g of 5% palladium-on-carbon. When no more hydrogen was absorbed, the catalyst was filtered off, and the filtrate was evaporated. The colorless, crystalline residue was recrystallized from solvent *A*; m.p. 129–130°, $[\alpha]_D^{25}$ −30.2° (*c* 0.5, ethyl acetate).

Anal. Calc. for $C_{22}H_{29}NO_{11}$: C, 54.65; H, 6.04; N, 2.89. Found: C, 54.63; H, 5.99; N, 3.12.

The obtained compound was dissolved in absolute pyridine (50 ml), and acetic anhydride (5 ml) was added at room temperature. After 16 h, the excess of the anhydride was decomposed by addition of water (3 ml). After 30 min, the mixture was diluted with 200 ml of water, made neutral by addition of sodium hydrogen carbonate, with stirring, and extracted with two 200-ml portions of dichloromethane. The extracts were combined, washed with 3M hydrochloric acid and sodium hydrogen carbonate solution, and dried (sodium sulfate). On evaporation of the solvent, a crystalline product (3.3 g) was obtained; this was recrystallized from solvent *A*, m.p. 144–145°, $[\alpha]_D^{25}$ − 31.2° (*c* 0.49, ethyl acetate).

Anal. Calc. for $C_{24}H_{31}NO_{12}$: C, 54.85; H, 5.95; N, 2.67. Found: C, 54.90; H, 6.09; N, 2.93.

2-Acetamido-5-ethoxyphenyl β-D-glucopyranoside (**2c**). — A solution of compound **2l** (2 g) in methanol (100 ml) was saturated at −10° with ammonia, and kept for 12 h at 25°; the mixture was evaporated, and the crystalline residue was recrystallized from a mixture of isopropyl alcohol (40 ml) and isopropyl ether (100 ml); m.p. 162–163°, $[\alpha]_D^{25}$ −66.2° (*c* 0.56, methanol).

Anal. Calc. for $C_{16}H_{23}NO_8$: C, 53.77; H, 6.49; N, 3.92. Found: C, 53.75; H, 6.79; N, 4.04.

2-Acetamido-5-ethoxyphenyl β-D-glucopyranosiduronic acid (**2e**). - 2-Acetamido-5-ethoxyphenyl β-D-glucopyranoside (**2c**; 5 g) was oxidized in water (300 ml) in the presence of platinum black (8 g) and sodium hydrogen carbonate (1.25 g) with oxygen (Vibro mixer) for 18 h at 80°.

The catalyst was filtered off by suction, and the filtrate was concentrated to ~ 100 ml. The free uronic acid was obtained by treatment of the solution with Amberlite IRC-120 (H^+) ion-exchange resin. The acidic solution obtained was concentrated at 35–40° *in vacuo* to 30–40 ml, and kept in a refrigerator; the free acid crystallized, m.p. 192–194° (dec.), $[\alpha]_D^{25}$ −67.3° (*c* 0.35, water).

Anal. Calc. for $C_{16}H_{21}NO_9$: C, 51.75; H, 5.70; N, 3.77. Found: C, 51.63; H, 5.68; N, 3.72.

Methyl (5-ethoxy-2-nitrophenyl 2,3,4-tri-O-acetyl-β-D-glucosid)uronate (**2g**). — 5-Ethoxy-2-nitrophenol (5 g) and methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate (13.5 g) were dissolved in freshly distilled quinoline (130 ml). A micronized mixture of dry silver oxide (6 g) and anhydrous calcium sulfate ("Siccon"-

Fluka, 13 g) was added, and the suspension was shaken for 18–20 h at 25° with protection from light. The mixture was diluted with dichloromethane (400 ml), and the inorganic salts were filtered off with suction and washed with dichloromethane (100 ml). The filtrate and washings were combined, washed successively with 2M hydrochloric acid (3 × 400 ml), sodium hydrogen carbonate solution (5%; 300 ml), and distilled water, and dried (anhydrous sodium sulfate). Evaporation of the solvent left a dark oil which soon crystallized. Purification of the product was achieved by chromatography on Florisil (350 g), with benzene and benzene–ether as eluants. Evaporation of the eluates gave crystals; these were recrystallized from solvent *A*; yield 9.6 g, m.p. 130–131°, $[\alpha]_D^{25}$ +8.9° (*c* 1.24, ethyl acetate).

Anal. Calc. for $C_{21}H_{25}NO_{13}$: C, 50.50; H, 5.05; N, 2.81. Found: C, 50.36; H, 5.10; N, 2.98.

Methyl (2-acetamido-5-ethoxyphenyl 2,3,4-tri-O-acetyl-β-D-glucosid)uronate (**2h**). — Methyl (5-ethoxy-2-nitrophenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid)uronate (**2g**; 3.2 g) was hydrogenated in ethyl acetate (60 ml) with 5% palladium-on-carbon (0.5 g) at 25°. The suspension was filtered, the filtrate was evaporated, and the residue was acetylated with acetic anhydride (3 ml) in absolute pyridine (60 ml). After 20 h, the mixture was poured into water (200 ml) with mechanical stirring. The precipitate was filtered off with suction, washed with water, dried over calcium chloride in a desiccator, and recrystallized from solvent *A*; yield 2.55 g, m.p. 156–157°, $[\alpha]_D^{25}$ −34.7° (*c* 0.52, ethyl acetate).

Anal. Calc. for $C_{23}H_{29}NO_{12}$: C, 54.01; H, 5.72; N, 2.74. Found: C, 54.14; H, 5.68; N, 2.99.

The compound can also be obtained from 2-amino-5-ethoxyphenyl β-D-glucopyranosiduronic acid (**2j**) by acetylation with acetic anhydride in pyridine, followed by formation of the methyl ester with diazomethane.

2-Acetamido-5-ethoxyphenyl β-D-glucopyranosiduronamide (**2k**). — A solution of methyl (2-acetamido-5-ethoxyphenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid)uronate (**2h**; 500 mg) in absolute methanol (20 ml) in a pressure tube was saturated at 0° with ammonia. The mixture was kept for 24 h at 25°; it was then evaporated, and the glassy residue was crystallized from isopropyl alcohol; m.p. 204°, $[\alpha]_D^{25}$ −82.0° (*c* 0.103, methanol).

Anal. Calc. for $C_{16}H_{22}N_2O_8$: C, 51.88; H, 5.99; N, 7.56. Found: C, 51.82; H, 5.99; N, 7.56.

Methyl [5-ethoxy-2-(trifluoroacetamido)phenyl 2,3,4-tri-O-acetyl-β-D-glucosid]uronate (**2i**). — Methyl (5-ethoxy-2-nitrophenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid)-uronate (**2g**; 4.5 g) in ethyl acetate (100 ml) was hydrogenated in the presence of Pd–carbon (0.5 g; 5% Pd) at 25°. The theoretical volume of hydrogen was absorbed within 3h. The catalyst was filtered off, the filtrate was evaporated to dryness at 30° *in vacuo*, the residue was dissolved in absolute pyridine (50 ml) and, at 0–5°, a solution of trifluoroacetic anhydride (1.00 ml) in benzene (20 ml) was slowly added, with mechanical stirring, during 10–12 min. The mixture was kept overnight in a refrigerator, and then the benzene was evaporated off *in vacuo* at 20°. The solution was

poured into ice–water (200 ml) with mechanical stirring, and the oily product which separated was extracted into dichloromethane (200 ml). The extract was washed successively with cold 2M hydrochloric acid (2×200 ml), 5% sodium hydrogen carbonate solution (200 ml), and water, dried (anhydrous sodium sulfate), and evaporated. The crystalline residue was recrystallized from solvent *A*; yield 3 g, m.p. 156.5–157.5°, $[\alpha]_D^{25}$ −17.0° (*c* 0.106, methanol).

Anal. Calc. for $C_{23}H_{26}F_3NO_{12}$: C, 48.85; H, 4.63; Found: C, 48.63; H, 4.56.

X-ray powder diffraction data (using CuKα radiation; relative intensities estimated visually, m = medium, s = strong, w = weak): 6.64 (s), 8.53 (m), 9.72 (s), 11.39 (w), 11.68 (w), 12.29 (m), 13.72 (w), 15.88 (w), 16.80 (w), 17,39 (w), 18.08 (w), 19.28 (m),20.52 (w), 21.15 (m), 21.44 (w), 22.50 (w), and 23.63 (w).

*8-Methyl-3,4-oxadiazolocoumarin 7-(3,4,6-tri-*O*-acetyl-α-*D*-glucopyranoside)* (**6**) *as an example of a stereospecific synthesis of a phenyl α-*D*-glucopyranoside.* — 7-Hydroxy-8-methyl-3,4-oxadiazolocoumarin (**4**) (4.3 g, obtained by reaction of 3-amino-7-hydroxy-8-methyl-2,4-chromandione with sodium nitrite in aqueous acetic acid; m.p. 204–205°) and the Brigl anhydride **5** (5.7 g) were dissolved in absolute toluene (120 ml), and the solution was heated for 48 h at 120°; it was evaporated to dryness at 40°, and the residue was purified by chromatography on silica gel or Florisil, elution being conducted with benzene–ethyl acetate; yield 2.1 g, yellow crystals, m.p. 192–193° (dec.), $[\alpha]_D^{25}$ +138.8° (*c* 0.835, chloroform).

Anal. Calc. for $C_{22}H_{22}N_2O_{12}$: C, 52.17; H, 4.38; N, 5.53. Found: C, 52.12; H, 4.36; N, 5.55.

*5-Ethoxy-2-nitrophenyl 3,4,6-tri-*O*-acetyl-α-*D*-glucopyranoside.* — A solution of 5-ethoxy-2-nitrophenol (3 g) and the Brigl anhydride[13] (5 g) in 50 ml of absolute toluene (purified by distillation from sodium wire) was refluxed for 24 h. After the solvent had been evaporated *in vacuo*, a solution of the residue was chromatographed on a column (150 g) of silica gel, with elution with 1:3 and 1:1 ether–benzene. The eluates were combined, and evaporated *in vacuo*, and the residue was recrystallized from solvent *A*; yield 1.7 g, m.p. 147–148°, $[\alpha]_D^{25}$ +192° (*c* 0.62, ethyl acetate).

Anal. Calc. for $C_{20}H_{25}NO_{12}$: C, 50.95; H, 5.35; N, 2.97. Found: C, 50.85; H, 5.32; N, 3.13.

Tetraacetate **3a** was prepared by reaction of this triacetate with acetic anhydride in pyridine; m.p. 140–141° (from solvent *A*), $[\alpha]_D^{25}$ +205.5° (*c* 0.1031, ethanol).

Anal. Calc. for $C_{22}H_{27}NO_{13}$: C, 51.46; H, 5.30; N, 2.73. Found: C, 51.66; H, 5.28; N, 2.78.

*2-Acetamido-5-ethoxyphenyl 2,3,4,6-tetra-*O*-acetyl-α-*D*-glucoside* (**3l**). — 5-Ethoxy-2-nitrophenyl 3,4,6-tri-*O*-acetyl-α-D-glucoside (1.2 g) was dissolved in ethyl acetate (40 ml) and hydrogenated at room temperature in the presence of 5% palladium-on-carbon (200 mg); 180 ml of hydrogen was absorbed within 3 h. The catalyst was filtered off by suction, and the filtrate was evaporated. The amino compound obtained was recrystallized from solvent *A*; m.p. 124–125°, $[\alpha]_D^{25}$ +192.5° (*c* 0.0983, methanol).

Anal. Calc. for $C_{20}H_{27}NO_{10}$: C, 54.42; H, 6.17; N, 3.17. Found: C, 54.15; H, 6.05; N, 3.25.

The compound (1 g) in absolute pyridine (20 ml) was acetylated with acetic anhydride (1 ml) for 16 h at room temperature. The mixture was evaporated at 40° *in vacuo*, and the residue was triturated with 50 ml of cold water. The product was extracted into dichloromethane (80 ml), and the extract was successively washed with cold 3M hydrochloric acid, sodium hydrogen carbonate solution, and water, dried (anhydrous sodium sulfate), and evaporated. The residue was chromatographed on Florisil (20 g). The pure product was eluted with 1:1 and 1:3 benzene–ether (200 ml), and the eluates were combined and evaporated; the residue was recrystallized from solvent *A*, m.p. 74–75°, $[\alpha]_D^{25}$ +121.8° (*c* 0.1045, methanol).

Anal. Calc. for $C_{24}H_{31}NO_{12}$: C, 54.86; H, 5.96; N, 2.67. Found: C, 54.81; H, 6.05; N, 2.86.

2-Acetamido-5-ethoxyphenyl α-D-glucopyranoside (**3c**). — 2-Acetamido-5-ethoxyphenyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucoside (**3i**; 400 mg) was dissolved in 20 ml of a 15% solution of ammonia in methanol. The mixture was kept overnight at room temperature, and evaporated *in vacuo* to dryness, and the residue was recrystallized from isopropyl alcohol–isopropyl ether; m.p. 171–172°, $[\alpha]_D^{25}$ +168° (*c* 0.1013, methanol).

Anal. Calc. for $C_{16}H_{23}NO_8$: C, 53.77; H, 6.99; N, 3.92. Found: C, 53.86; H, 6.45; N, 4.02.

Methyl (2-acetamido-5-ethoxyphenyl 2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate (**3h**). — A solution of 2-acetamido-5-ethoxyphenyl α-D-glucopyranoside (**3c**; 2 g) in 200 ml of water was placed in a 4-necked, 500-ml flask (fitted with a mechanical stirrer, a gas inlet-tube, a reflux condenser, and a thermometer), and oxidized with oxygen in the presence of platinum black (1.5 g) and sodium hydrogen carbonate (0.47 g). The oxidation was conducted during 18 h at 80°, with a vigorous stream of oxygen and intensive mechanical stirring. The hot mixture was then filtered with suction, and the filtrate was evaporated *in vacuo* at 40° to dryness. The residue was extracted with methyl alcohol (2 × 100 ml), and the extracts were combined, shaken with Amberlite IRC-120 (H^+) ion-exchange resin, filtered, and treated with an ethereal solution of diazomethane. After 60 min, the solution was evaporated *in vacuo* at 40°, and the residue (800 mg) was dissolved in absolute pyridine (20 ml) and acetylated with acetic anhydride (1 ml). After 20 h at 22°, the mixture was diluted with 100 ml of water, and extracted with 100 ml of dichloromethane. The extract was dried (anhydrous sodium sulfate), and evaporated, and the residue was purified by chromatography on Florisil (20 g). The pure methyl ester was eluted with 3:7 and 1:1 ether–benzene (100 ml), and the combined eluates were evaporated. The crystalline product was recrystallized from solvent *A*; needles, m.p. 115–116°, $[\alpha]_D^{25}$ +115.2° (*c* 0.1042, methanol).

Anal. Calc. for $C_{23}H_{29}NO_{12}$: C, 54.01; H, 5.72; N, 2.74. Found: C, 53.73; H, 5.73; N, 2.81.

ACKNOWLEDGMENT

The authors are indebted to Professor H.-J. Cantow, Institute of Macromolecular Chemistry of the University of Freiburg i.Br. (Germany) for permission to use a Varian 220-MHz n.m.r. spectrometer.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

Note

Novel aromatization of a trihydroxycyclohexanetrione (triketoinositol) to a dibenzo-*p*-dioxin derivative*

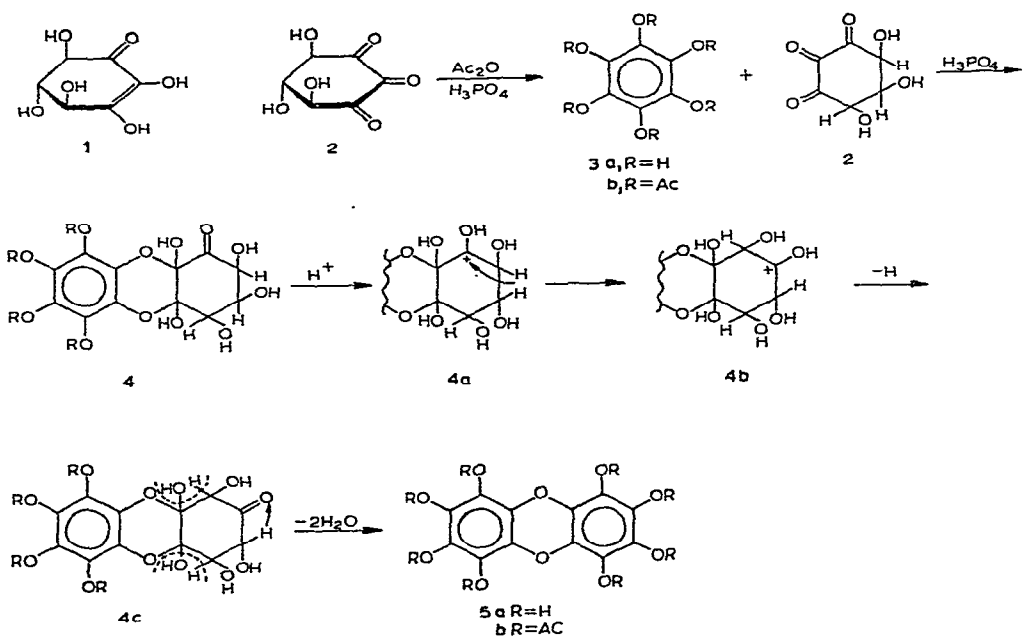
ALEXANDER J. FATIADI

Division of Analytical Chemistry, Institute for Materials Research, National Bureau of Standards, Washington, D. C. 20234 (U. S. A.)

(Received June 30th, 1969)

The first triketoinositol, namely, 4,6/5-trihydroxy-1,2,3-cyclohexanetrione (**2**), was prepared[1] by oxidation of the enolic form of 4,6/3,5-tetrahydroxy-1,2-cyclohexanedione (**1**), a diketoinositol obtained by oxidation of 1,2,3,5/4,6-tetrahydroxycyclohexane (*myo*-inositol), with either nitric acid[1] or bromine[2]. It was found[1] that, on acetylation in the presence of pyridine, compound **2** is aromatized to benzenehexol hexaacetate (**3b**) in high yield.

I now report that acetylation of **2** in the presence of an acidic catalyst (100% phosphoric acid) gives a new adduct, the fully hydroxylated dibenzo-*p*-dioxin **5a**,



*Part V. Methods in Inositol Chemistry. For Part IV, see *Carbohyd. Res.*, 9 (1969) 177. The nomenclature is in accordance with the IUPAC–IUB Tentative Nomenclature for Cyclitols [*Europ. J. Biochem.*, 5 (1968) 1], with the trivial name in parentheses.

isolated as its octaacetate **5b**. The formation of **5b** can be envisaged as an acid-catalyzed, nucleophilic attack of **3a** on 4,6/5-trihydroxy-1,2,3-cyclohexanetrione (**2**) to give a bishemiacetal intermediate **4**. Aromatization of the "right-hand half" of a molecule of the intermediate **4**, under the acidic conditions used in this experiment, is consistent with the following consecutive reactions: (*a*) protonation of a carbonyl group to give the oxonium intermediate **4a**, (*b*) a hydride shift to give **4b**, (*c*) elimination of a hydrogen ion to produce **4c**, and (*d*) elimination of two molecules of water to give the aromatic compound **5a**, followed by acetylation to **5b**, or of acetylation followed by elimination of two molecules of acetic acid to give **5b**.

Formation of a different dibenzo-*p*-dioxin derivative, for example, from catechol and *o*-benzoquinone[3], apparently proceeds by a nucleophilic addition. However, interaction of *N*-methylindole-5,6-diol with dehydro-L-ascorbic acid produces a benzo-*p*-dioxin derivative[4] having a hemiacetal structure, and a hemiacetal structure was also proposed for a dimer of the dioxin type obtained from dehydro-L-ascorbic acid alone[5].

Although the biosynthesis of phenolic compounds may proceed by the Birch synthetic path[6,7] from acetate *via* polyketo acids (or esters), the formation of **5a**, or its derivative described here, may be one way in which possible dibenzo-*p*-dioxin derivatives can be synthesized in Nature. (See Ref. 8 for a related discussion.)

EXPERIMENTAL

Melting points were determined with a Mel-Temp* apparatus and are corrected. N.m.r. spectra were obtained at 60 MHz on a Varian A-60 spectrometer, with tetramethylsilane ($\tau = 10.00$) as the internal standard. I.r. spectra were recorded with a Perkin–Elmer Model 257 spectrophotometer; u.v. spectra were recorded with a Beckman DK-2 or a Cary 14 spectrophotometer. The mass spectra were recorded with an LKB-9000 spectrometer. Analyses were made by William Schmidt of the Microchemical Analysis Section, National Bureau of Standards.

Preparation, and proof of structure, of 1,2,3,4,5,6,7,8-octaacetoxydibenzo-p-dioxin (**5b**). — A mixture of **2** monohydrate[1] (1 g, 5.17 mmoles) with acetic anhydride–phosphoric acid reagent[9] (12 ml of 100% syrupy H_3PO_4 and 48 ml of acetic anhydride, homogeneously mixed) was stirred for 1 h at 85°, giving a somewhat brownish solution that was decomposed with ice–water to afford **5b**, yield 1.3 g (77.5%). A slightly higher yield of **5b** (82.0%) was obtained if the mixture was stirred for 24 h at room temperature (about 95% of **2** dissolved). The crude product was recrystallized from warm acetic acid, and a sample was dried for 2 h at 110°/0.1 mmHg; m.p. 300–302° (298–300°, slow heating); λ_{max}^{MeOH} 232 (ε ~47,500), 278 (ε ~4,500), 295 (sh) nm (ε ~3,000).

Anal. Calc. for $C_{28}H_{24}O_{18}$: C, 51.85; H, 3.70; CH_3CO, 53.01. Found: C, 51.82; H, 3.72; CH_3CO, 53.37.

*Certain commercial instruments are mentioned in this paper; this does not imply recommendation or endorsement by the National Bureau of Standards.

The structure of **5b** was confirmed by i.r., n.m.r., and high-resolution mass spectroscopy; ν_{max}^{KBr} 1785 (vs) (C = O), 1650 (m) (C = C, aromatic), 1615 (w), 1480 (vs) (phenyl ring), 1210 (vs), 1180 (vs) (acetate), and 1062 (vs) cm^{-1} (C—O—C, aryl ether).

The n.m.r. spectrum of **5b** (~ 12% solution in methyl sulfoxide-d_6, with tetramethylsilane as the internal standard) showed only two singlets narrowly spaced and of equal intensity (separation 5 Hz), at τ 7.69 and 7.65, arising from two groups of methyl protons of the acetyl groups on O-1, O-4, O-5, and O-8, and O-2, O-3, O-6, and O-7.

The mass spectrum (70 eV) of **5b** showed a molecular-ion peak (parent ion) at *m/e* 648.00. (Calc. for $C_{28}H_{24}O_{18}$, 648.492). The spectrum also showed seven ion-peaks (moderate to strong intensities), due to the consecutive loss of ketene ($CH_2 = C = O$) group from the acetate groups, at *m/e* 606, 564, 522, 480, 438, 396, 354, and 312*.

Hydrolysis of octaacetate **5b** with 85% phosphoric acid containing a small proportion of sodium hydrosulfite ($Na_2S_2O_4$) at 90° gave the parent octahydroxy compound **5a** as a purple, microcrystalline powder (80% yield). Compound **5a** was difficultly soluble in most organic solvents; it was purified by recrystallization from aqueous methyl sulfoxide. The compound did not melt, but slowly decomposed on heating above 380°.

Anal. Calc. for $C_{12}H_8O_{10}\cdot H_2O$: C, 43.64; H, 3.05. Found: C, 43.89; H, 2.88*

Compound **5a** was reconverted into octaacetate **5b** with acetic anhydride–perchloric acid at 65°, showing that there had been no rearrangement during the hydrolysis.

ACKNOWLEDGMENT

The author thanks Dr. Henry M. Fales, National Heart Institute, National Institutes of Health, Bethesda, Maryland 20014, for recording the mass spectrum of **5b**, and for useful comments.

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*The spectrum also showed eight ion-peaks, arising from possible molecular rearrangements, at *m/e* 296, 280, 263, 246, 232, 216, 198 (weak), and 184. Other ion-peaks due to electron-impact fragmentation were seen at *m/e* 167, 158, 142, 128, 90, 60, and 40.

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Note

The reaction of lysozyme with 2-(bromoacetamido)-2-deoxy-D-glucose

A. CASSERA AND ZEHRA ALI

Department of Biochemistry, The Institute of Science and Technology, University of Manchester, Manchester (Great Britain)

(Received June 2nd, 1969)

We report a method by which 2-acylamino-2-deoxy-D-glucose residues have been attached to lysozyme (*N*-acetylmuramyl hydrolase, EC 3.2.1.17) molecules to form synthetic glycoproteins. The experiments were carried out in an attempt to find an "active-site directed irreversible inhibitor[3]" for lysozyme. 2-(Bromoacetamido)-2-deoxy-D-glucose was found not to be such a compound, since the substituted lysozymes were found to be of equal, or slightly greater, enzymic activity than native lysozyme, when assayed[4] by their action on *Micrococcus lysodeikticus*. It has been observed[5,6] that, when lysozyme is treated with iodoacetic acid at pH 9.0, carboxymethyl-substituted lysozymes are formed that are unaltered or even enhanced in their enzymic action.

EXPERIMENTAL

Hen egg-white lysozyme (60 mg) and 2-(bromoacetamido)-2-deoxy-D-glucose (60 mg) were dissolved in water (10 ml), and the pH was brought to 9.0 with N NaOH. The solution was then maintained for 3 h at 40°, the pH being held at 9.0 by the addition of more alkali. At the end of the period, the reaction was stopped by the addition of a few drops of acetic acid, and the protein fraction was isolated by passage of the mixture down a column of Sephadex G-10, followed by freeze-drying of the eluate. Chromatography of the treated lysozyme on Amberlite CG-50 resin, with 0.2M NaH_2PO_4 adjusted to pH 7.2 with 5N NaOH, showed three new protein peaks that were eluted from the column much more rapidly than was native lysozyme. The peaks were collected and purified by re-running on the chromatographic column. Analysis[1] of these proteins for 2-amino-2-deoxy-D-glucose, after hydrolysis for 10 h in 6N HCl at 110°, showed that they each contained 1 mole of amino sugar per mole (14,400 g) of protein. Examination of the products of acid hydrolysis, using an amino acid analyser, showed the presence of 1 mole each of ε-monocarboxymethyl-lysine and of 2-amino-2-deoxy-D-glucose per mole of each protein. Each analysed for five lysine residues per mole (native lysozyme analysed for six). All of the other amino acids were unchanged from native lysozyme. It is therefore concluded that, in these proteins, the amino sugar residues were attached, *via* $CO—CH_2$ linkages, to differing ε-amino groups of lysyl side chains of the lysozyme.

2-(Bromoacetamido)-2-deoxy-D-glucose was prepared by using a modification of Roseman and Ludowieg's[2] method for 2-acetamido-2-deoxy-D-glucose. Bromoacetyl bromide (2.0 ml) was added over 30 min to an ice-cold solution of 2-amino-2-deoxy-D-glucose hydrochloride (5.0 g) in 10% aqueous methanol (100 ml). The pH was maintained between 8.0 and 9.0 during the reaction by the addition of De-Acidite FF (CO_3^{2-}) resin. After 30 min, the filtered solution was passed through a column of Zeocarb 225 (H^+) resin. The eluate was freeze dried to give 2-(bromoacetamido)-2-deoxy-D-glucose (0.71 g, 10%), m.p. 153–154° (dec.), $[\alpha]_D^{24}$ +27 ±2° (equil., *c* 1.0, water) (Found: C, 32.4; H, 5.0; Br, 26.9; N, 4.7. $C_8H_{14}BrNO_6$ calc.: C, 32.0; H, 4.7; Br, 26.6; N, 4.7%). When the compound was repeatedly recrystallized from water, it decomposed with loss of hydrobromic acid.

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Note

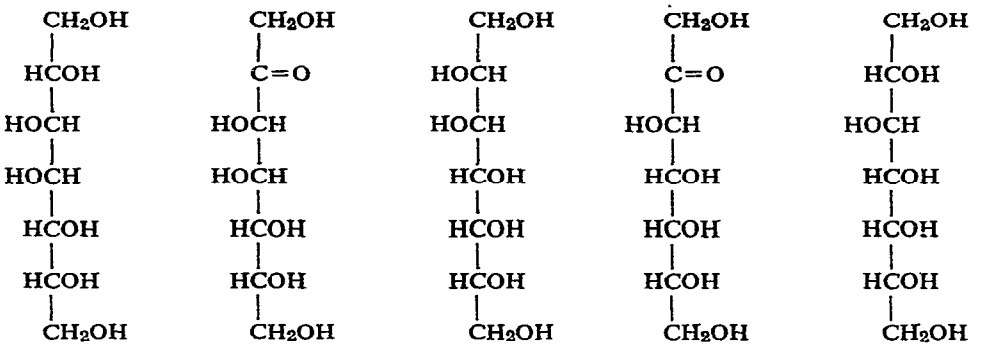
The isolation of volemitol and other polyhydric alcohols from avocado seeds

NELSON K. RICHTMYER

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. 20014 (U. S. A.)

(Received June 7th, 1969)

Perseitol (D-*glycero*-D-*galacto*-heptitol, **1**) was first isolated from the avocado by Avequin[1] in 1831. The closely related D-*manno*-heptulose (**2**) was discovered in the avocado by LaForge[2] in 1917. Sedoheptulose (D-*altro*-heptulose, **4**) was first isolated from *Sedum spectabile* Bor. by LaForge and Hudson[3] in 1917, and the closely related β-sedoheptitol (D-*glycero*-D-*gluco*-heptitol, **5**) was found in *Sedum* species by Charlson and Richtmyer[4] in 1960. Compounds **1** and **2** are related by oxidation



Perseitol	D-*manno*-Heptulose	Volemitol	Sedoheptulose	β-Sedoheptitol
1	**2**	**3**	**4**	**5**

and reduction, as are compounds **5** and **4** also. The fifth compound whose formula is shown is volemitol (D-*glycero*-D-*manno*-heptitol, **3**); it was discovered in the mushroom *Lactarius volemus* Fr. by Bourquelot[5] in 1889. It is related, similarly, to both D-*manno*-heptulose (**2**) and sedoheptulose (**4**). Nordal and Öiseth[6] isolated both volemitol (**3**) and sedoheptulose (**4**) from *Primula elatior* (L.) Hill, and obtained paper-chromatographic evidence for the probable presence of D-*manno*-heptulose (**2**) also. Begbie and Richtmyer[7], in 1966, described a large-scale study of *Primula officinalis* Jacq. in which they isolated not only D-*manno*-heptulose (**2**) and sedoheptulose (**4**) but also volemitol (**3**) and β-sedoheptitol (**5**).

The present paper reports the isolation of volemitol (3) from the avocado, where it occurs together with perseitol (1) and D-*manno*-heptulose (2). Glycerol, D-arabinitol, galactitol, *myo*-inositol, and D-*erythro*-D-*galacto*-octitol[4] were also isolated during the course of the investigation. According to two recent reviews[8], D-arabinitol is known to occur in fungi and lichens, but this seems to be the first time that it has been isolated from a higher plant.

EXPERIMENTAL

General. — Paper chromatography was performed on Whatman No. 1 filter paper by the descending method at room temperature. The following solvent systems were used: A, ethyl acetate–acetic acid–formic acid–water (18:3:1:4); B, butyl alcohol–ethyl alcohol–water (40:11:19); and C, butyl alcohol–pyridine–water (6:4:3). The spray reagents used were aniline hydrogen phthalate for aldoses, orcinol–hydrochloric acid for ketoses, and ammoniacal silver nitrate for alditols and other polyhydroxy compounds in general.

Isolation of polyhydric alcohols from avocado seeds. — The seeds from four hundred ripe avocados (Californian Calavo, Hass variety[9]) were crushed in a meat grinder and stored for several months at −5°. The meal (22,650 g) was then extracted twice with hot water, and the extract was deproteinized by the addition of aqueous zinc sulfate solution followed by saturated aqueous barium hydroxide solution until the solution was neutral to phenolphthalein. The precipitate was removed, and the filtrate was deionized by passage through columns of Amberlite IR-120 and Duolite A-4 ion-exchange resins. The eluate was concentrated *in vacuo* until considerable crystallization occurred, and the product was then filtered off and washed, first with 1:1 water–methanol and then with methanol. The air-dried perseitol (1) weighed 217 g; it melted at 187–188°, in agreement with the value reported by Hann and Hudson[10] for pure perseitol. Concentration of the mother liquor yielded 80 g of perseitol, m.p. 186–188°. Paper-chromatographic examination of the resulting mother liquor indicated the probable presence of D-fructose, D-glucose, and D-*manno*-heptulose, together with very small proportions of an aldopentose, a second aldohexose, and a second heptulose, besides several polyhydric alcohols. After concentration of the solution to remove methanol, the reducing sugars were decomposed by heating the aqueous solution in an open, stainless-steel container with an excess of barium hydroxide for about 20 h. The excess of alkali was then neutralized with carbon dioxide, and the mixture was filtered, deionized with Amberlite IR-120 and Duolite A-4 ion-exchange resins, and concentrated *in vacuo*; 14 g of perseitol, m.p. 185–187°, was obtained by crystallization. The mother liquor, when concentrated and examined by paper chromatography, appeared to contain lactones (revealed through the use of alkaline hydroxylamine followed by acid ferric chloride spray reagents). The solution was, therefore, passed slowly through a column of the strongly basic resin Amberlite IRA-400. Paper-chromatographic examination of the concentrated eluate showed that it contained a large proportion of perseitol and at least five other components having greater mobilities.

Separation of the polyhydric alcohols on cellulose columns. — One third (8.4 g) of the mixture of polyhydric alcohols thus obtained was mixed with powdered cellulose, and transferred to the top of a column (34 × 3.7 cm) containing washed cellulose powder (Whatman). Elution was begun with quarter-saturated aqueous butyl alcohol and, with the aid of an automatic fraction-collector, 14-ml fractions were collected as soon as a sample of the eluate left a residue when the solvent was evaporated. Thus, fractions 1–50 yielded 2.7 g of a syrup having the same mobility as glycerol on a paper chromatogram. Complete identification as **glycerol** was established by converting a portion of it into its tris-*p*-nitrobenzoate, m.p. 192–193° alone and when mixed with authentic ester[11].

Fractions 126–175 (0.168 g) yielded crystals (29 mg) having m.p. 99–102°, raised to 101–102° by recrystallization from absolute ethyl alcohol. The position of this compound on paper chromatograms suggested that it was a pentitol; in solvents A and B, it was indistinguishable from ribitol and D- and L-arabinitol (all of which melt at 102°); however, in solvent C, it migrated at the same rate as the arabinitols but more slowly than ribitol. Its i.r. spectrum (Nujol mull) agreed with that of the arabinitols. It was identified as **D-arabinitol** through mixed melting points: undepressed when the compound was mixed with authentic material, but depressed when it was mixed with either ribitol or L-arabinitol.

Fractions 301–625 (0.231 g) yielded 75 mg of crystalline material, m.p. 125–130°. Upon recrystallization from 30 ml of methanol, clusters of small, heavy prisms separated, together with a few needle-like crystals that were removed by decantation. The prisms melted at 180–188°; a second recrystallization from methanol gave prisms (22 mg) of a compound identified as **galactitol** because it had m.p. and mixed m.p. of 187–188°, and i.r. spectrum (Nujol mull) and paper-chromatographic behavior (solvents A and B) identical with those of authentic material.

The rest of the mixture of polyhydric alcohols (16.8 g) was fractionated similarly, and some of the resulting fractions, together with the corresponding ones from the first fractionation, were combined into (*a*) a "hexitol–volemitol" fraction and (*b*) a "perseitol–octitol" fraction for further examination.

The "hexitol–volemitol" fraction (0.35 g) was transferred to a column of cellulose, and at first eluted with eighth-saturated aqueous butyl alcohol, and later with quarter-saturated aqueous butyl alcohol, 16-ml fractions being collected. The first portions of eluate (fractions 301–750) contained galactitol and a small amount of a second substance (m.p. 137–138°) that was not identified. Fractions 1201–1825 (67 mg of syrup) were, from paper-chromatographic comparisons, believed to contain **volemitol** (3) and, on inoculation with that compound, yielded a product that, after recrystallization from methanol, melted at 153–154°, alone, and when mixed with authentic volemitol. Its identity was verified by its i.r. spectrum (Nujol mull).

The "perseitol–octitol" fraction (5.9 g) was refractionated on a larger cellulose column (83 × 5 cm) by elution with half-saturated aqueous butyl alcohol, the eluate being collected in 16-ml fractions. The first fractions, which appeared to contain perseitol only, were discarded; the middle fractions (1251–2375) contained perseitol

and an octitol; and the final fractions, obtained by elution with methanol, yielded 0.4 g of the readily identified *myo*-inositol, having m.p. and mixed m.p. 224–225°.

Fractions 1251–2375 (1.7 g) were refractionated on the larger cellulose column in the same way. The new fractions 1251–1500 (0.68 g) were combined, treated with Darco X decolorizing carbon, and evaporated to give 0.68 g of solid residue. Recrystallization from aqueous methanol afforded 0.49 g of clusters of small, prismatic needles having m.p. 167–168°; the mother liquor was combined with fractions 1501–1850 (0.12 g) to give an additional 0.15 g of a compound having m.p. 166–168°. This compound was identified as the anhydrous modification of **D-*erythro*-D-*galacto*-octitol**[4] by mixed m.p., i.r. spectrum, and the fact that it lost no weight when heated at 100° overnight in a high vacuum.

ACKNOWLEDGMENTS

The author thanks Mr. Edward W. Tracy of this Laboratory of Chemistry for assistance in the preliminary isolation of the polyhydric alcohols, and Mrs. Anne H. Wright, also of this Laboratory, for making the i.r. spectral comparisons.

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Note

The isolation of perseitol and volemitol from *Sedum*, and some other observations on *Sedum* constituents

NELSON K. RICHTMYER

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. 20014 (U. S. A.)

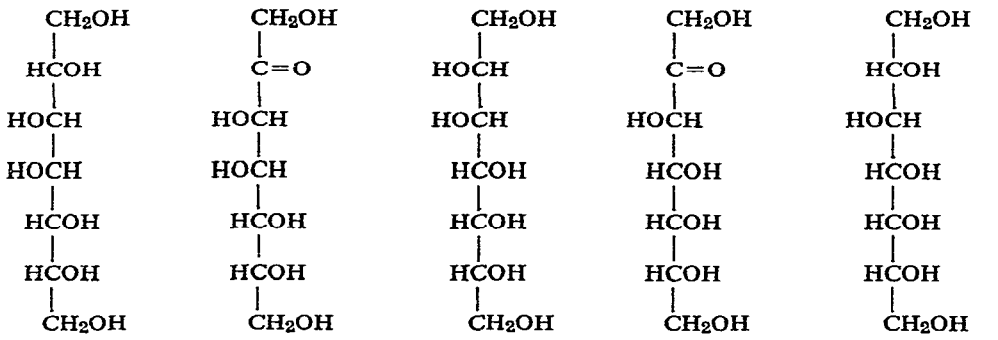
(Received June 7th, 1969)

The preceding paper[1] discussed the occurrence, in plants, of D-*manno*-heptulose (**2**) and sedoheptulose (D-*altro*-heptulose, **4**), and of the heptitols that are closely related to them by reduction, namely, perseitol (D-*glycero*-D-*galacto*-heptitol, **1**), volemitol (D-*glycero*-D-*manno*-heptitol, **3**), and β-sedoheptitol (D-*glycero*-D-*gluco*-heptitol, **5**). For example, compounds **1**, **2**, and **3** have been isolated from the avocado[1] (family, Lauraceae) and compounds **2**, **3**, **4**, and **5** from *Primula officinalis* Jacq.[2] (family, Primulaceae). The present paper describes the isolation from *Sedum* species (family, Crassulaceae) of perseitol (**1**) and volemitol (**3**), in addition to sedoheptulose (**4**, as the crystalline anhydride sedoheptulosan) and β-sedoheptitol (**5**), whose isolation from *Sedum* has been reported earlier[3]. Thus, it is seen that D-*manno*-heptulose (**2**) in the avocado is accompanied by heptitols **1** and **3**, and that sedoheptulose (**4**) in *Primula* and *Sedum* is accompanied by heptitols **3** and **5**. Inasmuch as D-*manno*-heptulose (**2**) and sedoheptulose (**4**) occur together in some plants, such as *Primula*, it is logical to predict that, eventually, all five of these compounds will be isolated from the same plant extract.

As a result of the earlier part of this investigation, Charlson and Richtmyer[3] reported the isolation from *Sedum* (as well as from the avocado) of D-*glycero*-D-*manno*-octulose. Subsequently, Sephton and Richtmyer[4] isolated, also, D-*glycero*-L-*galacto*-octulose, D-*erythro*-L-*gluco*-nonulose, and D-*erythro*-L-*galacto*-nonulose from the avocado; they proved the structure of, and synthesized, each of the last three higher-carbon sugars. Begbie and Richtmyer[2] isolated from *Primula* roots, and independently proved the structure of, each of the two octuloses and two nonuloses. Because these investigations[2,4] were so thorough, it no longer seems important to continue with the tedious separation, purification, and identification of the other 8- and 9-carbon ketoses in *Sedum*, and work on the problem has been abandoned.

Because D-*erythro*-D-*galacto*-octitol is closely related to D-*glycero*-D-*manno*-octulose and both have been found in the avocado[3], and because that octulose has been found also in *Sedum*[3], a special search was made for the octitol in *Sedum*. To this end, 37.5 kg of *S. spectabile* Bor. was processed as described in the Experimental section, and finally fractionated on a column of Dowex 50W-X8 (Ba^{2+}) ion-exchange

resin; no more than a trace, if any, of the octitol could be detected by paper chromatography.



EXPERIMENTAL

Further fractionation of Sedum *extract.* — In 1960, Charlson and Richtmyer[3] described (*a*) the extraction of 385 kg of *Sedum* plants (principally *S. spectabile*) with water, and (*b*) subsequent treatments of the extract with methanol to precipitate gums, with yeast to remove fermentable sugars, with dilute acid to convert sedoheptulose into sedoheptulosan (of which 2.4 kg was isolated), and with bromine water to decompose the aldoses. The resulting product was 345 g of syrup that contained at least a dozen ketoses, their anhydrides, and polyhydric alcohols. This material was fractionated on cellulose columns by eluting with mixtures of benzene and methanol containing 1% of water. The eluate was divided into eleven fractions and, as described earlier[3], from a 3.6-g portion of fraction 9 were separated D-*glycero*-D-*manno*-octulose and β-sedoheptitol. The remainder of fractions 8–11 weighed 33.5 g, and a description of its further investigation was promised.

That investigation was started by putting the 33.5 g of syrup that contained the octulose and nonulose portions of the extract on a column of cellulose (55 × 7.5 cm) and eluting with ethyl acetate–acetic acid–water (from 36:5:4 to 3:1:1). The octulose fraction (as determined by paper chromatography) was set aside, and the nonulose fraction was heated with 0.2M aqueous hydrochloric acid to hydrolyze a compound that appeared to be a sedoheptulose-containing disaccharide. After being deionized and concentrated, this material (16 g) was then refractionated on a cellulose column by eluting with aqueous butyl alcohol, to yield an additional octulose fraction. At this point, the octulose fractions were combined (6.2 g), put on top of a new cellulose column (88 × 5 cm), and eluted with quarter-saturated aqueous butyl alcohol. After 7090 ml of eluate had been separated as a forerun, the use of an automatic fraction-collector was begun, and 16-ml fractions were collected. These fractions were combined, and concentrated, in groups of 25; crystalline material was deposited in some of the combined fractions.

Fractions 551–950 yielded 0.84 g of crystals that were recrystallized twice from methanol containing a few drops of water. The product thus obtained (0.20 g of needles) melted at 150–152° and was identified as **volemitol (3)** by a mixed m.p. of 151–152° (the authentic volemitol melted at 152–153°) and by comparison of its i.r. spectrum (Nujol mull) with that of authentic volemitol. The mother liquor from the volemitol began to crystallize spontaneously, and deposited **β-sedoheptitol (5)** which was identified by m.p. and mixed m.p. of 128–130°.

Fractions 1951–2575 yielded 22 mg of crystals. These were recrystallized from aqueous methanol, in clusters of small, elongated prisms. A melting point and mixed m.p. of 187–188°, together with a comparison of i.r. spectra, identified the product as **perseitol (1)**.

A search for D-erythro-D-galacto-*octitol in* Sedum spectabile. — A quantity (77.2 kg) of *S. spectabile* was extracted with water; the extract was concentrated, and poured into methanol; the mixture was filtered, and the filtrate was concentrated, deionized with Amberlite IR-120 and Duolite A-4 ion-exchange resins, and evaporated to yield 600 g of syrup. An aqueous solution of this syrup in a stainless-steel pot was heated for 32 h on a steam bath with a total of 750 g of barium hydroxide octahydrate to maintain alkalinity (to decompose all reducing sugars). The solution was made neutral with carbon dioxide, the suspension filtered, and the filtrate deionized with Amberlite IR-120 and Duolite A-4 ion-exchange resins, and finally with Amberlite IRA-400 (to remove any resistant lactones). Evaporation of the solution gave 30 g of syrup that showed 6 heavy spots and 4 light spots on paper chromatograms (6:4:3 butyl alcohol–pyridine–water; visibilized with ammoniacal silver nitrate at room temperature). Crystallization began spontaneously in the syrup, and was aided by the addition of methanol and cooling. The first crop weighed 2.0 g and, after recrystallization from aqueous methanol, gave 1.8 g of *myo*-inositol having m.p. and mixed m.p. of 224–225°. The mother liquor from the first crop was concentrated in a current of air to yield, spontaneously, a second crop of crystals that weighed 13.5 g; recrystallization from hot methanol yielded, in 3 crops, a total of 12.1 g of anhydrous sedoheptulosan having m.p. and mixed m.p. of 154–155°. The identifications of the *myo*-inositol and sedoheptulosan were further confirmed by paper chromatography.

The mother liquor from the crystallization of these two compounds was evaporated to a dry syrup that weighed 13.8 g. A solution of a portion of this syrup (6.7 g, equivalent to 37.5 kg of *S. spectabile*) in water was concentrated to a small volume, and placed on the top of a column (93 × 4.5 cm) of Dowex 50W-X8 (Ba^{2+}) ion-exchange resin. The column was eluted slowly with water, with use of an automatic fraction-collector. Although the fractionation failed to give any clear-cut separation into the nine or ten components that were evident on paper chromatograms, there was no more than a faint trace of a spot visible in the area where the octitol should appear, even when the chromatograms sprayed with ammoniacal silver nitrate were kept in the dark for two weeks.

ACKNOWLEDGMENTS

The author thanks Mr. Edward W. Tracy of this Laboratory of Chemistry for technical assistance, and Mrs. Anne H. Wright, also of this Laboratory, for making the i.r. spectral comparisons.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

Note

Araucaria bidwillii gum
Part III*. Partial acid hydrolysis of the gum

G. O. ASPINALL, J. A. MOLLOY, AND C. C. WHITEHEAD
Department of Chemistry, Trent University, Peterborough, Ontario (Canada)
(Received May 19th, 1969; in revised form, June 27th, 1969)

INTRODUCTION

In Parts I[1] and II[2] the major component of *Araucaria bidwillii* gum has been shown to be a highly branched, acidic polysaccharide. During the examination of oligosaccharides formed on partial acetolysis of the carboxyl-reduced polysaccharide it was observed that those containing (1→6)-linked linkages had high positive specific rotations[2]. Definite evidence was presented that one disaccharide was 6-*O*-α-D-glucopyranosyl-D-galactose. Since this disaccharide appeared to correspond to the aldobiouronic acid, 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose (**3**), which had been characterised as a product of partial acid hydrolysis of another sample of the gum[1], and since the parent polysaccharide had a negative specific rotation indicative of a preponderance of β-D-glycosidic linkages, it was suggested that depolymerisation during acetolysis was accompanied by anomerisation of a high proportion of the (1→6)-β-D-glycosidic linkages[2]. We have now sought additional evidence on this point by further examination of the (1→6)-linked oligosaccharides formed on partial hydrolysis of the gum with dilute mineral acid.

RESULTS AND DISCUSSION

The water-soluble portion of the gum[2] was subjected to partial hydrolysis with dilute mineral acid under different conditions to yield (*i*) neutral oligosaccharides and (*ii*) acidic disaccharides. In the first series of experiments, chromatographic separation of the products furnished two oligosaccharides containing (1→6)-linkages having the chromatographic mobilities of 6-*O*-β-D-galactopyranosyl-D-galactose (**1**) and the polymer-homologous trisaccharide (**2**). Gas–liquid chromatography of the cleavage products from the methylated oligosaccharides confirmed the nature of the linkages. The disaccharide was characterised by conversion into the crystalline phenylosazone. The n.m.r. signals assigned to the anomeric proton[3] (τ 5.39; J = 7.3 Hz) in the disaccharide alditol corresponded to those of a β-D-galactoside.

*For Parts I and II see refs. 1 and 2, respectively.

The changes in specific rotation during periodate oxidation[4] of the disaccharide alditol and of gentiobiitol further indicated the presence of a β-D-glycosidic linkage. *O*-β-D-Galactopyranosyl-(1→6)-*O*-β-D-galactopyranosyl-(1→6)-D-galactose (**2**) was characterised as the crystalline trisaccharide.

In the second series of partial hydrolysis experiments, chromatographic separation of the products afforded the two aldobiouronic acids, 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose (**3**), and 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose (**4**). Both acidic disaccharides were converted into their crystalline, fully methylated derivatives. In the case of the latter disaccharide, location of the methyl ether substituent was confirmed by paper chromatography of the periodate oxidation products[5] of the 4-*O*-methylglucose formed on hydrolysis after reduction of the methyl ester methyl glycosides with sodium borohydride. Both aldobiouronic acids gave alditols that showed n.m.r. signals assignable to anomeric protons[3] (τ 5.39, $J = 7$ Hz; and τ 5.39, $J = 6.5$ Hz) of β-D-glycosidic linkages.

β-D-Gal*p*-(1→6)-D-Gal (**1**)
β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-D-Gal (**2**)
β-D-G*p*A-(1→6)-D-Gal (**3**) 4-*O*-Me-β-D-G*p*A-(1→6)-D-Gal (**4**)

The characterisation of the neutral (**1** and **2**) and acidic (**3** and **4**) oligosaccharides formed on partial hydrolysis of *Araucaria bidwillii* gum with dilute mineral acid provides clear evidence for the configuration of the (1→6)-β-D-glycosidic linkages. As far as we are aware, there are no recorded instances of anomerisation of glycosidic linkages taking place during formation of oligosaccharides by partial hydrolysis of polysaccharides with aqueous mineral acid. We conclude, therefore, that the configurations of the linkages in the above oligosaccharides are genuinely representative of those in the parent polysaccharide. Although these and earlier studies on the gum do not exclude the possibility that both α-D- and β-D-glycosidic linkages between similarly substituted, sugar residues may be present in the polysaccharide, it is more probable that the oligosaccharides having α-D-glycosidic linkages that were formed on partial acetolysis of the carboxyl-reduced gum[2] resulted from anomerisation during the acid-catalysed depolymerisation. These results show that care should be exercised in assessing the significance of configurations of glycosidic linkages in oligosaccharides formed during acetolysis of polysaccharides.

EXPERIMENTAL

General procedures. — The general experimental procedures were as described[2] in Part II. P.m.r. spectra were obtained with a Varian A-60 spectrometer by using solutions in deuterium oxide of samples in which hydroxyl protons had been previously exchanged. Unless otherwise stated, optical rotations were observed for water solutions at *ca.* 22°. The acidic polysaccharide was isolated from the gum as described[2] in Part II.

Partial acid hydrolysis of the acidic polysaccharide and isolation of neutral oligosac-

charides. — Acidic polysaccharide (17.5 g) in 0.05M sulphuric acid (650 ml) was heated for 4 h under reflux. Degraded polysaccharide was precipitated by the addition of acetone to the cooled solution and the precipitate was removed by centrifugation. The supernatant liquid was concentrated to remove acetone, neutralised with barium carbonate, centrifuged, treated with Amberlite resin IR-120 (H^+) to remove barium ions, and concentrated to a syrup. The syrup was adsorbed on a column (20 g, 40 × 3 cm) of *O*-(2-diethylaminoethyl)-Sephadex A-25 (formate form), and neutral sugars were eluted with water. After concentration, the eluted solution was adsorbed on a column (37 × 5 cm) of charcoal–Celite (1:1). The column was eluted with water (4 l) to remove monosaccharides, and then with water containing a gradient of 0–5% of ethanol (4 l), 5% of ethanol (2 l), and a gradient of 5–10% of ethanol (4 l), to give fractions containing oligosaccharides. Fractions containing components having the chromatographic mobilities of 6-*O*-β-D-galactopyranosyl-D-galactose, $R_{Galactose}$ 0.36 and 0.22 in solvents *A* and *C*, and the polymer-homologous trisaccharide, $R_{Galactose}$ 0.24 and 0.10 in solvents *B* and *A*, were further separated by filter-sheet chromatography to give oligosaccharides **1** and **2**.

Oligosaccharide **1**. — Oligosaccharide **1** (102 mg), $[\alpha]_D$ +30.2° (*c* 1.1), gave only galactose on hydrolysis. The methanolysis products from the methylated oligosaccharide were examined by g.l.c. on column (*c*) and had the retention times of methyl glycosides of 2,3,4,6-tetra-, and 2,3,4- and 2,3,5-tri-*O*-methylgalactose (tetra- and combined tri-*O*-methyl derivatives in approximately equimolar proportions). Samples of the derived alditol (10 mg) and of gentiobiitol (16 mg) were each oxidised in water (2 ml) containing sodium metaperiodate (75 mg); constant optical rotations were observed after 4 h, the products having $[\alpha]_D$ +103° and +101°, respectively[4]. The p.m.r. spectrum of the alditol in deuterium oxide showed a doublet (τ 5.39, *J* 7.3 Hz) attributable[3] to the anomeric proton of the aldosyl moiety. Oligosaccharide **1** was characterised as 6-*O*-β-D-galactopyranosyl-D-galactose by conversion into its phenylosazone; m.p. and mixed m.p. (with a sample prepared from a previously characterised specimen of the disaccharide[6]) 180–182°.

Oligosaccharide **2**. — Oligosaccharide **2** (19 mg), $[\alpha]_D$ +22° (equil.) (*c* 0.5), gave only galactose on hydrolysis, and galactose and oligosaccharide **1** on partial hydrolysis. Comparison of the colorimetric reactions of the sugar and the derived alditol with the phenol–sulphuric acid reagent[7] indicated that it was a trisaccharide. The methanolysis products from the methylated trisaccharide were examined by g.l.c. on column (*c*) and had the retention times of methyl glycosides of 2,3,4,6-tetra-, and 2,3,4- and 2,3,5-tri-*O*-methylgalactose. The trisaccharide crystallised from ethanol–water and was characterised as *O*-β-D-galactopyranosyl-(1→6)-*O*-β-D-galactopyranosyl-(1→6)-D-galactose[8] by m.p. and mixed m.p. 137–142°, and by X-ray powder photography.

Partial acid hydrolysis of the acidic polysaccharide and isolation of acidic oligosaccharides. — Acidic polysaccharide (16 g) in 0.5M sulphuric acid (1000 ml) was heated for 4 h under reflux. The cooled solution was neutralised with barium hydroxide and barium carbonate, barium sulphate was removed by filtration, and the

filtrate was concentrated, treated with Amberlite resin IR-120 (H^+) to remove barium ions, and adsorbed on a column (20 × 3 cm) of *O*-(2-diethylaminoethyl)-Sephadex A-25 (formate form). Neutral sugars were eluted with water and acidic sugars (3.3 g) with 0.05M formic acid. A portion (1.5 g) of the mixture of acidic sugars was fractionated by filter-sheet chromatography in solvent *C* to give oligosaccharides **3** and **4**.

Oligosaccharide **3**. — Oligosaccharide **3** (340 mg), $R_{Galactose}$ 0.23 in solvent *C*, had $[\alpha]_D$ −2° (*c* 1.0) and gave glucuronic acid and galactose on hydrolysis. The aldobiouronic acid was characterised as 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose by conversion into the methyl ester methyl glycoside hexamethyl ether[9], identified by m.p. 90° and mixed m.p. (with a sample of m.p. 89–90°) 89–90°, and by X-ray powder photography. The p.m.r. spectrum of the disaccharide alditol in deuterium oxide showed a doublet (τ 5.39, *J* 7 Hz) attributable[3] to the anomeric proton of the aldosyl moiety.

Oligosaccharide **4**. — Oligosaccharide **4** (160 mg), $R_{Galactose}$ 0.60 in solvent *C*, had $[\alpha]_D$ 0° (*c* 1.0) and gave 4-*O*-methylglucuronic acid and galactose on hydrolysis. Reduction of the derived methyl ester methyl glycosides with sodium borohydride, followed by hydrolysis, gave 4-*O*-methyl-glucose and galactose. The identity of the 4-methyl ether was established by chromatography of its periodate-oxidation products[5], which were indistinguishable from those derived from 4-*O*-methyl-D-mannose. The aldobiouronic acid was further characterised by conversion into the methyl ester methyl glycoside hexamethyl ether of 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose[9], identified by m.p. and mixed m.p. 85–87°, and by X-ray powder photography. The p.m.r. spectrum of the disaccharide alditol in deuterium oxide showed a doublet (τ 5.39, *J* 6.5 Hz) attributable[3] to the anomeric proton of the aldosyl moiety and a three-proton singlet at τ 6.50 (—OMe).

ACKNOWLEDGMENTS

The authors thank the National Research Council of Canada for financial support of this work, and Dr. M. B. Perry for recording the n.m.r. spectra.

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Note

Oxidative demethylation with chromium trioxide in acetic acid

S. J. ANGYAL AND K. JAMES
School of Chemistry, The University of New South Wales, Kensington, N. S. W. 2033 (Australia)
(Received June 16th, 1969)

The removal of *O*-methyl groups from sugars has always been regarded as an unrewarding operation. Recently, the use of boron trichloride[1] made this reaction possible under mild conditions, but this reagent removed most other *O*-substituents as well.

The purpose of this note is to draw attention to the recently described[2] oxidative demethylation with chromium trioxide in glacial acetic acid. This method is potentially very useful in carbohydrate chemistry, because it is carried out under mild conditions and the hydroxyl groups can be protected by acylation. Under the conditions of the reaction, esters are not attacked. The methyl ether is converted into a formic ester which is stable under the conditions used. However, glycosides[3] and acetals[4] are attacked by the reagent, and should not therefore be present.

As an example of the reaction, the oxidation of 1,2,4,6-tetra-*O*-acetyl-3-*O*-methyl-β-D-glucopyranose to 1,2,4,6-tetra-*O*-acetyl-3-*O*-formyl-β-D-glucopyranose, in 64% yield, is described. The α-D anomer is similarly converted into the formate in 66% yield. The 1-*O*-acetyl derivatives of β-D-aldopyranoses are attacked[4] by the reagent, but this reaction is much slower than the demethylation and does not seem to interfere with the latter. Attempts to hydrolyse the formic ester selectively have not been successful.

Similarly, benzyl ethers are oxidized by chromium trioxide to benzoic esters. This reaction could be useful for the debenzylation of compounds containing groups which are affected by catalytic hydrogenation or by reduction with dissolving metals. Thus, 1,2,4,6-tetra-*O*-acetyl-3-*O*-benzyl-β-D-glucopyranose is converted into the 3-*O*-benzoyl derivative, identical with a sample prepared by acetylation of 3-*O*-benzoyl-D-glucose.

The pentaacetate of quebrachitol (L-2-*O*-methyl-*chiro*-inositol) is readily oxidised[5] to the 2-formate which can be hydrolysed to L-*chiro*-inositol.

EXPERIMENTAL

*1,2,4,6-Tetra-*O*-acetyl-3-*O*-formyl-β-D-glucopyranose.* — 1,2,4,6-Tetra-*O*-acetyl-3-*O*-methyl-β-D-glucopyranose[6], m.p. 95–96° (2.0 g), was added to a stirred suspension of chromium trioxide (6.0 g) in glacial acetic acid (60 ml) at ambient temperature.

Stirring was continued for 1 h, at the end of which time g.l.c. analysis [4′ glass column of LAC I.R. 296 (1.5%) on Chromosorb W (100–200 mesh) at 215°] indicated that the reaction was complete. The resulting, dark-red suspension was poured into chloroform (150 ml), and the mixture was washed sequentially with water, saturated, aqueous sodium hydrogen carbonate, and water, and dried ($MgSO_4$). The pale-green solution was treated once with decolourising carbon to remove the remaining chromium salts and evaporated under diminished pressure to a colourless, viscous oil (1.8 g) which was crystallised from ether–light petroleum (b.p. 60–80°) and then from 95% ethanol to give the formate (1.35 g, 64%), m.p. 104–105°, $[\alpha]_D^{24}$ +5.3° (*c* 1.64, chloroform); n.m.r. data (Varian A-60 in chloroform-*d*): δ 8.05 (singlet, formyl proton), 5.78 (quartet showing the typical distortion due to virtual coupling, as observed in the spectrum of penta-*O*-acetyl-β-D-glucopyranose[7], $J_{1,2}$ *ca.* 7.4 Hz, H-1); no *O*-Me signal.

Anal. Calc. for $C_{15}H_{20}O_{11}$: C, 47.87; H, 5.32. Found: C, 47.87; H, 5.46.

1,2,4,6-Tetra-O-acetyl-3-O-formyl-α-D-glucopyranose. — A solution of 1,2,4,6-tetra-*O*-acetyl-3-*O*-methyl-β-D-glucopyranose (4.0 g) in acetic anhydride (28 ml) was added[7] to an ice-cold solution of conc. sulphuric acid (0.2M) in acetic anhydride (28 ml). After 1.5 h at room temperature, the solution was poured into chloroform and washed sequentially with water, saturated, aqueous sodium hydrogen carbonate, and water, and dried ($MgSO_4$). Evaporation of the chloroform solution under diminished pressure gave a colourless oil which, after purification on silicic acid with ether–light petroleum (b.p. 60–80°) (6:4), gave 1,2,4,6-tetra-*O*-acetyl-3-*O*-methyl-α-D-glucopyranose (3.4 g, 85%) as an oil[7] which was shown to be homogeneous by both t.l.c. and g.l.c.

A suspension of chromium trioxide (6.0 g) and 1,2,4,6-tetra-*O*-acetyl-3-*O*-methyl-α-D-glucopyranose (2.0 g) in glacial acetic acid (60 ml) was stirred for 1 h at room temperature. The product (1.8 g) was isolated as a colourless, viscous oil by a procedure similar to that described above. Crystallization of this oil from benzene–light petroleum (b.p. 60–80°) gave the formate (1.4 g, 66%), m.p. 76–77°, $[\alpha]_D^{24}$ + 113.2° (*c* 0.58, chloroform); n.m.r. data: δ 8.05 (singlet, formyl proton), 6.32 (doublet, $J_{1,2}$ 3.2 Hz, H-1), 5.60 (triplet, $J_{2,3} = J_{3,4}$ 9.5 Hz, H-3); no *O*-Me signal.

Anal. Calc. for $C_{15}H_{20}O_{11}$: C, 47.87; H, 5.32. Found: C, 47.56; H, 5.34.

1,2,4,6-Tetra-O-acetyl-3-O-benzoyl-β-D-glucopyranose. — 1,2,4,6-Tetra-*O*-acetyl-3-*O*-benzyl-β-D-glucopyranose[8] {m.p. 102–103°, $[\alpha]_D^{23}$ −1.25° (*c* 1.45, chloroform)} (1.4 g) was added to a stirred suspension of chromium trioxide (3.8 g) in glacial acetic acid (40 ml) at ambient temperature. Stirring was continued for 30 min, and the reaction mixture was then poured into chloroform. The product was isolated, by the procedure described above, as a colourless oil (1.3 g) which, on crystallization from 95% ethanol, gave 1,2,4,6-tetra-*O*-acetyl-3-*O*-benzoyl-β-D-glucopyranose (0.7 g, 50%), m.p. 154–155°, $[\alpha]_D^{23}$ +10.6° (*c* 1.16, chloroform); n.m.r. data: δ 8.11–7.82 and 7.67–7.25 (two and three aromatic protons, respectively), 5.88 (ill-defined quartet, owing to virtual coupling[7], $J_{1,2}$ *ca.* 7.2 Hz, H-1). By contrast, the starting material has a well-resolved spectrum: δ 7.30 (singlet, aromatic protons), 5.68 (doublet,

$J_{1,2}$ 8.0 Hz, H-1), 5.15 (two, nearly overlapping triplets, $J_{2,3} = J_{3,4}$ *ca.* 9 Hz, H-2 and H-4), 4.63 (singlet, benzylic H), 3.77 (triplet, H-3).

The m.p. of the benzoate was not depressed when admixed with a sample prepared by the acetylation of 3-*O*-benzoyl-D-glucose with acetic anhydride and pyridine. The 3-*O*-benzoyl-D-glucose was obtained from 3-*O*-benzoyl-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose by hydrolysis with 90% trifluoroacetic acid[9].

Anal. Calc. for $C_{21}H_{24}O_{11}$: C, 55.52; H, 5.37. Found: C, 55.76; H, 5.31.

ACKNOWLEDGMENT

This investigation was supported by a grant from the Australian Research Grant Committee.

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Note

1,3:4,6-Di-*O*-benzylidene-D-mannitol

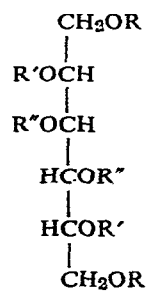
H. B. SINCLAIR

Northern Regional Research Laboratory, Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Peoria, Illinois 61604 (U. S. A.)

(Received April 22nd, 1969; in revised form, July 28th, 1969)

During an investigation of products resulting from the acid-catalyzed condensation of bromoacetaldehyde diethyl acetal and D-mannitol[1], 2,5-di-*O*-methyl-D-mannitol (**1**) (or its peracetate **2**) was needed for structural confirmation. The procedures for preparing this dimethylhexitol required either the preparation of three intermediates[2] (five steps in all) or the graded hydrolysis of 1,3:2,5:4,6-tri-*O*-ethylidene-D-mannitol[3], entailing a laborious separation of tri-, di-, mono-*O*-acetals, and free D-mannitol, methylation, and hydrolysis. As either procedure was lengthy and difficult, a simpler method was sought.

Acid-catalyzed condensation of aldehydes with D-mannitol usually yields a 1,3:2,5:4,6-tri-*O*-alkylidene-D-mannitol[3–5]. From the experimental conditions reported in the literature it appeared that product insolubility favored tricyclic acetal formation. Since the last ring to be formed is a seven-membered ring, reaction in a homogeneous medium might favor formation of the dicyclic acetal with six-membered rings. This was shown when D-mannitol was treated with benzaldehyde in acidified methyl sulfoxide to give a di-*O*-benzylidene-D-mannitol **3**. Proof that this product was a di-*O*-acetal was obtained by treatment with excess methanesulfonyl chloride in pyridine to give a di-*O*-methanesulfonate **4**, and by methylation with barium oxide–methyl iodide in *N*,*N*-dimethylformamide (Kuhn's procedure) to give a di-*O*-methyl ether **5**. Evidence for a 1,3:4,6-di-*O*-acetal structure was established by acid hydrolysis of compound **5** to give **1**, which was not isolated but directly converted into its tetra-*O*-acetyl ester **2**.



1 R = R″ = H, R′ = Me

2 R = R″ = Ac, R′ = Me

3 R+R″ = PhCH, R′ = H

4 R+R″ = PhCH, R′ = $MeSO_2$

5 R+R″ = PhCH, R′ = Me

EXPERIMENTAL

T.l.c. was performed on Silica Gel G* with air-equilibrated plates of 0.25-mm thickness and the solvent systems as specified. The spots were detected by spraying with 5% ethanolic sulfuric acid and heating until charred. U.v. spectra were measured with a Cary Model 14 spectrophotometer. I.r. spectra were determined with a Perkin–Elmer Model 621 spectrophotometer. N.m.r. spectra were obtained with a Varian Model HA-100 spectrometer. Chemical shifts were compared against internal tetramethylsilane. Melting points were determined on a Mel-Temp or a Mettler FP-1 apparatus. Analytical samples were dried in a 1–10 mm vacuum in presence of sodium hydroxide and sulfuric acid for 24–36 h.

1,3:4,6-Di-O-benzylidene-D-mannitol (**3**). — After D-mannitol (20 g) was dissolved in a mixture of benzaldehyde (40 ml) and methyl sulfoxide (200 ml), conc. sulfuric acid (10 ml) was added dropwise (requiring *ca.* 20 min) during which time the mixture warmed. After being kept 24 h at room temperature, the mixture was poured onto an ice–water slush (3 l). An oil separated which slowly turned solid as the ice melted. This solid was separated by filtration and washed with hexane (200 ml); it was then covered with water (4–6 l) and heated to boiling. Sodium carbonate was added to maintain the pH at 7–8 (paper). A small amount of solid remained insoluble at the boiling point and was removed by filtration; it melted at 214–216° and is probably a tri-*O*-benzylidene-D-mannitol[6]. On cooling, the filtrate slowly deposited crystals, which were separated by filtration to yield 4.96 g (12%) of **3**, m.p. 146–149°; $[\alpha]_D^{23}$ −7° (*c* 1.063, acetone); λ_{max}^{KBr} 730 cm^{-1} (phenyl); λ_{max} in 95% ethanol, 256 nm (ε 435); R_F 0.21 (1:1 carbon tetrachloride–ethyl acetate); n.m.r.: δ 5.56 (benzyl CH), ratio of aromatic to benzyl to aliphatic protons 9.9:1.9:10.0 (theory 10:2:10).

Anal. Calc. for $C_{20}H_{22}O_6$: C, 67.03; H, 6.19. Found: C, 67.32; H, 6.17.

1,3:4,6-Di-O-benzylidene-2,5-di-O-methylsulfonyl-D-mannitol (**4**). — Methanesulfonyl chloride (0.3 ml) was added to a cooled pyridine solution (2 ml) containing **3** (275 mg). After being kept for 64 h at 0–5°, the reaction mixture was worked up in the usual manner. The resulting solid was recrystallized from acetone–water to give **4**, 330 mg (83%), m.p. 187–189° (decomp.); $[\alpha]_D^{22}$ −30° (*c* 0.416, acetone); λ_{max}^{KBr} 1370, 1340, 1185, 1175 (SO_3), and 700 cm^{-1} (phenyl); λ_{max} in 95% ethanol, 256 nm (ε 440); R_F 0.57 (1:1 carbon tetrachloride–ethyl acetate); n.m.r.: δ 5.50 (benzyl CH), 2.96 (CH_3), ratio of aromatic to benzyl to aliphatic to methyl protons 5.2:1.0:4.2:3.0 (theory 5:1:4:3).

Anal. Calc. for $C_{22}H_{26}O_{10}S_2$: C, 51.35; H, 5.09; S, 12.46. Found: C, 51.48; H, 5.16; S, 12.38.

1,3:4,6-Di-O-benzylidene-2,5-di-O-methyl-D-mannitol (**5**). — A mixture of *N,N*-dimethylformamide (20 ml), methyl iodide (5 ml), barium oxide (5 g), and **3** (790 mg) was stirred vigorously for 3 days at room temperature, diluted with chloroform (25 ml), and filtered. The solid remaining on the funnel was washed with chloro-

* The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar produts not mentioned.

form, and the washings were combined with the filtrate. After being washed with water, the organic layer was dried and concentrated to give impure **5** (802 mg), m.p. 140–146°. Recrystallization from 95% ethanol (20 ml) gave matted needles of pure **5** (710 mg, 83%), m.p. 144–145.5°; $[\alpha]_D^{23}$ −22° (*c* 0.842, acetone); ν_{max}^{KBr} 2830 (OMe), and 730 cm^{-1} (phenyl); λ_{max}, in 95% ethanol, 256 nm (ε 420); R_F 0.75 (2:1 carbon tetrachloride–ethyl acetate); n.m.r.: δ 5.61 (benzyl CH), 3.46 (OMe), ratio of aromatic to benzyl to aliphatic to methoxyl protons 10.0:2.0:8.6:5.7 (theory 10:2:8:6).

Anal. Calc. for $C_{22}H_{26}O_6$: C, 68.37; H, 6.78. Found: C, 68.72; H, 6.95.

1,3,4,6-Tetra-O-acetyl-2,5-di-O-methyl-D-mannitol (**2**). — A suspension of **5** (533 mg) in 70% acetic acid (50 ml) was warmed until dissolution. The solution was kept for 16 h at room temperature, and concentrated in vacuum to a syrup. This syrup, acetic anhydride (10 ml), and anhydrous sodium acetate (1 g) were heated for 2 h at 100°, cooled to room temperature, and added to vigorously stirred ice (150 ml). After the ice melted, the solution was extracted with three 20-ml portions of chloroform. The extracts were washed with 100-ml portions of water, 10% sodium hydrogen carbonate, and water, dried, and concentrated to a thick oil. A solution of this oil in hot ethanol (10 ml) deposited crystalline **2** on cooling, 272 mg (52%), m.p. 107–108.8°; $[\alpha]_D^{24}$ +42° (*c* 0.224, methanol); ν_{max}^{KBr} 2840 (OMe), 1745, and 1735 cm^{-1} (ester); R_F 0.48 (1:1 carbon tetrachloride–ethyl acetate); n.m.r.: δ 3.38 (OMe), 2.09, and 2.06 (OAc). (Lit.[3]: m.p. 107–108°, $[\alpha]_D^{23}$ +39.5°).

Anal. Calc. for $C_{16}H_{26}O_{10}$: C, 50.79; H, 6.92. Found: C, 51.00, H, 6.84.

ACKNOWLEDGMENTS

Acknowledgment is made to Clara E. McGrew, Bonita R. Heaton, and Karen A. Jones for the microchemical analyses, and to W. A. Boyd and L. W. Tjarks for the n.m.r. data.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

Preliminary communication

A comparison of the structures of the α-D-glucose–urea and D-glucitol–pyridine hydrogen-bond complexes

R. L. SNYDER, R. D. ROSENSTEIN, H. S. KIM, AND G. A. JEFFREY
Crystallography Laboratory, University of Pittsburgh, Pittsburgh, Pa. 15213 (U. S. A.)
(Received September 12th, 1969; in revised form, November 13th, 1969)

The acid character of many carbohydrates enables them to form complexes with a wide variety of compounds. Numerous complexes between carbohydrates and alkali and alkaline-earth salts have been reported[1], and many other adducts involving such bases as urea[2], pyridine[3], and various amides[4] are known. Another adduct, which has not been considered in this light, is water; many carbohydrates, of course, form hydrates. Studies of X-ray crystal structures of a number of adducts involving metallic salts[5] have been made, but the nature of the bonding in the carbohydrate-base addition compounds has not till now been investigated. Nucleosides, which are condensation products of purine and pyrimidine bases with sugars, have been studied by X-ray diffraction. We have undertaken structural analyses of adducts and here report the preliminary results for the α-D-glucose–urea and D-glucitol–pyridine complexes.

The 1:1 addition compound α-D-glucose–urea has been reported[6] to crystallize in the orthorhombic space group $P2_12_12_1$, with $a = 6.913$, $b = 9.152$, $c = 16.509$ Å and four formula weights in the unit cell. In our analysis, MoKα diffractometer data were collected using an unequal interval nine-point θ–2θ scan technique[7], and the structure was solved using a combination of the symbolic-addition[8] and tangent-refinement[9] approaches. All non-hydrogen atoms were located in a three-dimensional E-synthesis, and the 16 hydrogens were found in the difference electron-density map. Full-matrix least squares refinement with anisotropic temperature parameters for the non-hydrogen atoms converged to a residual of 5.1 percent for 1140 reflections. The α-D-glucose and urea molecules are linked by a hydrogen bond network as shown in Fig. 1. The ureas, in the cylinders, form infinite rows staggered about the two-fold screw axis along *a*, with their planes nearly normal to it. Each glucose in the infinite rows of glucose molecules is linked with a pair of urea cylinders. It also bridges a pair of urea molecules within each of the two cylinders. Each glucose row is held together by hydrogen bonds from the anomeric hydroxyl of one glucose to the primary alcohol of the next molecule in the row. The remaining hydrogen bonds not shown interconnect the rows of glucose molecules. Every oxygen and nitrogen atom is hydrogen bonded, with nine bonds accepted and nine bonds donated, involving each formula unit in 18 hydrogen bonds.

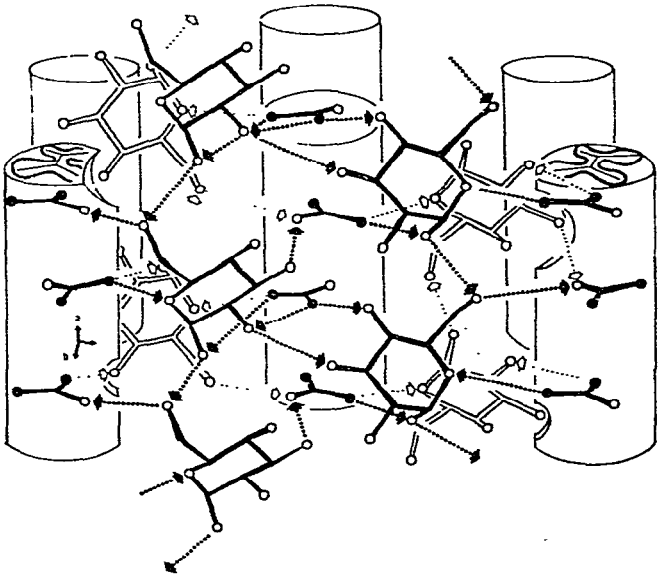

Fig. 1. Schematic representation of the crystal structure of the α-D-glucose–urea addition compound, showing a complete set of hydrogen bonds (dotted lines) between α-D-glucose and urea. For purposes of clarity, most of the inter-glucose hydrogen bonds have been omitted. The projections of the urea molecules are drawn at the top of 2 of the forward cylinders.

The D-glucitol–pyridine complex crystallizes in space group $P2_1$ with $a = 4.652$, $b = 10.207$, $c = 13.439$ Å, $\beta = 90°30'$ and two formula weights in the unit cell; 1165 reflections were recorded by a diffractometer with CuKα radiation using a θ–2θ scan technique. The structure was solved in the same way as that of the α-D-glucose–urea complex. Refinement on all coordinates and anisotropic thermal parameters for all non-hydrogen atoms has led to a residual of 4.8 percent.

The D-glucitol and pyridine molecules, as in the α-D-glucose–urea structure, are held together in a hydrogen bond network as shown in Fig. 2. But here the complex has alternating layers of pyridine and D-glucitol molecules and relies only on van der Waals attractions to hold together the pyridine molecules within the infinite sheets, rather than the completely three-dimensional hydrogen bonding found in the α-D-glucose–urea. It is interesting to note that the pyridine molecules within each sheet stack in the typical aromatic herringbone arrangement, whereas the ureas in the α-D-glucose–urea structure form no urea–urea hydrogen bonds, quite unlike the structure of the pure substance.

The most striking effect of complex formation, in both of the compounds under discussion and in the recently reported D-glucose hydrate[10], is a change in the conformation of the carbohydrate molecules. In the D-glucitol complex, both of the terminal hydroxyls in the glucitol molecule are anti-parallel to hydrogen atoms; whereas, in pure D-glucitol[11], they are anti-parallel to carbon atoms. That is, in the Klyne-Prelog notation[12,13], O-1 is Msc to O-2 and Ap to C-3, and O-6 is Psc to O-5 and Ap to C-4 in pure D-glucitol; but in D-glucitol–pyridine O-1 is Msc to

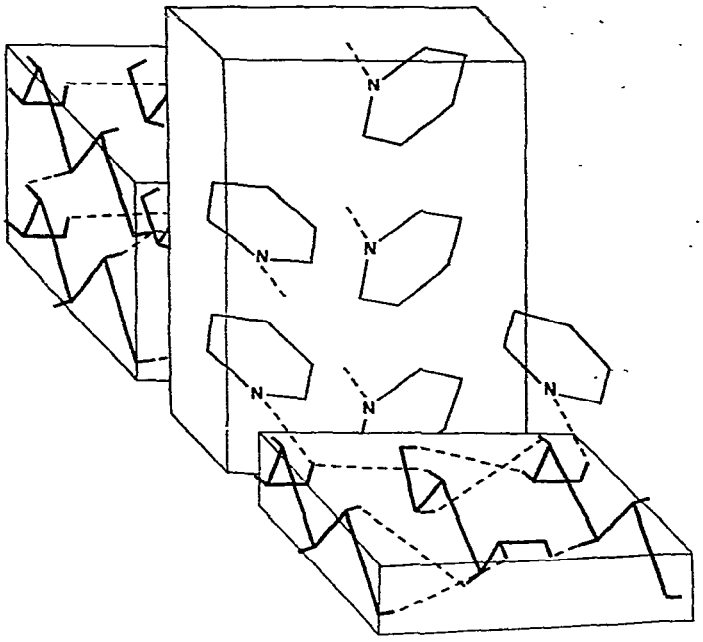

Fig. 2. Schematic representation of the crystal structure of the D-glucitol–pyridine addition compound.

C-3 and Psc to O-6, and O-6 is Msc to O-5, and Psc to C-4, a rotation of 120° around the terminal C–C bonds at both ends. The conformation of the primary-alcohol hydroxyl group with respect to the ring oxygen in the glucose molecules in both α-D-glucose–urea and D-glucose–water is Msc (anti-parallel to hydrogen). In pure α-D-glucose[14], it is Psc (anti-parallel to carbon). The Msc conformation is sterically unfavorable, making the bond distances of the ether linkage "significantly" unequal. In contrast, these two C–O bond lengths are the same in pure α-D-glucose.

It is clear that the primary driving force in the formation of the two complexes reported here and in that of the D-glucose hydrate is the ability to form an exceptionally stable hydrogen-bonding arrangement. In the case of the D-glucose adducts, this arrangement is sufficiently stable to overcome the considerable non-bonding repulsions present in the observed conformation.

ACKNOWLEDGMENTS

We would like to thank Dr. A. McL. Mathieson for supplying us with seed crystals of the α-D-glucose–urea complex. This research was supported by a grant (GM-11293) from the U. S. Public Health Service, National Institute of General Medicine.

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Carbohyd. Res., 12 (1970) 153–156

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

CONFORMATION OF ACETYLATED CYCLODEXTRINS AND AMYLOSE*†

B. CASU, M. REGGIANI,
Istituto Scientifico di Chimica e Biochimica G. Ronzoni, 20133 Milano (Italy)
G. G. GALLO AND A. VIGEVANI
Laboratori Ricerche Lepetit S.p.A., 20158 Milano (Italy)
(Received April 14th, 1969; in revised form, July 1st, 1969)

ABSTRACT

The conformation of the monomeric units and of the polymeric chain of the peracetates of α- and β-cyclodextrin and amylose have been investigated by i.r. and p.m.r. spectroscopy. The spin–spin coupling constants of the ring protons and the i.r. spectra indicate the *C1* conformation for the D-glucopyranose units. The i.r. dichroism of an oriented film of amylose triacetate is consistent with a helical conformation of the chain.

The chemical shift difference of the signals for H-1 and H-4 in β-cyclodextrin triacetate (assumed to be in a "quasi-eclipsed" chain conformation) and amylose triacetate is consistent with a rotation of the monomeric units of amylose triacetate around the C-1–O and C-4–O bonds. The solvent effect on the chemical shift of the ring and acetyl protons has also been studied. Assignments for the acetyl signals of the triacetates of cyclodextrins and amylose are proposed.

INTRODUCTION

A number of physico-chemical properties of amylose and its cyclic analogues (cyclodextrins) are dependent on the conformations of the D-glucopyranose units and of the polymer chain or the macrocycle. The D-glucopyranose ring of the above compounds has been conclusively demonstrated by X-ray[1], p.m.r.[2,3], and i.r. studies[3,4] to be in a substantially undistorted, *C1*(D) chair conformation. Such a conclusion is also supported by theoretical calculations of the conformational energies[5]. Due to the rigidity of the monomeric units in the chair form, the flexibility of the amylose macromolecule (which allows at least three stable chain conformations[6]) is then attributed to the rotation of the units around the "glycosidic" C-1–O and C-4–O bonds. In the case of cyclic oligomers of α-D-glucose, such rotation is largely prevented by steric restrictions imposed by the macrocyclic structure.

*Part IV of the Series *Conformation of amylose and its derived products.* Part III: Ref. 9.
†Dedicated to Professor Dr. E. Husemann for her sixtieth birthday.

The stability of the *C1* conformation of α-D-glucopyranose is higher than that of any other possible forms[7], even in *O*-methyl[8,9] and *O*-acetyl[10,11] derivatives. On the other hand, in dimeric and polymeric structures, the *C1* conformation might be destabilized by nonbonded and dipolar interactions between bulky substituents on adjacent units, especially in cyclodextrins, where steric interactions between contiguous units cannot be relieved by rotation of the units about the glycosidic bonds.

We have recently shown by p.m.r. and i.r. spectroscopy that *O*-methylation does not appreciably modify the *C1* conformation of the D-glucopyranose units of cyclodextrins and amylose[9]. Since the above conformation was found for per-*O*-methylated cyclodextrins as well as for 2,6-di-*O*-methylated cyclodextrins, in which the 3-hydroxyl group is hydrogen bonded to O-2 of the contiguous unit, it is probable that the intramolecular hydrogen-bond between contiguous units, already demonstrated for unsubstituted cyclodextrins[1,3], is only an additional factor that stabilizes the *C1*(D) conformation, but does not determine it. These findings are in keeping with recent calculations of conformational energies on unsubstituted cyclodextrins, which suggest that only the nonbonded interactions present can account for the actual molecular conformation of these products[12].

In the present work, the i.r. and p.m.r. spectroscopic investigation has been extended to acetylated derivatives of cyclodextrins and amylose, mainly to ascertain whether the acetyl groups, whose steric requirements are more severe than those of methyl groups, destabilize the *C1* conformation of the D-glucopyranose units.

The i.r. approach used is based on established correlations between some spectral features and the orientation (equatorial or axial) of the C-1–H bond[13] and of the acetoxyl groups[14] relative to the plane of the D-glucopyranose units. Information about the chain conformation has been sought in measurements with polarized i.r. radiation on oriented samples[15].

The p.m.r. approach has mainly utilized the well-known dependence of the chemical shifts and coupling constants of the hydrogen atoms of the pyranose rings of acetylated monosaccharides on their relative orientation[10,11]. Indirect evidence about the conformation of the pyranose rings has been obtained from the chemical shift of the acetoxyl signals[10]. Particular consideration has been given to the probability that the chemical shift of the anomeric protons of a polysaccharide depends, among other factors, on the relative orientation of the contiguous unit[9,16]. That is, the C-4–O bond appears to shield H-1 to an extent that depends on the angle of internal rotation of the monomeric units about the C-1–O bond. Similarly, the extent of shielding of H-4 by the C-1–O bond appears to depend on the angle of internal rotation around the C-4–O bond. Accepting these premises, information can be obtained about the chain conformation of polyglucoses by estimating the contribution of the diamagnetic anisotropy of C-4–O and C-1–O bonds to the chemical shift of H-1 and H-4, respectively.

The triacetates of α- and β-cyclodextrin and amylose have been studied in the solid state (amorphous and crystalline) by i.r. spectroscopy, and in solution by p.m.r. spectroscopy. The acetylated monomer (α-D-glucose pentaacetate) and dimer

(β-maltose octaacetate), as well as β-D-glucose pentaacetate, have also been examined for comparison purposes.

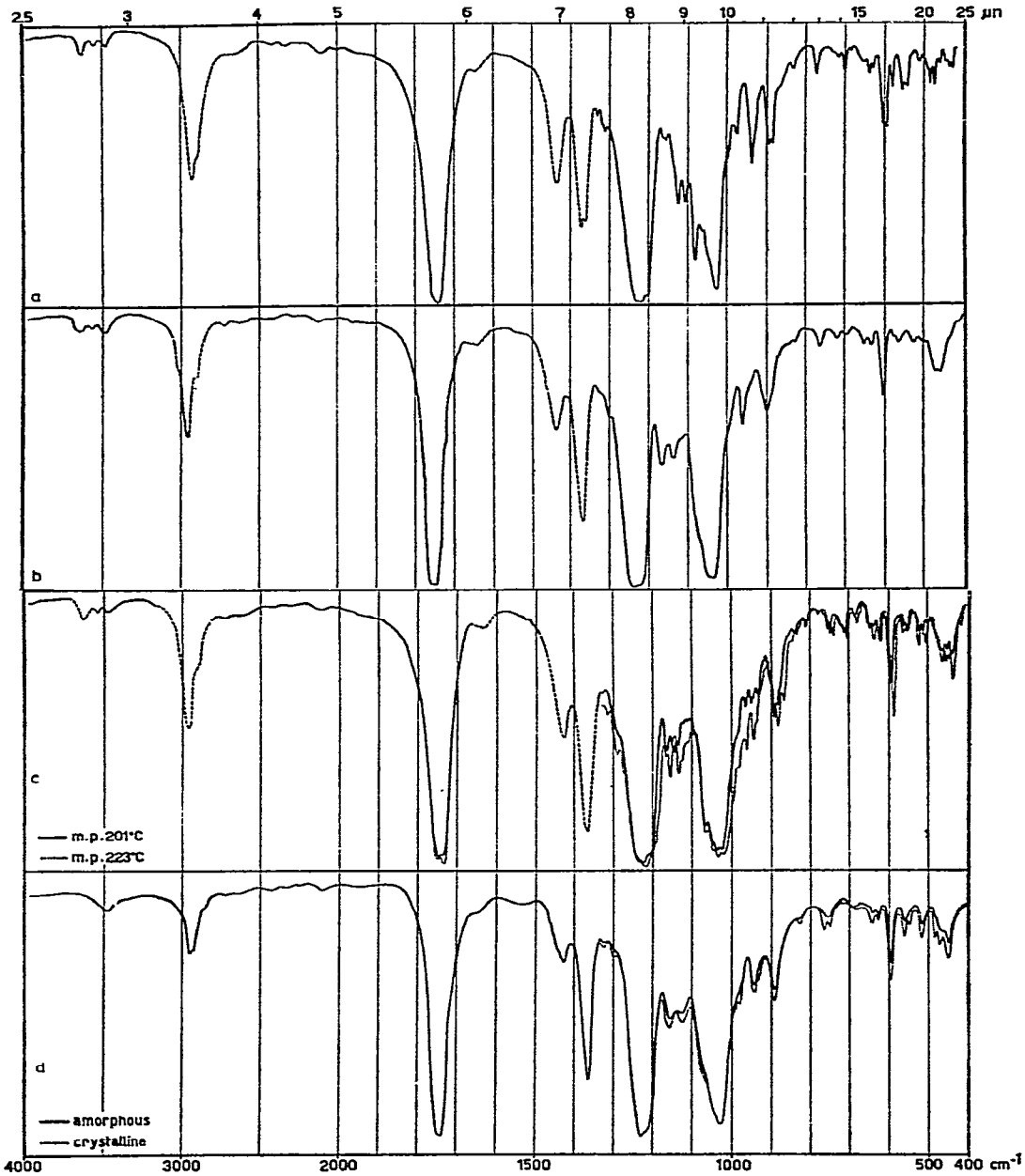

Fig. 1. I.r. spectra of β-maltose octaacetate (a), α- and β-cyclodextrin triacetates (Nujol mulls) (b and c), and amylose triacetate (film) (d).

RESULTS

I.r. spectra

The i.r. spectra of the peracetates of β-maltose, α- and β-cyclodextrin, and amylose in the solid phase are shown in Fig. 1. The main bands were easily assigned to C–H stretching vibrations (~2960 cm^{-1}), C=O stretching (~1750 cm^{-1}), C–O–C stretching of the acetoxyl groups (~1230 and 1040 cm^{-1}), C–H bending (~1430 and 1370 cm^{-1}), and C–O–C asymmetric stretching of the glycosidic bridge (~1160 cm^{-1}). Absorptions due to the acetoxyl groups are the most intense, whereas the bands associated with C–O, C–C, and C–H vibrational motions of the pyranose units and of the chain skeleton are of medium or weak intensity.

The bands due to "ring vibrations" and "C-1-group vibration"[13] are evident in the spectrum of α-D-glucose pentaacetate[13,14] in the regions near 920 and 850 cm^{-1}, indicating the *C1*(D) conformation (H-1 equatorial). In the spectrum of β-maltose octaacetate, besides the ring and the C-1-group bands typical of α-D derivatives having the *C1* conformation, a medium intensity band is observed near 890 cm^{-1}. This band, also shown by β-D-glucose pentaacetate and by a number of β-D-glucose derivatives[13,14], is probably due[13] to the C-1-group of the ring having H-1 axial. The i.r. spectrum of β-maltose octaacetate is thus consistent with the *C1*(D) conformation of both units (H-1 equatorial in the A ring and axial in the B ring).

The triacetates of cyclodextrins and amylose show a pattern in the 1000–750 cm^{-1} range substantially similar to that of β-maltose octaacetate. The splitting of a few bands, particularly evident in the case of β-cyclodextrin triacetate, is probably due to crystallinity effects. The C-1-group band, although weak, is still observable. It is worth noting that the frequency of the band near 950 cm^{-1}, attributable to a ring vibration[13], moves towards higher frequencies in going from β-maltose octaacetate (941 cm^{-1}) to the triacetates of amylose (954 cm^{-1}) and of cyclodextrins (α: 959; β: 964 cm^{-1}). This trend parallels that of a similar band characteristic of unsubstituted maltosides[4] and can be ascribed to vibrational coupling between groups belonging to contiguous units.

The polarized i.r. spectrum of an oriented amylose triacetate film is shown in Fig. 2. It exhibits little or no polarization for most bands, including those attributed to the acetyl C=O and C–O bonds. A few bands of medium or low intensity are, nevertheless, well polarized, *e.g.*, the doublet near 760 cm^{-1}.

P.m.r. spectra

P.m.r. data are given in Table I for the ring protons, and in Table II for the acetoxyl protons of peracetylated α-D-glucose, β-maltose, α- and β-cyclodextrin, and amylose in solution in deuteriochloroform.

The 100 MHz spectra of peracetylated α-D-glucose and β-maltose are shown in Fig. 3, and those of triacetylated cyclodextrins and amylose in Fig. 4. Assignments for most signals are given in the tables and the figures, those for the ring protons of α-D-glucose pentaacetate having previously been made by Lemieux and Stevens[11].

TABLE I

CHEMICAL SHIFTS (τ) AND COUPLING CONSTANTS (Hz, GIVEN IN PARENTHESES) OF THE RING PROTONS OF PERACETYLATED α-D-GLUCOSE AND ITS α-(1→4)-LINKED DIMER, POLYMER, AND CYCLIC OLIGOMERS IN $CDCl_3$ AT 100 MHz

Compounds	*H-1* *doublet*	*H-2* *pair of doublets*	*H-3* *pair of doublets*	*H-4* *pair of doublets*	*H-5* *pair of doublets*	*H-6* *pair of doublets*	*H-6′* *pair of doublets*
Penta-*O*-acetyl-α-D-glucose	3.69 (4.0)	4.92 (4.0; 9.8)	4.51 (9.8; 9.5)	4.87 (9.5; 8.8)	5.88[a] (10.0[a])	5.82; 5.86; 5.88[b]	5.72 (12.2; 4.0)
Octa-*O*-acetyl-β-maltose							
ring A	4.59 (3.9)	5.14 (3.9; 10.5)	4.62 (10.5; 9.0)	4.95 (9.0; 9.7)	5.82[c]	5.77 (12.2; 3.9)	5.51 (12.2; 3.9)
ring B	4.25 (7.8)	5.03 (7.8; 9.0)	4.69 (9.0; 7.8)	5.96 (7.8; 8.0)	5.89[b]	5.77 (12.2; 3.9)	5.51 (12.2; 3.9)
Amylose triacetate	4.66 (~4)	5.25[d]	4.56[d]	6.01[d]	6.01[d]	5.51; 5.63; 5.74[b]	
α-Cyclodextrin triacetate	4.93 (3.5)	5.24 (3.5; 9.9)	4.43 (9.9; 8.2)	6.21 (8.2; 8.6)	5.80[e]	5.56[b]	
β-Cyclodextrin triacetate	4.92 (3.9)	5.21 (3.9; 9.2)	4.68 (9.2; 8.1)	6.29 (8.1; 8.4)	5.79; 5.91[b]	5.35; 5.47; 5.66; 5.69[b]	

[a]Data from Ref. 11, taken on the 6,6′-dideuterio derivative. [b]Most-prominent peaks of a complex multiplet. [c]Most-prominent peak observed upon irradiating the signal for H-4A. [d]Unresolved peak. [e]Peak arising from a doublet, observed upon irradiating the signal for H-4.

TABLE II

ACETOXYL RESONANCES (τ) OF PERACETYLATED D-GLUCOSES, β-MALTOSE, AMYLOSE, AND CYCLODEXTRINS IN $CDCl_3$ AT 100 MHz

Penta-*O*-acetyl-α-D-glucose	7.83;			7.92;		7.97;	7.98;	7.99;			
Penta-*O*-acetyl-β-D-glucose		7.89;		7.92;		7.97;	7.97;	7.99;			
Octa-*O*-acetyl-β-maltose		7.88;	7.91;	7.92;		7.96;	7.98;	7.99;	8.00;	8.01;	
Amylose triacetate	7.84;									8.02;	8.06;
α-Cyclodextrin triacetate	7.85;				7.94;	7.96;					
β-Cyclodextrin triacetate			7.90;	7.92;		7.97;					

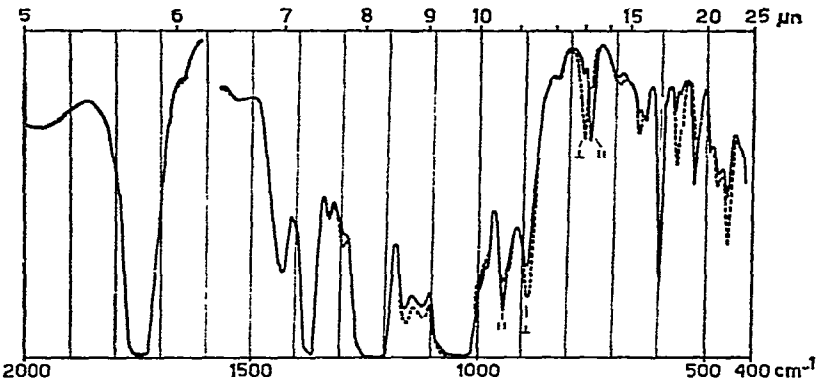

Fig. 2. I.r. polarized spectrum of an oriented film of amylose triacetate. (∥ = polarized light parallel to the direction of stretching; ⊥ = polarized light perpendicular to the direction of stretching).

For assignment of the signals of the peracetylated dimer, cyclic oligomers, and polymers of α-D-glucose, advantage has been taken of some generalizations worked out by Lemieux *et al.*[10,11] for acetylated monosaccharides. The assignments were confirmed by spin-decoupling experiments.

Data for solutions in Me_2SO-d_6 (see Table III) permit a comparison of the chemical shift of the *ring protons* of the unsubstituted carbohydrates[3,17] with those of the corresponding peracetylated derivatives. As expected, on account of the influence of the acetyl group, the resonances of H-2, H-3, and H-6 of α-(1→4)-linked oligo- and poly-D-glucoses are consistently shifted downfield on acetylation of the hydroxyl groups at C-2, C-3, and C-6. The signal for H-1 of peracetylated glycosides is therefore no longer the one farthest downfield. As the resonances of the ring protons of peracetylated α-(1→4)-linked di-, oligo-, and poly-D-glucoses are spread over a range of more than 2 p.p.m., most signals approach a first-order pattern and allow an approximate evaluation of the coupling constants directly from the signal splitting. Such values are fully consistent with those expected for the *C1* conformation of the D-glucopyranose units, wherein H-1 and H-2 are in a *trans* (equatorial, axial) orientation, and H-2, H-3, and H-4 are *trans*-axially oriented with respect to the next proton in the ring.

From data in Table I, it appears that the ring protons of peracetylated α-(1→4)-linked di-, oligo-, and poly-D-glucoses resonate at somewhat different field relative to the pentaacetylated monomer. Besides the above-mentioned upfield shift of the signals for H-1 and H-4, due to replacement of the acetyl groups at O-1 and O-4 by the glycosidic bridge, a slight, upfield shift is observed for the signals of H-2 and H-3 of β-maltose and particularly for the "internal" ones, designated H-2A and H-3B. As for the chemical shift differences between the dimer, the oligomers, and the polymer, an upfield shift is clearly shown by the resonances for H-1 and H-4 in going from maltose to amylose to cyclodextrins, as shown in Fig. 5. Furthermore, the signal for H-3 of α-cyclodextrin is found at significantly low field relative to β-maltose, amylose, and β-cyclodextrin.

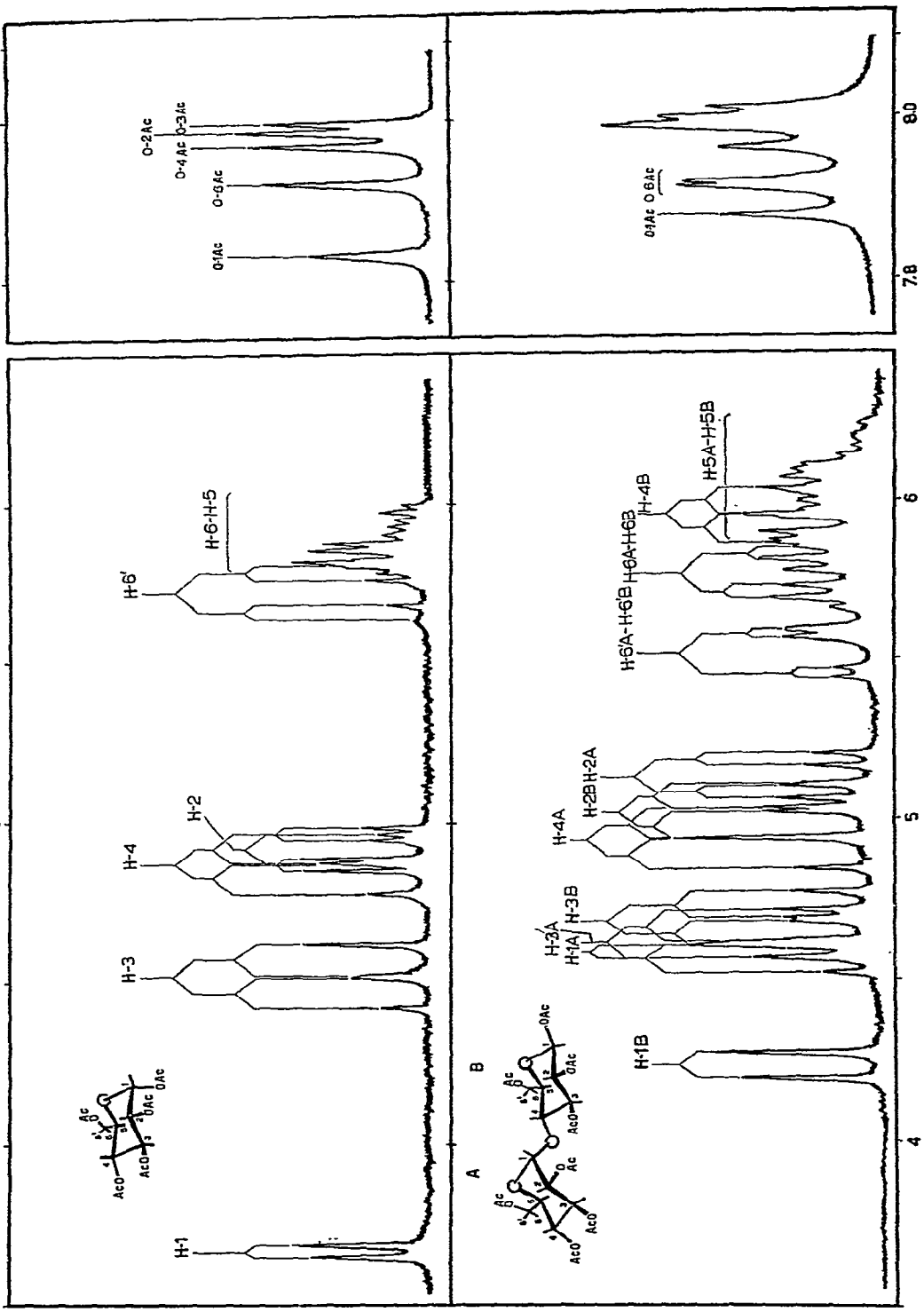

Fig. 3. P.m.r. spectra of α-D-glucose pentaacetate and β-maltose octaacetate in $CDCl_3$ solution at 100 MHz.

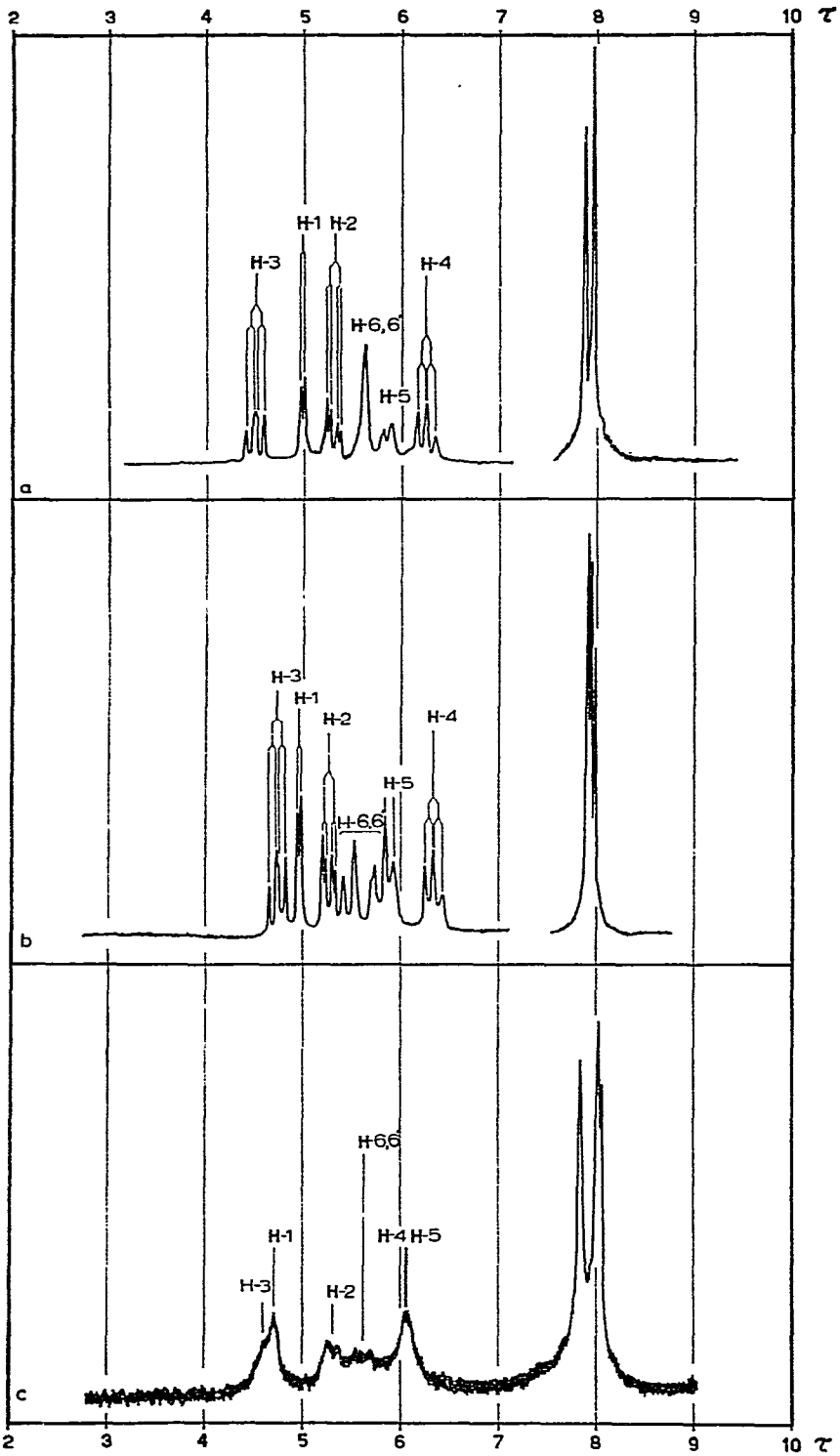

Fig. 4. P.m.r. spectra of α- and β-cyclodextrin triacetate (a and b) and amylose triacetate (c) in $CDCl_3$ solution at 100 MHz.

TABLE III

SOLVENT EFFECTS ON THE CHEMICAL SHIFT[a] OF RING AND ACETYL PROTONS OF THE PERACETATES OF α-D-GLUCOSE AND OF α- AND β-CYCLODEXTRIN

Compounds	*Solvents*	*Ring protons*								*Acetyl protons*					
		H-1		*H-2*		*H-3*		*H-4*		*O-3*		*O-2*		*O-6*	
		τ	Δτ	τ	Δτ	τ	Δτ	τ	Δτ	τ	Δτ	τ	Δτ	τ	Δτ
Penta-*O*-acetyl-α-D-glucose	Acetone-d_6	3.72	+0.06	4.96	+0.05	4.46	−0.03	4.83	−0.01	7.97	+0.07	8.00	+0.04	8.00	+0.04
	Me_2SO-d_6	3.81	+0.15	4.97	+0.06	4.56	+0.07	4.92	+0.08	8.00	+0.10	8.00	+0.04	8.00	+0.04
	C_6D_6	3.42	−0.24	4.76	−0.15	4.20	−0.29	4.65	−0.19	8.22	+0.32	8.30	+0.34	8.36	+0.40
	Pyridine-d_5	3.25	−0.41	4.51	−0.40	4.04	−0.45	4.45	−0.39	7.91	+0.01	7.96	0.00	8.00	+0.04
α-Cyclodextrin triacetate	Acetone-d_6	4.87	−0.16	5.27	+0.02	4.49	+0.06	6.02	−0.19	7.90	+0.08	7.96	+0.04	7.96	+0.04
	Me_2SO-d_6	4.93	0.00	5.30	+0.05	4.57	+0.14	5.92	−0.29	7.95	+0.13	7.98	+0.06	7.98	+0.06
	C_6D_6	4.77	−0.16	4.96	−0.29	4.24	−0.19	6.15	−0.06	8.04	+0.22	8.07	+0.15	8.12	+0.20
	Pyridine-d_5	4.35	−0.58	4.72	−0.53	3.90	−0.53	5.73	−0.48	7.69	−0.13	7.84	−0.08	7.88	−0.04
β-Cyclodextrin triacetate	Acetone-d_6	4.73	−0.18	5.13	−0.09	4.50	−0.18	6.03	−0.26	7.87	−0.01	7.87	−0.03	7.87	−0.07
	Me_2SO-d_6	4.90	−0.01	5.21	−0.01	4.78	+0.10	6.13	−0.16	7.94	+0.06	7.94	+0.04	7.94	0.00
	C_6D_6	4.65	−0.26	4.90	−0.32	4.28	−0.40	6.14	−0.15	7.94	+0.06	7.94	+0.04	8.01	+0.07
	Pyridine-d_5	4.45	−0.46	4.78	−0.44	4.25	−0.43	5.80	−0.49	7.80	−0.08	7.80	−0.10	7.80	−0.14

[a] τ values measured at 60 MHz; Δτ values relative to $CDCl_3$ solution.

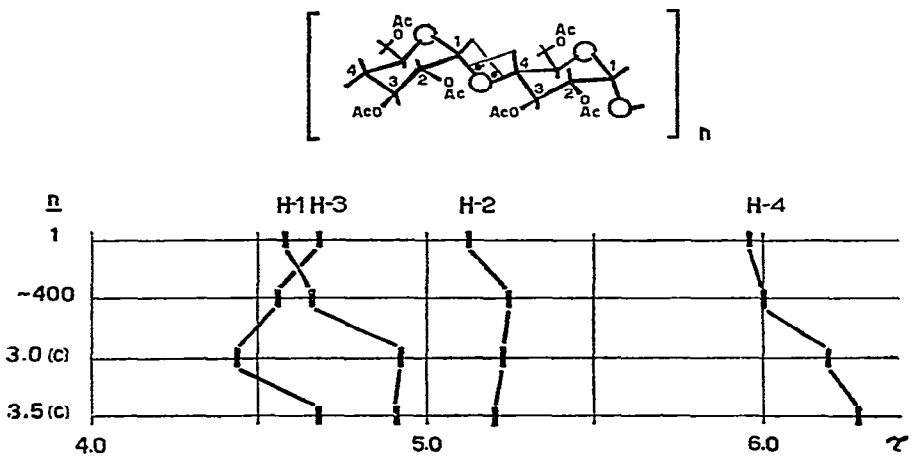

Fig. 5. Variation of the chemical shift of H-1, H-2, H-3, and H-4 signals going from β-maltose octaacetate ($n = 1$) to amylose triacetate ($n = \sim 400$) to α-cyclodextrin triacetate [$n = 3(c)$], and to β-cyclodextrin triacetate [$n = 3.5$ (*c*)] in $CDCl_3$ solution. For β-maltose octaacetate H-1 and H-2 signals of the ring *A*, and H-3 and H-4 signals of the ring *B* are considered.

Assignment of the *acetoxyl resonances* of α-D-glucose pentaacetate (Fig. 3) was made by comparison with that recently reported for β-D-glucose pentaacetate[18], based on the chemical shift observed for some tetraacetylated β-D-glucoses whose unsubstituted hydroxyl groups were subsequently deuterioacetylated. Only two acetoxyl signals of α-D-glucose pentaacetate change on altering the anomeric configuration. The largest shift (0.06 p.p.m. upfield) is observed for the O-1 acetyl peak, easily identifiable as the methyl signal at the lowest field[10]. The other signal of α-D-glucose pentaacetate, which differs from the corresponding one of the β-D anomer (upfield shift of 0.01 p.p.m.), was assigned to the acetyl group at O-2 on the assumption that this group is affected by the change in configuration. From data in Table II, it appears that the triacetates of amylose and α-cyclodextrin display one acetoxyl signal at a rather low field (τ 7.84 and 7.85, respectively) compared to the usual range[19] of the equatorial acetoxyl resonances in pyranoses.

Table III reports the chemical shift of the ring and acetyl protons of α-D-glucose pentaacetate and of the triacetates of α- and β-cyclodextrin in a number of solvents (acetone-d_6, Me_2SO-d_6, benzene-d_6, and pyridine-d_5), and the $\Delta\tau$ relative to chloroform-*d*.

The following observations can be made: (*a*) The signal for H-1 is found at slightly lower field for β- than for α-cyclodextrin, except in pyridine, where the situation is reversed. (*b*) The signal for H-2 is at higher field for α-cyclodextrin than for α-D-glucose and is at somewhat lower field for β- than for α-cyclodextrin, except in pyridine, where the signal produced at lower field is that of α-cyclodextrin. (*c*) The signal for H-3 moves slightly towards higher field in going from the monomer to α- and to β-cyclodextrin, in benzene and in acetone, whereas in methyl sulphoxide it is displayed at about the same field for α-D-glucose and α-cyclodextrin, but shifts

more than 0.2 p.p.m. upfield for β-cyclodextrin. In pyridine, the H-3 shift, relative to that of α-D-glucose, displays the same trend as in chloroform, *i.e.*, it is negative (*ca.* −0.1 p.p.m.) for α-cyclodextrin, and positive (*ca.* +0.2 p.p.m.) for β-cyclodextrin. (*d*) H-4 absorbs at somewhat higher field for β- than for α-cyclodextrin, except in benzene where the chemical shift is nearly the same. (*e*) No comparison was made for the signals for H-5, H-6, and H-6′, because they are superimposed to some extent.

As regards the solvent effect on the acetyl protons, the following observations can be made (the O-1 and O-4 acetyl signals are not considered): (*a*) Relative to the signals for α-D-glucose in chloroform, there is a general upfield shift in the order: pyridine (*ca.* +0.02 p.p.m.) <acetone and Me_2SO (*ca.* +0.05 p.p.m.) <benzene (*ca.* +0.35 p.p.m.). (*b*) The same trend is observed for α-cyclodextrin in acetone, in Me_2SO, and in benzene, and for β-cyclodextrin in Me_2SO and in benzene, whereas a downfield shift with respect to chloroform is observed for α-cyclodextrin in pyridine and for β-cyclodextrin in acetone and in pyridine. All of the acetoxyl signals are found at higher field for α- than for β-cyclodextrin, except in pyridine, where one peak of α-cyclodextrin is consistently downfield with respect to β-cyclodextrin. Since a parallel shift is given by H-3 of α- relative to β-cyclodextrin in the same solvent, this acetoxyl resonance for α-cyclodextrin (τ 7.69 in pyridine) and also the corresponding one for β-cyclodextrin (τ 7.80 in pyridine), is tentatively attributed to the O-3 acetyl group.

DISCUSSION

Whereas the coupling constants of the ring protons of di-, oligo-, and polyglucoses are dependent essentially on the conformation of the glucopyranose units, their chemical shifts are considered to be affected also, to a variable extent, by the chain conformation, because they are influenced by the magnetic anisotropy of the C–O and C–C bonds of the adjacent units. With the acetylated derivatives, a definite contribution to the chemical shift of the ring protons is expected also from the acetoxyl groups, and the extent of this contribution is dependent on the conformation of such groups. Owing to non-bonded and dipolar interactions between the substituents on adjacent units, the average conformation of the acetoxyl groups is expected to vary in going from the monomer to the polymer, and is also expected to be different for different relative orientations of the contiguous units.

To estimate the order of magnitude of the chemical shift change of the ring protons for a given change in the chain conformation, triacetylated cyclodextrins were taken as reference structures. In such structures, the C-1–H and C-4–H bonds of each pair of contiguous monomeric units lie approximately on the plane bisecting the macrocycle through the glycosidic bridges. As shown in Fig. 5, in such a chain conformation, the projected angle between H-1 and the centre of the C-4–O bond (ϑ) is larger than 55°, and therefore H-1 lies within the shielding cone of the C-4–O bond[20]. Similarly, H-4 lies within the shielding cone of the C-1–O bond. Any depar-

ture from the "quasi-eclipsed" conformation of cyclodextrins, by rotation of the monomeric units about the C-1–O and/or C-4–O bonds, will then lead to deshielding of H-1 and/or H-4, respectively. As the H-1 and H-4 signals both move towards lower field in going from cyclodextrins to amylose and to β-maltose, it is accordingly inferred that, in "linear" structures, the removal of the steric restrictions imposed by the macrocycle is accomplished by rotation of the monomeric units about both the C-1–O and C-4–O bonds. A similar trend had already been observed for the H-1 signal of unsubstituted[3] and *O*-methylated[9] compounds. In the peracetylated derivatives, the extent of such rotation is higher for the dimer than for the polymer, as observable also for *O*-methylated compounds[9]. Thus, in our case, the conformation of the acetoxyl groups appears to affect essentially only the protons on the carbon atoms to which they are attached, *i.e.*, C-2, C-3, and C-6, but has little influence on the chemical shift of H-1 and H-4.

Among the factors that may contribute to alter the local magnetic field at each proton, the electrostatic and magnetic anisotropic effects associated with the lone pairs of electrons of the glycosidic oxygen atom probably cannot be neglected, since the orientation of their orbitals with respect to H-3 and H-5 is dependent on the relative orientation of the contiguous units*. In the present instance, this contribution cannot be evaluated readily, because the H-3 resonance is influenced also by the conformation of the O-3 acetyl group, and the chemical shift of H-5 cannot be obtained accurately from our spectra. Furthermore, although no evidence for inclusion has been obtained so far for triacetylated cyclodextrins, the possibility that there are localized solvent effects, due to inclusion of solvent molecules within the macrocycle, is not improbable.

The assignment of the signals of the acetyl protons of the triacetates of cyclodextrins and amylose is uncertain because of the large differences in chemical shift that are expected to be brought about by small changes in the conformation of the acetyl groups relative to the reference monomer. A tentative interpretation was made by assuming that any large shift of the signals for H-2, H-3, and H-6, on going from the dimer, to the cyclic oligomers, to the polymer, or on changing the solvent, should be parallelled by a similar shift of the corresponding acetoxyl signals. On this basis, the most downfield methyl signal of α-cyclodextrin triacetate was assigned to the O-3 acetyl group. Moreover, since the separation of the signals for H-3 and H-2 of α-cyclodextrin triacetate increases on going from benzene to methyl sulphoxide to acetone to chloroform to pyridine, and parallels the separation between the O-3 acetyl peak and the next acetyl peak at higher field, this latter peak is probably attributable to the O-2 acetyl group. A similar assignment of the acetoxyl signals (*i.e.*, to the O-3, O-2, and O-6 acetyl groups, in the order towards higher field) probably holds also for amylose triacetate.

*The importance of the lone pairs of electrons on oxygen in determining the chemical shift of some ring protons (such as H-3 and H-5 of α-D-glucopyranose) has been pointed out by Lemieux[16], in connection with the deshielding of axial hydrogen atoms by an opposing oxygen atom in axial orientation.

It is noteworthy that the O-3 acetyl group of the triacetates of α-cyclodextrin and amylose resonates at a field that is typical for axially oriented groups in monosaccharides[19]. Since the *C1* conformation of the D-glucopyranose units rules out axial acetoxyl groups, the correlations worked out for monosaccharides are no longer valid for macromolecular structures, where non-bonded and dipolar interactions between adjacent units may strongly affect the conformation and hence the magnetic shielding of the acetoxyl groups.

Concerning amylose triacetate, the i.r. dichroism of its oriented film (Fig. 2) confirms the recently reported evidence, from X-ray data[21], that this polymer exists in a helical conformation. In fact, the lack of polarization of the C=O and C–O acetyl absorptions in the sample (that shows a good X-ray fibre pattern and a remarkable polarization of a few minor i.r. bands) suggests an "average" orientation of the above bonds along the polymer axis. The i.r. polarization of the cellulose triacetate, which displays a strong perpendicular dichroism of the acetyl C=O and C–O bands and a parallel dichroism of the C–O glycosidic band[22], is, on the contrary, consistent with a substantially linear structure with the acetoxyl groups projected in a plane normal to the chain axis. The dichroism observed for a few medium or weak bands of amylose triacetate most probably arises from lateral vibrational interactions between adjacent polymer chains.

EXPERIMENTAL

The i.r. spectra were obtained with a Perkin–Elmer Model 125 grating spectrophotometer. The i.r. polarized spectrum of the oriented film of amylose triacetate was measured with a Perkin–Elmer AgCl polarizer.

The p.m.r. spectra were taken on Varian A-60 and HA-100 spectrometers, with tetramethylsilane as internal standard, on solutions containing 2–15% of carbohydrate. The apparent coupling constants were measured directly from the signal splitting. The probe temperature was 38° for the measurements at 60 MHz and 28° for those at 100 MHz.

Amylose triacetate was obtained by the Mullen and Pacsu procedure with acetic anhydride in pyridine[23] from amylose of mol. wt. 150,000 (Koch–Light, Colnbrook, Bucks, England). Amorphous films of amylose triacetate for the i.r. measurements were cast from solutions in $CHCl_3$ or CH_2Cl_2. The crystalline film for the unpolarized spectrum was obtained by heating the amorphous film for 2 h at 200°. The crystalline, oriented film for measurements with i.r. polarized light was obtained by stretching[24] the amorphous film seven times its length in glycerol at 170°; its X-ray diffraction diagram is substantially the same as that reported by Sarko and Marchessault[21].

α-Cyclodextrin triacetate (m.p. 244–245°) and β-cyclodextrin triacetate (m.p. 223–224°) were prepared, by the same procedure as for amylose triacetate, from unsubstituted cyclodextrins[25]. Reference samples of the triacetylated cyclodextrins and a polymorphic form of β-cyclodextrin triacetate (m.p. 200–201°) were kindly provided by Prof. H. Schlenk of the University of Minnesota.

β-Maltose octaacetate, m.p. 159°, $[\alpha]_D^{20}$ +62.8° (chloroform), was prepared according to Whistler and Hickson[26].

ACKNOWLEDGMENTS

We express our appreciation to Mr. M. Psallidi and Mr. A. Ripamonti for technical assistance, to Dr. A. Segre of the Politecnico, Milano, for the use of the HA-100 instrument, and to Dr. A. S. Perlin for kindly revising the manuscript. Two of us (B.C. and M.R.) gratefully acknowledge the U.S. Department of Agriculture for a Research Grant [UR-E-15 (10) 32 FG-It-115].

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

COMPARATIVE STUDIES OF LORENZINI JELLY FROM TWO SPECIES OF ELASMOBRANCH

PART II. STRUCTURAL STUDIES OF GLYCOPEPTIDES

M. J. How*, J. V. S. Jones†, and M. Stacey
Department of Chemistry, The University, Edgbaston, Birmingham 15 (Great Britain)

(Received July 14th, 1969)

ABSTRACT

The structures of glycopeptides obtained from pronase-digests of the Lorenzini jelly of two species of elasmobranch have been studied by periodate oxidation and alkaline degradation. The carbohydrate–peptide linkage region in all of the glycopeptides was 2-acetamido-2-deoxy-D-galactosylthreonine, with a majority of the 2-acetamido-2-deoxy-D-galactosyl residues substituted at O-3 and O-6 by oligosaccharides of various sizes. Some were substituted by oligosaccharides at O-6 only. Structural features of the oligosaccharide substituents have been investigated.

INTRODUCTION

In the previous paper[1], we reported the isolation and analysis of glycopeptides from pronase-digests of the hyaline jelly from the organs of Lorenzini of two species (*Raja clavata* and *Cetorhinus maximus*) of elasmobranch fish. The glycopeptides contained D-galactose, 2-amino-2-deoxyglucose, 2-amino-2-deoxy-D-galactose, fucose, acetyl groups, sulphate, and peptide. Quantitative analyses showed that, for each species, the glycopeptides could be represented by a spectrum of molecules from those of high contents of 2-amino-2-deoxy-D-galactose and peptide and low contents of 2-amino-2-deoxyglucose and sulphate, to those of low contents of 2-amino-2-deoxy-D-galactose and peptide and high contents of 2-amino-2-deoxyglucose and sulphate. Gross structural features of the glycopeptides described in the previous paper[1] have been investigated by degradation with alkali and oxidation with periodate.

MATERIALS AND METHODS

The preparation, purification, and analysis of glycopeptides from the Lorenzini jelly of *Raja clavata* and *Cetorhinus maximus* are described in the previous paper[1]. Glycopeptides R3 (from *R. clavata*) and S1, S2, and S3 (from *C. maximus*) were

*Present address: Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford, Great Britain.
†Present address: Department of Chemistry, Queen Elizabeth College, Campden Hill Road, London, W. 8, Great Britain.

used for structural studies. Analytical methods used, in addition to those described in the previous paper[1], are indicated in the descriptions of appropriate experiments.

Treatment of glycopeptides with 0.5N sodium hydroxide. — In a typical experiment, a solution of the glycopeptide (12 mg) in water (1 ml) was mixed with N sodium hydroxide (1 ml) at 25°. Half of the sample was neutralised immediately with dry Dowex-50 X8 (H^+) resin and used as a control; the remainder was saturated with nitrogen and incubated in a stoppered tube for 48 h at 25° prior to neutralisation as described above. Control and alkali-treated solutions were adjusted to 5.0 ml, and aliquots of each were analysed for absorption at 241 nm, for Morgan–Elson chromogen (by a modification of the method of Reissig *et al.*[2], in which the heating step after the addition of potassium tetraborate reagent was omitted), and for amino acids and amino sugars liberated on hydrolysis (6N hydrochloric acid, 23.3 h, 111°; and 2N hydrochloric acid, 20 h, 100°, respectively). In addition, aliquots (1 ml) of the test and control solutions were eluted with water from columns (40 × 2 cm) of Bio-Gel P-2, and the eluate was scanned by using the phenol–sulphuric acid assay[3]. Appropriate fractions were combined, concentrated by rotary evaporation, and tested for Morgan–Elson chromogen.

Treatment of glycopeptide S2 with alkali and borohydride. — Glycopeptide S2 (6 mg) in water (1 ml) was treated with 2M sodium borohydride (1 ml) for 20 min at 25°. N Sodium hydroxide (2 ml) was added, and the solution was saturated with nitrogen and incubated in a stoppered tube for 45 h at 25°. The solution was neutralised [Dowex-50 X8 (H^+) resin], and concentrated to dryness by rotary evaporation, and boric acid was removed by repeated distillation of methanol from the residue. Aliquots (0.5 ml) of a solution of the residue in water (5 ml) were analysed for amino acids and amino sugars. Further aliquots (1 ml and 0.25 ml, respectively) were tested for Morgan–Elson chromogen and for reaction with D-galactose oxidase[4], and the remainder was fractionated on Bio-Gel P-2 (40 × 2 cm) by elution with water.

Further investigation of the alkaline-degradation products from S2. — Glycopeptide S2 (3 mg) in water (3 ml) was incubated with N sodium hydroxide (3 ml) at 25°. The solution was analysed for absorbance at 241 nm, for Morgan–Elson chromogen, and for reaction with D-galactose oxidase at intervals during 65 h. In a separate experiment, glycopeptide S2 was treated with 0.5N sodium hydroxide for 34 h at 25°, and the neutralised (N hydrochloric acid) solution was eluted with water from Bio-Gel P-2 (100 × 2 cm), previously calibrated by fractionating Blue Dextran (mol. wt. 2×10^6, Pharmacia), lactose, galactose, Morgan–Elson chromogen (from 2-acetamido-2-deoxy-D-glucose), and cupric sulphate. Appropriate fractions were combined, and concentrated by rotary evaporation. The totally excluded material (fraction *A*) was subsequently eluted with water from Bio-Gel P-150 (68 × 1.5 cm).

Oxidation of glycopeptides with periodate. — In a typical experiment, glycopeptide (10 mg) was treated with 0.01M sodium metaperiodate in the dark at 4°. Consumption of periodate was measured by titration with arsenite[5]. After 10 h, the excess of periodate was destroyed with ethylene glycol, and the solution was

dialysed against 0.1M sodium chloride followed by distilled water at 4°. The non-diffusible material was concentrated, and sodium borohydride (40 mg) was added during 3 h. The pH of the solution was maintained at 7–8 by using carbon dioxide. After 18 h at 4°, the pH was adjusted to 5 with acetic acid, and the solution was dialysed. "Oxidised and reduced" glycopeptide (5 mg) was recovered by freeze-drying, and was analysed for sugars.

Acid hydrolysis of "oxidised and reduced" glycopeptide S2. — (*a*) *Total hydrolysis.* "Oxidised and reduced" glycopeptide S2 was hydrolysed with 2N hydrochloric acid for 6 h at 100°, mannitol was added as an internal standard, and the hydrolysate was analysed [Pye 104, temperature-programmed, gas–liquid chromatograph with a column packing of 10% SE30 on siliconised Celite (Pye–Unicam, Ltd.), maintained at 75° for 20 min and heated to 210° at 4°/min] for alditols and sugars after *O*-trimethylsilylation.

(*b*) *Partial hydrolysis.* "Oxidised and reduced" glycopeptide S2 (2.6 mg) was hydrolysed with 0.5N hydrochloric acid (0.25 ml) for 8 h at room temperature, and an aliquot (0.2 ml) of the hydrolysate was eluted with water from Sephadex G-10 (100 × 1 cm). Fractions (0.5 ml) were scanned by using the cysteine–sulphuric acid reagents[6], and for acetaldehyde[7] and formaldehyde[8] liberated on oxidation with periodic acid.

RESULTS

Alkaline degradation of glycopeptides. — Treatment of each of the glycopeptides with 0.5N sodium hydroxide for 48 h at 25° resulted in increased absorption at 241 nm, attributable to the formation of unsaturated amino acid(s), and the formation of the Morgan–Elson chromogen. Threonine and 2-acetamido-2-deoxy-D-galactose

TABLE I

CHEMICAL ANALYSES OF GLYCOPEPTIDES S1, S2, S3, AND R3 BEFORE (B) AND AFTER (A) TREATMENT WITH 0.5N SODIUM HYDROXIDE FOR 48 H

Component (μmole/mg)	*Glycopeptide*							
	S1		*S2*		*S3*		*R3*	
	B	A	B	A	B	A	B	A
Threonine	1.05	0.32	0.89	0.26	0.14	0.03	0.26	0.09
Serine	0.04	0.06	0.04	0.04	0.06	0.03	0.05	0.05
Glutamic Acid	0.03	0.05	0.05	0.04	0.06	0.06	0.16	0.16
Proline	0.29	0.30	0.33	0.26	0.13	0.12	0.07	0.07
Glycine	0.03	0.05	0.05	0.02	0	0	0.05	0.05
Alanine	0.06	0.06	0.08	0.06	0.13	0.08	0.08	0.07
2-Amino-2-deoxy-D-galactose	1.09	0.38	0.64	0.24	0.21	0.08	0.20	0.10
2-Amino-2-deoxyglucose	0.31	[a]	0.60	0.59	1.43	[a]	1.38	[a]
Isoleucine	0.30	0.30	0.21	0.18	0.02	0.01	0.06	0.05
Lysine	0.33	0.13	0.29	0.09	0.03	0	0.05	0.01

[a]Not determined.

residues were extensively destroyed by treatment of the glycopeptides with alkali, whereas the other amino acid components, except lysine, were unchanged (Table I). Destruction of lysine was consistent with the formation of two basic, ninhydrin-positive components that were eluted immediately prior to lysine on the autoanalyser. These components were not present in acid hydrolysates of the native glycopeptides.

Fractionation on Bio-Gel P-2 of glycopeptides before and after treatment with 0.5N sodium hydroxide at 25° showed that extensive degradation had occurred (Figs. 1 and 2). Some of the products of smaller molecular weight contained Morgan–Elson chromogen. The amount of chromogen formed was greatest for S1 and S2.

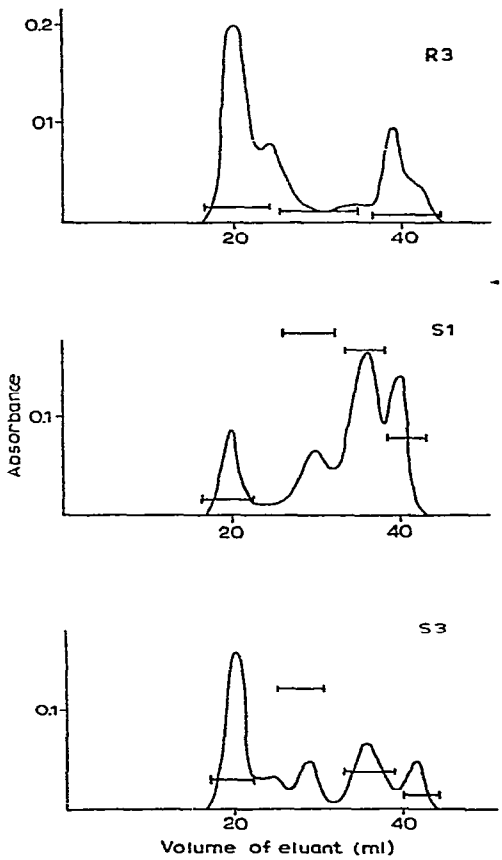


Fig. 1. Elution of alkali-treated Lorenzini glycopeptides R3, S1, and S3 from Bio-Gel P-2. Fractions assayed by using phenol–sulphuric acid reagents (————). Combined fractions assayed for Morgan–Elson chromogen (|————|).

After treatment with alkali and borohydride, glycopeptide S2 liberated, on acid hydrolysis, a component that was tentatively identified, from its elution point on autoanalysis, as 2-aminobutyric acid. This component represented *ca.* 14% of the theoretical value calculated from the amount of threonine destroyed. A second, new component was also present in the hydrolysate.

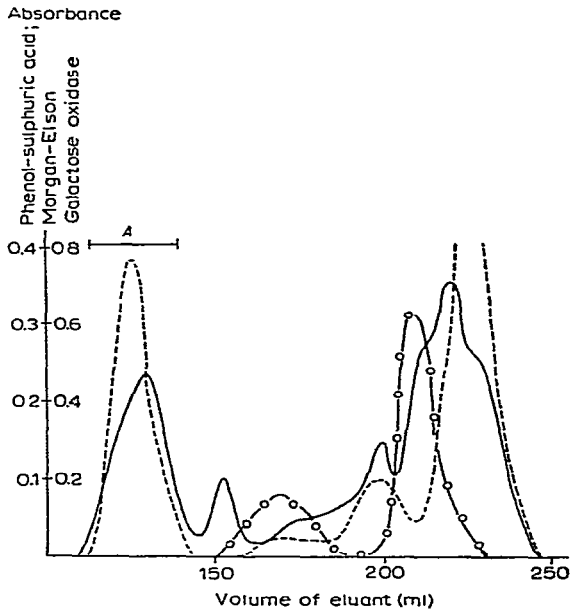


Fig. 2. Elution of alkali-treated Lorenzini glycopeptide S2 from Bio-Gel P-2. Fractions assayed by using phenol–sulphuric acid (————), D-galactose oxidase (- - - -), and Morgan–Elson reagents (—O—O—).

Fractionation on Bio-Gel P-2 of S2 that had been treated with alkali and borohydride showed that a large proportion of material of small molecular weight was produced, with a considerable diminution in the effect of D-galactose oxidase on the products.

The effect of D-galactose oxidase on the products of alkaline degradation of glycopeptide S2 remained constant for approximately 30 h, during which time the absorbance at 241 nm and formation of Morgan–Elson chromogen increased to maximal values and remained constant (Fig. 3). After 30 h, the reaction with D-galactose oxidase decreased rapidly. The rate of formation of chromogen was directly proportional to the rate of destruction of threonine, as measured by increased absorbance at 241 nm.

The reaction mixture after 34 h contained material excluded from Bio-Gel P-2, as well as products of smaller molecular weight (Fig. 2). Morgan–Elson chromogen was present exclusively in the "included" region in two fractions, which, from their elution positions, probably contained tri- or tetra-saccharides and higher oligosaccharides, respectively. The D-galactose oxidase and Morgan–Elson chromogen scans were not coincident, and the elution point of the substances of smaller molecular weight that reacted with D-galactose oxidase indicated that they contained one or two sugar units.

Some of the material in alkali-degraded S2 was totally excluded by Bio-Gel P-2 and was also totally excluded by Bio-Gel P-150 (Fig. 4). Analysis of this fraction

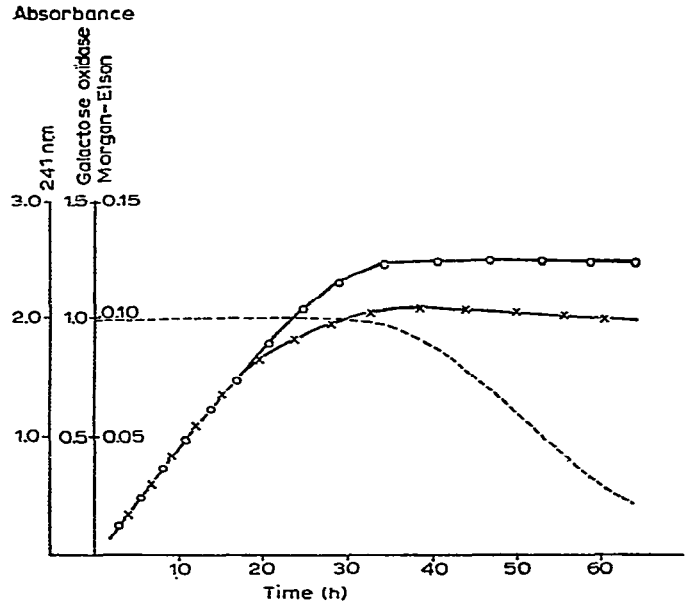


Fig. 3. Reaction of Lorenzini glycopeptide S2 with 0.5N sodium hydroxide. Aliquots of solution analysed by using D-galactose oxidase (-----) and Morgan–Elson reagents (—O—O—), and for absorbance at 241 nm (—×—×—).

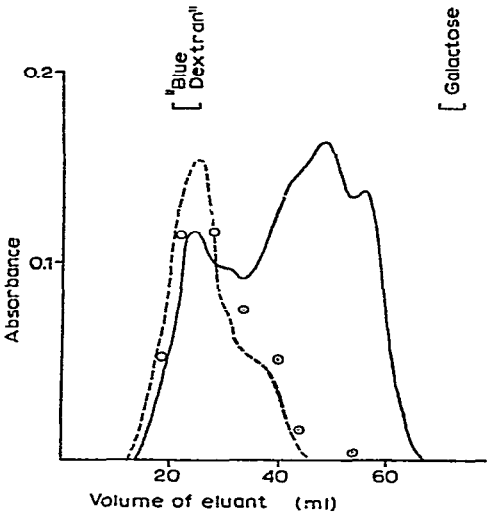


Fig. 4. Elution of Fraction *A* (see Fig. 2) from Bio-Gel P-150. Fractions assayed by using phenol–sulphuric acid (————) and D-galactose-oxidase reagents (-----) and for 2-amino-2-deoxy-hexose (⊙⊙).

indicated a ratio of galactose to total amino sugar similar to that in untreated S2. The retarded component(s) on Bio-Gel P-150 contained only small proportions of amino sugar and reacted with phenol–sulphuric acid, but not with D-galactose oxidase.

Periodate oxidation of glycopeptides S1, S2, and S3. — All of the monosaccharide constituents of the glycopeptides were susceptible, to some extent, to oxidation (Table II). The consumption of periodate, maximal after approximately 10 h, and destruction of sugars indicated that a large number of sugar residues consumed more than one mole of periodate per mole. Reduction of periodate-oxidised S2 with borohydride at pH 7–8 gave a product ("oxidised and reduced" S2) which, unlike the un-oxidised glycopeptide, did not react with D-galactose oxidase. All three glycopeptides contained equimolar amounts of sulphate, and 2-amino-2-deoxyglucose that was not oxidised by periodate.

TABLE II

CHEMICAL ANALYSES OF GLYCOPEPTIDES S1, S2, AND S3 BEFORE (B) AND AFTER (A) OXIDATION WITH PERIODATE

Component (μ mole/mg)	*Glycopeptide*					
	S1		*S2*		*S3*	
	B	A	B	A	B	A
Fucose	0.12	0.02	0.20	0.06	0.25	0.22
Galactose	1.85	0.33	1.77	0.65	1.99	1.16
2-Amino-2-deoxy-D-galactose	1.09	0.77	0.71	0.44	0.27	[a]
2-Amino-2-deoxyglucose	0.31	0.18	0.68	0.30	1.43	[a]
Sulphate	0.18		0.31		1.31	

[a]Not determined.

Hydrolysis of "oxidised and reduced" S2 with 2N acid for 6 h at 100° liberated glycerol, propane-1,2-diol, and very small proportions of threitol. Hydrolysis with 0.5N acid for 8 h at room temperature liberated propane-1,2-diol (equivalent to *ca.* 65% of the fucose content of S2 that had been oxidised by periodate) and glycerol, but no threitol.

DISCUSSION

Preliminary structural studies of the glycopeptides isolated[1] by pronase-digestion of the Lorenzini jellies of *Raja clavata* and *Cetorhinus maximus* indicated the presence of terminal, non-reducing residues of D-galactose or 2-acetamido-2-deoxy-D-galactose, and of alkali-labile linkages between carbohydrate and peptide.

The present work indicates that all of the glycopeptides contain 2-acetamido-2-deoxy-D-galactose residues linked glycosidically to the β-hydroxy group of threonine. Significant contributions from structures such as 2-acetamido-2-deoxy-D-galactosyl-D-galactosyl-threonine may be discounted since, on treatment of, for example, the glycopeptide S2 with alkali, the destruction of 2-acetamido-2-deoxy-D-galactose residues, as measured by the appearance of the Morgan–Elson chromogen, occurs *immediately* (Fig. 3).

In general, alkaline degradation of the glycopeptides to molecules of smaller molecular weight occurs with the production of α-aminocrotonic acid from threonine, and of the Morgan–Elson chromogen from 2-acetamido-2-deoxy-D-galactose. Gel-filtration analysis of the products of alkaline degradation shows that the chromogen is attached to an oligosaccharide, which, in the intact glycopeptide, must be linked to a 2-acetamido-2-deoxy-D-galactose residue at O-6. Substitution of the latter residue at O-4 would preclude chromogen formation, and a substituent at O-3 would be eliminated during alkaline degradation. Indeed, chromogen formation is negligible, under the conditions used[9], unless O-3 of the 2-acetamido-2-deoxy-D-galactose residue is substituted. It is concluded, therefore, that *ca.* 50% of these sugar residues in R3 and 65, 63, and 62% in S1, S2, and S3, respectively, are 1,3,6-linked. Substitution at O-6 of 2-acetamido-2-deoxy-D-galactose residues precludes their reaction with D-galactose oxidase, and the reaction of the Lorenzini glycopeptides with D-galactose oxidase is therefore most probably due to the presence of terminal, non-reducing D-galactose residues. The absence of free Morgan–Elson chromogen in the alkaline digests of any of the glycopeptides suggests that none of the 2-acetamido-2-deoxy-D-galactose residues involved in the alkali-labile linkage to threonine is substituted at O-3 only.

Some of the threonine and 2-acetamido-2-deoxy-D-galactose residues, that were not destroyed on treatment of the glycopeptides with alkali, may be internal residues in the peptide and oligosaccharide chains, respectively. Alkaline degradation studies of synthetic serine glycosides have shown[10] that, for β-elimination to occur, both the amino and the carboxylic acid groups of the serine residue must be substituted. Since the Lorenzini glycopeptides were obtained by proteolytic digestion of the whole jelly, they might be expected to contain some amino acid residues, which, if linked glycosidically to a sugar residue, would be alkali-stable.

Equimolar amounts of threonine and 2-acetamido-2-deoxy-D-galactose were destroyed on alkaline treatment of all of the glycopeptides, except S2, in which more threonine than 2-acetamido-2-deoxy-D-galactose, on a molar basis, was destroyed. This could be explained in terms of a second type of monosaccharide linked glycosidically to threonine, or, alternatively, to the presence in S2 of some 2-acetamido-2-deoxy-D-galactose residues linked glycosidically to threonine but not substituted at O-3.

The destruction of lysine residues, and the concomitant formation of two ninhydrin-positive components of similar basicity to lysine, on treatment of the glycopeptides with alkali is, as far as we are aware, the first report of such a reaction. The β-elimination of an *O*- or *S*-substituent occurs when *O*-substituted seryl or threonyl or *S*-substituted cysteinyl derivatives in peptides or in substituted proteins are treated with alkali at room temperature. Thus, treatment of ribonuclease with alkali resulted in the formation of dehydroalanine residues which subsequently reacted with the ε-amino group of an adjacent lysine residue[11]. Hydrolysis of the alkali-treated protein liberated *N*-(DL-2-amino-2-carboxyethyl)-L-lysine which was eluted immediately prior to lysine on the autoanalyser. The production of two new

amino acids, at the expense of lysine, on treatment of the Lorenzini glycopeptides with alkali may be explained similarly in terms of a reaction involving the ε-amino group of a lysine residue and α-aminocrotonic acid with the formation of two diastereoisomeric lysine derivatives [*N*-(DL-1-methyl-2-amino-2-carboxyethyl)-L-lysine] which would be liberated on hydrolysis of the alkali-treated glycopeptide with acid.

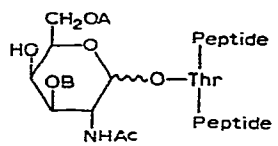
Treatment of S2, the major glycopeptide component isolated from *Cetorhinus maximus*, with alkali and borohydride, followed by acid hydrolysis, liberated, among other products, 2-aminobutyric acid and a component tentatively identified, from its position of elution from the autoanalyser, as the reduced form of the Morgan–Elson chromogen. This component has been reported in acid hydrolysates of keratan sulphate and *N*-acetylchondrosine[9], after treatment with alkali and borohydride. The absence of Morgan–Elson chromogen in S2 after treatment with alkaline borohydride supports the view that such residues had been reduced. 2-Acetamido-2-deoxy-D-galactitol, which might be produced by the reduction with borohydride of liberated 2-acetamido-2-deoxy-D-galactose residues, was not resolved from 2-amino-2-deoxy-glucose by using the autoanalyser. Low yields of 2-aminobutyric acid have been reported in similar studies of this type of carbohydrate–peptide linkage in other glycoproteins[12]. Improved yields have been obtained by using a more efficient hydrogenation process[13].

The loss in ability to react with D-galactose oxidase, which occurs on treatment of S2 with alkali, or alkali and borohydride, most probably indicates the destruction of the terminal, non-reducing D-galactose residues. The O-3 substituent of 2-acetamido-2-deoxy-D-galactose residues that formed part of the alkali-labile, carbohydrate–peptide linkage-region might comprise single D-galactose residues, and/or oligosaccharides having terminal, non reducing residue(s) of D-galactose. On treatment with alkali, such O-3 substituents would be liberated and subsequently further degraded by alkali or reduced by borohydride. Modification by alkali of terminal, non-reducing residues of D-galactose in oligosaccharide chains substituted at O-3 of a 2-acetamido-2-deoxy-galactose residue in the carbohydrate–peptide linkage implies "peeling" of the oligosaccharide chains subsequent to their elimination from O-3. The complex mixture of products obtained from S2, after reaction with alkali for 34 h, contained components of high molecular weight, which probably included some unreacted S2. Another product of high molecular weight contained neither Morgan–Elson chromogen nor amino sugar and did not react with D-galactose oxidase. Its reaction in the phenol–sulphuric acid assay indicated that it was mainly a galactan, presumably eliminated from O-3 of the amino sugar residue involved in the carbohydrate–peptide linkage-region. The lack of reactivity with D-galactose oxidase may be attributed to the presence of terminal, non-reducing fucose and/or galactose sulphate units. Other fractions present after reaction for 30 h were of smaller molecular weight and showed considerable variation in the ratio of hexose to amino sugar. The structural significance of some such products is difficult to interpret, since they may have been produced by alkali-mediated "peeling" reactions of larger oligosaccharides. Such a reaction is prevented, however, in those oligosaccharide

chains that are terminated by a Morgan–Elson chromogen residue. The point of elution of this type of oligosaccharide unit on Bio-Gel P-2 implies that the O-6 substituents of 1,3,6-linked 2-acetamido-2-deoxy-D-galactose residues that are glycosidically linked to threonine must be relatively small oligosaccharides.

Oxidation by periodate of 29 and 38%, respectively, of the 2-acetamido-2-deoxy-D-galactose residues in S1 and S2 indicates that such residues are not substituted at O-3 or O-4. This is consistent with the destruction by alkali of 65 and 63% of 2-acetamido-2-deoxy-D-galactose residues in S1 and S2, since these latter residues are assumed to be 1,3,6-linked. A large number of the oxidised sugar residues consumed more than one mole of periodate per sugar residue, which could account for the oxidation of terminal, non-reducing D-galactose residues and the concomitant loss of reaction with D-galactose oxidase. The absence of significant proportions of threitol in acid hydrolysates of S2 after sequential periodate oxidation and reduction of the product with borohydride indicates the absence in S2 of 1,4-linked D-galactose residues. The presence of propane-1,2-diol is diagnostic of terminal, non-reducing, fucose residues in S2.

Generalised structural features of the glycopeptides which are consistent with these results are shown below.



Glycopeptide S2, in which threonine comprises approximately 50% of the peptide moiety, must contain oligosaccharide groups substituted frequently along the peptide chain. The nature of A and B can only be discussed in general terms. For all of the glycopeptides, A is a relatively short-chain oligosaccharide (2–8 sugar units) containing D-galactose and 2-acetamido-2-deoxyglucose. In glycopeptide S2, B probably includes a galactan of relatively high molecular weight, as well as small oligosaccharide units containing two or three sugar units that include a terminal D-galactose residue. The presence of the latter type of residue in S2 has been indicated from immunochemical studies[14]. Structures in which B=H may also be present.

Certain structural features are common to all of the glycopeptides, irrespective of the species from which they were derived. Earlier work showed that the glycopeptides S1, S2, and S3 contained a spectrum of molecules from those of high contents of 2-amino-2-deoxy-D-galactose and peptide and low contents of 2-amino-2-deoxyglucose and sulphate to those of high contents of 2-amino-2-deoxyglucose and sulphate and low contents of 2-amino-2-deoxy-D-galactose and peptide. In view of this, it is important to stress that the present studies can only give generalised structural features of these complex molecules.

ACKNOWLEDGMENTS

The authors thank Mr. J. Doyle, Scottish Marine Biological Association, Millport, for samples of Lorenzini jelly and for valuable discussion. The Science Research Council is thanked for financial support.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

L-IDURONIC ACID, A MAJOR CONSTITUENT OF HEPARIN

A. S. PERLIN AND G. R. SANDERSON*
Department of Chemistry, McGill University, Montreal, Quebec (Canada)
(Received July 19th, 1969)

ABSTRACT

Heparin has been degraded by a sequence of several reactions to give as the major identifiable products, 1,6-anhydro-L-idopyranose (**2**) and 2,5-anhydro-D-mannitol (**3**), these products being attributed originally to L-idosyluronic acid and the 2-amino-2-deoxy-D-glucopyranosyl residues, respectively. Products such as methyl α-D-glucopyranoside (**4**), derivable from D-glucosyluronic acid residues, were relatively minor, the ratio of **2** to **3** to **4** being approximately 5:5:1. These findings are supported by periodate-oxidation experiments, which have led to the detection of threitol (5 parts) and erythritol (1 part). The latter results show also that the L-idosyluronic (as well as the D-glucosyluronic) acid residues are substituted at position 4, and other evidence indicates that a hexosaminyl residue frequently is the 4-substituent. By reducing carboxyl groups of heparin (as methyl esters) with borohydride in tritiated water, evidence has been obtained to indicate that little, if any, of **2** is derived through isomerization of D-glucosyluronic acid residues. By contrast, and as required by the above information, the use of borohydride-*t* affords isotopically labelled **2**. It is suggested that the presence of L-idosyluronic acid residues as major components of heparin helps to explain what heretofore has been regarded as an anomalously low positive rotation for the polymer.

INTRODUCTION AND DISCUSSION

The p.m.r. spectra of heparins from a variety of sources are sufficiently similar to indicate that all are basically alike, although they demonstrate that minor differences exist[1,2]. Moreover, these spectral data and associated chemical evidence have led to the proposal[2] that heparins contain a substantial proportion (perhaps one-third) of L-idosyluronic acid residues, in addition to the D-glucosyluronic acid and 2-amino-2-deoxy-D-glucose residues long recognized as important constituents. Relatively little chemical information is available as to the respective proportions of the two uronic acid moieties[3–10], although it has been inferred most commonly that the D-*gluco* isomer is the preponderant one. Hence, the overall composition of heparins

*Postdoctoral Fellow, 1967–69.

has remained incompletely defined. Problems such as the mode of linkage and configuration of the L-idosyluronic acid residues also have not been settled. The present paper considers these and related questions.

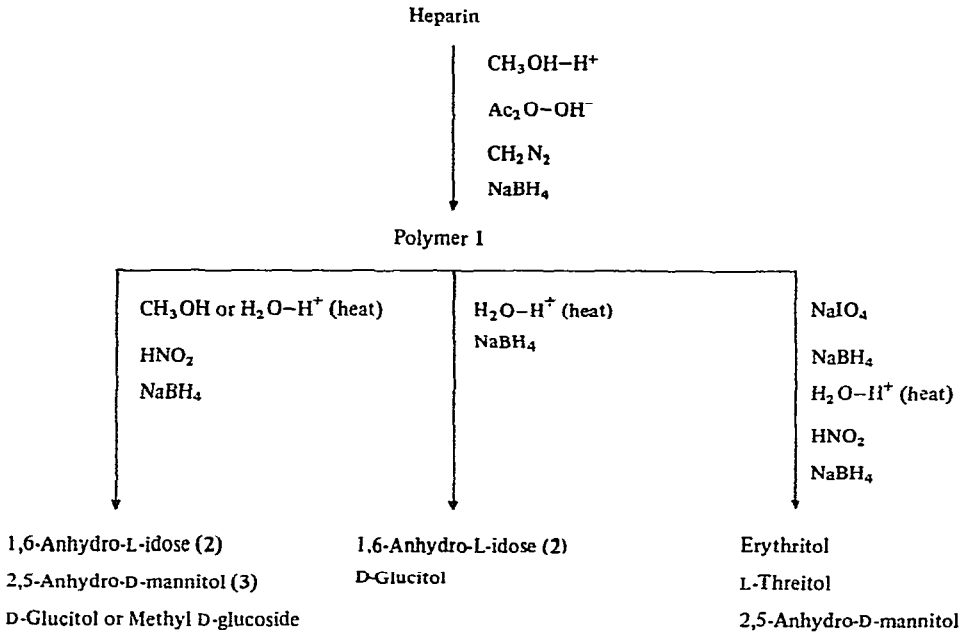

Fig. 1. Sequences of reactions used in degrading heparin to products of low molecular weight.

In re-examining heparin by chemical means, several well-known reactions have been utilized in the overall scheme outlined in Fig. I. To begin with, a succession of treatments with methanolic hydrogen chloride[11] was used to remove *N*-sulfate groups and a major proportion of the *O*-sulfate groups, the liberated amino groups being *N*-acetylated as an intermediate step in this desulfation sequence[4]. The methyl ester of the polysaccharide was then reduced in two treatments with sodium borohydride (*cf.* ref. 5*a*) affording a product (polymer **1**, $[\alpha]_D+76°$) that contained only a trace of uronic acid and approximately one sulfate group per 4 hexose residues. Product **1**, therefore, corresponded closely to a material that has already been prepared from heparin in a similar manner by Wolfrom *et al.*[4].

The modified heparin (**1**) was heated under reflux with 5% methanolic hydrogen chloride (Fig. I), which effected partial degradation and liberated most of the amino groups. Deaminative degradation with nitrous acid[3] was then carried out, and the product of this reaction was reduced with sodium borohydride. After final work-up, the ion-free, syrupy material (1.1 g from 2.5 g of **1**) was found by paper chromatography to contain at least five monosaccharide components, and a minor proportion of higher molecular-weight material. This mixture was fractionated partially by column chromatography on cellulose, and further separation was effected by selective tritylation as described in the Experimental section. The major products

obtained were 1,6-anhydro-L-idose (**2**), characterized as the crystalline triacetate, and crystalline 2,5-anhydro-D-mannitol (**3**). D-Glucose (**4**), in the form of the mixed methyl glycosides and D-glucitol, was a minor product; it was characterized as crystalline methyl α-D-glucopyranoside. The formation of **2**, **3**, and **4** is attributed to the presence of residues of L-idosyluronic acid, 2-amino-2-deoxy-D-glucopyranose, and D-glucosyluronic acid, respectively, in the original heparin.

These findings were supported, and more quantitative data obtained, by smaller-scale experiments in which the final products were peracetylated and analyzed by g.l.c. Yields were estimated by introducing a known quantity of a reference compound (L-arabinose) at the outset of each degradative sequence. Four typical sets of results are presented in Table I, which lists the yields of **2**, **3**, and **4** obtained when polymer **1** was degraded in slightly different ways. In these experiments also, 1,6-anhydro-L-idose and 2,5-anhydro-D-mannitol were preponderant, whereas relatively little D-glucose was detected.

TABLE I

DEGRADATION PRODUCTS (%) FROM MODIFIED HEPARIN (**1**)

Degradation sequence[a]	*1,6-Anhydro-L-idose* (**2**)	*2,5-Anhydro-D-mannitol* (**3**)	*D-Glucitol or methyl D-glucoside*	*L-Iditol or methyl L-idoside*
H, R	6	0	1.5	—
M, D, R	18	13	1.5	1.0
M, D, R, H, R	14	12	3	1.5
H, D, R	17	13	3	1.0
H, D, R, H, R	15	9	3	2.5

[a]M, methanolysis; D, deamination; R, reduction; H, acid hydrolysis.

Overall yields from **1** were uniformly low, amounting to 35% at best. Most of the unrecovered material appears to have been lost during treatment of the reaction mixtures with ion-exchange resins—for example in the preparative experiment the yield of neutral products corresponded to only 40% of theory. Hence the low yields are attributed largely to the removal of those residues that still contain a sulfate, carboxyl, or free amino group, together with other residues that are attached to them. As noted above, an average of one residue in four of polymer **1** contains a particularly stable sulfate group, which may or may not be hydrolyzed during the ensuing treatment. It could be possible, therefore, that a preferential loss of material occurs and that the ratios listed in Table I are not representative. Inasmuch as the sulfate groups are concerned, it appears likely (see below) that they would contribute to a diminished yield of 2,5-anhydro-D-mannitol, rather than of **2** or **4**. A low yield of **3** can be ascribed also to the fact that *N*-deacetylation was incomplete, and the probability that subsequent deamination was not quantitative. However, even assuming that there is no significant loss of 1,6-anhydro-L-idose, the fact that this product accounts for about 20% of **1** shows that L-iduronic acid must be regarded as more than a minor constituent of heparin.

Consideration has been given to the possibility that at least part of the 1,6-anhydro-L-idose originates from isomerization of methyl D-glucosyluronate residues during the alkaline borohydride reduction—a matter discussed earlier as well[2]. During the current study, this possibility has been examined by using tritiated water as solvent for borohydride reduction of the partially desulfated methyl ester of heparin. Thus, if isomerization were to have involved the abstraction of H-5 and transient formation of a C-5 carbanion—which appears to be a plausible mechanism—tritium should have been incorporated. However, since the product was virtually inactive, it is concluded that isomerization occurs to a negligible extent during the reduction process.

By contrast, when tritium-labelled sodium borohydride was used to reduce the methyl uronate residues, extensive incorporation of isotope occurred. The tritiated specimen of polymer **1** was then degraded and a paper chromatogram of the products was scanned for radioactivity. As expected, the zone containing 2,5-anhydro-D-mannitol was inactive, whereas the two zones containing 1,6-anhydro-L-idose and a mixture of D-glucitol and L-iditol, respectively, were highly active. Moreover, the *ido:gluco* ratio of activity was approximately 3:1, again showing the preponderance of *ido* components in the mixture. These results also serve to eliminate the remote possibility that the 1,6-anhydro-L-idose is derived initially from a neutral sugar residue rather than, via the latter, from L-iduronic acid.

The current indication that heparin is richer in L-iduronic acid than in D-glucuronic acid, derives support from glycol-cleavage oxidation experiments. When treated with an excess of sodium periodate, polymer **1** reduced 0.5 moles of the oxidant per mole in 24 h; no formaldehyde was produced, and only a trace of formic acid was detected. This result is in general accord with periodate-oxidation data obtained by others with variously modified heparin preparations[3,12–14]. Since the *N*-acetylated 4-substituted hexosaminyl residues[5,13,14] should not react, the uptake of periodate must be due to the L-idosyl and D-glucosyl residues which, by implication therefore, cannot be extensively sulfated.

The "polyaldehyde" obtained by periodate oxidation (Fig. 1) was reduced with sodium borohydride, and this product was hydrolyzed with acid. Deamination of the hydrolyzate with nitrous acid was then effected, followed by a second reduction. This sequence afforded a mixture of products, which after peracetylation was analyzed by g.l.c.: a threitol and erythritol* were found, in a ratio of 5:1. As would be expected, **3** also was a major product, whereas only traces of **2** and **4** were detected. Since threitol is related to the *ido* configuration and erythritol to the *gluco*, these results again indicate that heparin contains far more L-idosyluronic acid than D-glucosyluronic acid residues. Furthermore, the formation of these tetritols is evidence that the 4-position of L-idosyluronic acid residues (assuming pyranosyl rings), as well of hexosaminyl and D-glucosyluronic acid[4,13,14], is involved in glycosidic bonding.

*Erythritol has been found also in earlier studies involving the degradation of periodate-oxidized heparin[14].

Based mainly on the 100-MHz p.m.r. spectra of heparin, it was suggested earlier[2] that the polymer is composed of about equal proportions of L-idosyluronic acid, 2-amino-2-deoxy-D-glucosyl, and D-glucosyluronic acid residues. The present chemical findings point to a lower content of the latter type of residue. It is pertinent, therefore, that 220-MHz spectra obtained recently[15]—which are much better resolved than those at 100 MHz—appear to be consistent with such a possibility, although a fully satisfactory correlation of all of the various data has yet to be made. In any event, it is clear that L-idosyluronic acid, as well as the hexosaminyl residues, is a major constituent of the heparin studied and, because the p.m.r. spectra of all heparins so far examined closely resemble each other, it is assumed that these findings apply to other heparins as well.

One aspect of the degradation of polymer **1** sheds some light on the question of the sequence of residues in heparin. That is, it was found that only about one-third of the 1,6-anhydro-L-idose is liberated by the preliminary acid hydrolysis or methanolysis of **1** (Fig. 1 and Table I), whereas most of it is released in the subsequent deamination step. This shows that few of the L-idosyl residues are linked together. Furthermore, it appears that these residues have relatively weak glycosidic bonds and, as selective partial hydrolysis of the polymer occurs, become end-residues of 1,6-anhydro-L-idose. Hexosaminyl residues remain glycosidically bound directly to these end-residues, which become detached only upon deaminative degradation. Hence, it is concluded that an important structural component of heparin may be represented by the biosyl unit **5** (sulfate groups are neglected for simplicity); the α-D-(1→4) glycosidic bond assignment follows from the studies of previous workers (see, for example, refs. 5 and 14).

5

The anomeric configuration of the L-idosyluronic acid residue in **5** is not assigned. However, the fact that a residue of this kind is present in heparin helps to explain what formerly was regarded as an anomalous optical rotation for the polymer[16]. Thus, whereas heparin possesses a molecular rotation ($M_D/100$) of about 150° (and polymer **1** of about 140°), compounds based solely on the α-D-*gluco* configuration exhibit much more positive molecular rotations, that is, about 300°. Since methyl α- and β-L-idopyranosides give values of −300 and +93°, respectively[17,18], an L-idosyluronic acid residue of either configuration in heparin would contribute to the relatively low positive value actually observed. However, until more complete data are available on the composition of heparin, and questions such as the conformation of L-idosyluronic acid residues are settled, the rotatory values cannot furnish reliable information about the configurations of the L-idosyluronic

acid residues. The possibility of obtaining this structural information by p.m.r. spectroscopy will be discussed elsewhere[19].

EXPERIMENTAL

Materials and methods. — Heparin (sodium salt) was obtained from Dr. E. L. Masson, Upjohn Co., Don Mills, Ontario. The p.m.r. spectrum of this material in deuterium oxide closely resembled those of Group B (trace of *N*-acetyl) heparins examined earlier[1,2]. A portion of the heparin was precipitated with cetyltrimethylammonium bromide, as described by Wolfrom *et al.*[4], but since the spectrum of the precipitate did not differ from that of the starting material, the latter was used for the following studies without further purification.

Paper chromatography was effected with 10:4:3 (v/v) ethyl acetate–pyridine–water or 4:1:5 (v/v) butyl alcohol–ethanol–water as the solvent, and with aniline oxalate[20] or ammoniacal silver nitrate[21] as the spray reagent. G.l.c. was performed with a Hewlett-Packard 5750 Research Chromatograph. Samples for analysis were peracetylated by heating with acetic anhydride and anhydrous sodium acetate; the solution was concentrated *in vacuo* to dryness, and a chloroform extract of the residue was injected directly into the chromatograph.

Uronic acids were determined by a modification[22] of the carbazole method[23].

Tritium was assayed as follows: the sample was burned in a combustion vessel, the water produced was then suspended in toluene (15 ml, containing a phosphor) and analyzed with a liquid scintillation-counter.

Partial desulfation and N-*acetylation of heparin.* — The overall procedure was closely similar to that described by Wolfrom *et al.*[4]. A suspension of sodium heparinate (25 g) in 60mM methanolic hydrogen chloride (250 ml)[11] was stirred vigorously for 18 h, the liquid was decanted, and this procedure was twice repeated with fresh portions of the acidified methanol. A fourth treatment was carried out, and this suspension was neutralized with diazomethane and concentrated to dryness (yield, 21.6 g). The powdery product was dissolved in water (300 ml) and treated at pH 10 and 0° with acetic anhydride (28 ml) to effect *N*-acetylation (as described by Roseman and Ludowieg[24]); the solution was then dialyzed for 72 h, and lyophilized, affording a white solid (19.5 g). In deuterium oxide solution, the latter produced a strong p.m.r. singlet at 2.0 p.p.m., corresponding to the presence of an *N*-acetyl group[1,2]. The *N*-acetylated material was then subjected to four additional treatments with methanolic hydrogen chloride (see ref. 4 and also as above). The final suspension was neutralized with diazomethane and dialyzed in aqueous solution, and then lyophilized; yield, 14.8 g.

Preparation of polymer **1.** — To a vigorously stirred solution of the partially desulfated, *N*-acetylated, methyl ester of heparin (10 g) in ice–water (165 ml), a solution of sodium borohydride (3.3 g) in ice–water (165 ml) was added over a period of 2 h. After a further 18 h at 5° the reaction mixture was dialyzed for 72 h, and then lyophilized; yield, 8.1 g. The uronic acid content of this product, relative

to that of the untreated ester, was 38%. Re-esterification was effected by twice shaking a suspension of the material (6.7 g) in 60mM methanolic hydrogen chloride (150 ml) for 18 h, following which the solid was recovered on a filter, washed well with ethereal diazomethane, and dried. After a second reduction with sodium borohydride (2.3 g) as above, a white powder (polymer **1**) was obtained (5.8 g), $[\alpha]_D$ +76° (*c*, 1.9 water).

Anal. Found: C, 40.0; H, 7.1; N, 3.2; S, 4.0.

Polymer **1** contained only a trace of uronic acid, as indicated by the carbazole test: by using 360 μg of sample per ml, the absorbance values at 520 nm were 0.393 and 0.029, before and after reduction, respectively. Similarly, an acidified solution of polymer **1** in deuterium oxide showed negligible carboxyl absorption (at 1725 cm^{-1}), whereas the original heparin absorbed very strongly at 1725 cm^{-1} under these conditions (although not prior to acidification). Polymer **1** also absorbed strongly at 1480 and 1628 cm^{-1} and showed a strong p.m.r. singlet at 2.0 p.p.m., consistent with the presence of an acetamido group. In addition, the p.m.r. spectrum exhibited a general upfield shift of signals relative to those of heparin, ascribable to the loss of carboxyl and sulfate groups.

Degradation of polymer **1** *and chromatographic fractionation of the products.* — A suspension of polymer **1** (2.5 g) in methanolic hydrogen chloride (5%, 125 ml) was heated for 24 h under reflux. The solid dissolved within one hour. The solvent was neutralized with ethereal diazomethane, and concentrated. Water (47 ml) was added to the syrupy residue (3.0 g), followed by sodium nitrite (1.9 g) and M hydrochloric acid[3] (15 ml). After 120 h, the reaction mixture was diluted, treated successively with Amberlite IR-120 (H^+ form) and Dowex-1 (hydrogen carbonate form) ion-exchange resins, and concentrated to a syrup (1.6 g). To a solution of this syrup in ice–water (75 ml) a solution of sodium borohydride (0.5 g) in ice–water (25 ml) was added slowly. After 18 h, the reaction mixture was worked up by treatment with Amberlite IR-120 resin to remove sodium ions, and with methanol to remove borate, and concentrated. The syrupy product (1.1 g) obtained was found by paper chromatography to contain at least five components; two of these were major and had the same mobilities as 1,6-anhydro-L-idose and 2,5-anhydro-D-mannitol, respectively.

The syrup was chromatographed on a column (4 × 40 cm) of cellulose powder, with butyl alcohol one-quarter-saturated with water as eluant at a flow rate of about 30 ml per h. The elution pattern was monitored by paper chromatography and several different fractions of product were recovered. Based on paper chromatograms and p.m.r. spectra, the main fractions appeared to consist of the following compounds (yields in parentheses, chirality not specified): Fraction A: methyl α-*plus* β-idoside (80 mg); Fraction B: 1,6-anhydroidose *plus* 2,5-anhydromannitol *plus* (trace of) unknown (420 mg); Fraction C: methyl α- *plus* β-glucoside (60 mg); Fraction D: glucitol *plus* iditol (43 mg).

By washing the column successively with ethanol and water, an additional 250 mg of material was recovered; this material travelled slowly, with streaking, on paper chromatograms, and was not examined further.

2,3,4-Tri-O-acetyl-1,6-anhydro-β-L-idopyranose. — A solution of Fraction B (150 mg) and chlorotriphenylmethane (320 mg) in pyridine (3 ml) was heated for 30 min under reflux, and the solution was poured into ice–water (150 ml), to afford a solid product (356 mg) (see next paragraph). The filtrate was extracted 4 times with chloroform, treated with mixed ion-exchange resins, and when concentrated yielded a syrup (72 mg) which, paper chromatographically, appeared to be a pure 1,6-anhydroidose. On acetylation by heating with acetic anhydride (5 ml) and anhydrous sodium acetate (120 mg) this syrup afforded 1,6-anhydro-2,3-4-tri-*O*-acetyl-β-L-idopyranose (45 mg). After recrystallization from ethanol the product had m.p. 67–68° (undepressed on admixture with an authentic specimen), $[\alpha]_D + 74°$ (*c* 1.3, chloroform). The mass spectrum was indistinguishable from that reported for the D-enantiomorph[25].

Anal. Calc. for $C_{12}H_{16}O_8$: C, 50.00; H, 5.55. Found: C, 49.9; H, 5.2.

2,5-Anhydro-D-mannitol. — A solution of the solid tritylation product (above) in 80% acetic acid (10 ml) was heated for 30 min at 95°, water (10 ml) was added, and the precipitate that formed was filtered off. On concentration, the filtrate gave a product (yield, 21 mg) which, after crystallization from ethanol, had m.p. 98–99° $[\alpha]_D + 60°$ (*c* 0.8, water). The product gave an X-ray powder diagram indistinguishable from that of authentic 2,5-anhydro-D-mannitol, and the mixed m.p. was undepressed.

Methyl α-D-glucopyranoside. — Fraction C partially crystallized from solution in ethanol when seeded, affording methyl α-D-glucopyranoside (16 mg), m.p. 171–172°, undepressed, $[\alpha]_D$ 158° (*c* 1.2, water).

Degradation of polymer **1**, *and gas–liquid chromatographic examination of the products.* — Preliminary methanolysis of polymer **1** was carried out in some experiments, as described above, and acid hydrolysis was employed in others, the duration of these treatments being varied from 4 to 24 h. In a typical experiment employing acid hydrolysis, a solution of polymer **1** (100 mg) in 0.5M sulfuric acid (10 ml) was heated for 4 h at 95°, an excess of barium carbonate was introduced, and the filtrate was concentrated to dryness. The residue was dissolved in water (2 ml) containing L-arabinose (9.0 mg), and the solution was divided into two 1-ml portions (*a* and *b*). Sodium borohydride (30 mg) was added to *a*, the reaction mixture was worked up after 18 h, and the reduction product was acetylated with hot acetic anhydride–sodium acetate. The second portion (*b*) was diluted with water (1 ml), M hydrochloric acid (0.6 ml) and sodium nitrite (75 mg) were added, and after 110 h the solution was deionized; the product was then successively reduced with sodium borohydride and acetylated (as above).

Chloroform extracts of the acetylated products from *a* and *b* were analyzed by g.l.c., by using a column of 10% BDS at 195°. By reference to the emergence times and quantitative response of authentic samples, the product from *a* was found to contain 1,6-anhydroidose and arabinitol in a ratio of 0.2–0.4:1, whereas in the product from *b* this ratio was 1.0–1.2:1.

Periodate oxidation of polymer **1**. — A solution of the polymer (200 mg) in 0.1M aqueous sodium metaperiodate (10 ml) was kept in the dark, and its periodate

content was assayed spectrometrically[26] at chosen intervals. Between 5 and 24 h the level of periodate uptake remained virtually constant at about 0.5 mole per mole. A separate solution of polymer **1** (50 mg) and sodium metaperiodate (40 mg) in deuterium oxide (0.3 ml) was examined at 5 h and 24 h by p.m.r. spectroscopy. A trace of formic acid was found to be produced, as shown by the appearance of a very weak signal at δ 8.6 p.p.m.[27] whereas no signal at δ 5.4 p.p.m., attributable to formaldehyde[27], was detected.

Oxidized polymer **1** (60 mg) was separated from inorganic ions by dialysis, and subjected to the following sequence of reactions by using procedures similar to those described above: borohydride reduction, hydrolysis with 0.5M sulfuric acid (4 h at 95°), deamination, borohydride reduction, and acetylation. On gas–liquid chromatographic examination, with a polyester LAC-728 liquid phase at 225° and the pertinent reference compounds as standards, the final reaction product was found to contain tetra-*O*-acetyl-erythritol and -threitol, present in a ratio of 1:5. By using a liquid phase of UC-W98 at 220°, the peracetates of 1,6-anhydroidose, 2,5-anhydromannitol, and glucitol *plus* iditol also were detected in the final product, their relative ratios being 1:9:1.4.

Borohydride reduction of modified heparin in tritiated water. — A sample (300 mg) of the partially desulfated, *N*-acetylated, methyl ester of heparin was treated once as described above (under "Polymer **1**") with sodium borohydride in tritiated water. The product (190 mg) contained 39% of the uronic acid content of the starting material.

A reference sample was prepared from 1,2:4,5-di-*O*-isopropylidene-β-D-*erythro*-hexopyranos-2,3-diulose[28] through exchange with a portion of the same tritiated water (admixed with pyridine), as described for preparation of the 4-deuterio compound[28].

A 25-mg portion of each product and of polymer **1** was analyzed for tritium. The following counts/min were recorded: polymer **1**, 28; tritium-treated polymer, 515; reference, 251,100. Based on a calculated value of approximately 100,000 counts/min for incorporation of tritium by each hexose residue produced, these results show that the incorporation of tracer during reduction amounted to about 0.5%.

Reduction of modified heparin with sodium borohydride-t. — A sample (300 mg) of the partially desulfated, *N*-acetylated, methyl ester of heparin was treated once as described above (under "Polymer **1**") with a mixture of sodium borohydride (100 mg) and sodium borohydride-*t* (4 mg; 10 mCi). The product (240 mg, uronic acid content 40% of that of the starting material) was degraded as above by sequential acid hydrolysis, nitrous acid deamination, and borohydride reduction. Paper-chromatographic examination of the degradation products, and gas–liquid chromatographic examination (column of UC-W98 at 220°) of their derived acetates, indicated the presence of a mixture of 1,6-anhydroidose, 2,5-anhydromannitol, glucitol, and iditol.

A paper chromatogram of the degradation products was cut horizontally into narrow strips, each of which was assayed for tritium. The results showed that zones

corresponding in mobility to 1,6-anhydroidose and to glucitol *plus* iditol (unresolved mixture) were radioactive, whereas that containing 2,5-anhydromannitol was inactive. The ratio of the total count for 1,6-anhydroidose relative to that for the mixture of hexitols was approximately 2:1; based on the gas–liquid chromatographic estimate (column of BDS at 195°) of the glucitol: iditol ratio, the radioactivity of 1,6-anhydroidose relative to that of glucitol was about 3:1.

ACKNOWLEDGMENTS

The authors express their gratitude to Dr. E. L. Masson and the Upjohn Company, Don Mills, Ontario for generously providing the heparin used in this study, to Dr. N. K. Richtmyer for a sample of 2,3,4-tri-*O*-acetyl-1,6-anhydro-β-D-idopyranose, and to Norman Ness for measurement of p.m.r. spectra.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

N.M.R. SPECTRA OF ACETYLATED 1-*O*-ACYL-D-GLUCOPYRANOSES AND ACETYLATED METHYL 1-*O*-ACYL-D-GLUCOPYRANURONATES. SHIELDING EFFECT OF THE AROMATIC SUBSTITUENT ON THE 2-ACETOXYL GROUP*

N. PRAVDIĆ AND D. KEGLEVIĆ

Tracer Laboratory, Institute "Ruđer Bošković", Zagreb (Yugoslavia)

(Received June 4th, 1969; in revised form, July 21st, 1969)

ABSTRACT

A study of the n.m.r. spectra of acetylated 1-*O*-acyl-D-glucopyranoses and acetylated methyl 1-*O*-acyl-D-glucopyranuronates has shown that, in derivatives containing an aryl- or indolyl-acetyl grouping at C-1, one of the acetyl-group signals appears at unusually high field (τ 8.18–8.43). It was found that the highest shift is associated with the β-D-linked 1-*O*-(indol-3-ylacetyl) substituent. The synthesis of 3,4,6-tri-*O*-acetyl-2-*O*-trideuteroacetyl-1-*O*-(indol-3-ylacetyl)-D-glucopyranose (**6**) allowed the assignment of the upfield-shifted signal to the 2-acetoxyl group. A rationalization of this specific shielding based on preferential, steric orientation is presented.

INTRODUCTION

In the course of a synthesis of 1-*O*-(indol-3-ylacetyl)-β-D-glucopyranose[1], a considerable upfield shift of the signal for one of the four acetoxyl groups was observed in the n.m.r. spectrum of the acetylated derivative. The signals of the acetoxyl-methyl protons in the n.m.r. spectrum (chloroform-*d*) of 2,3,4,6-tetra-*O*-acetyl-1-*O*-(indol-3-ylacetyl)-β-D-glucopyranose (**1**) appeared at τ 7.93, 7.99, 8.03, and 8.42. Three of the signals are in the range (7.88–8.03) characteristic for equatorial acetoxyl groups[2,3], but the signal at 8.42 is at unusually high field. Although it has been noted[3–7] that the acetoxyl group is shielded when an aryl substituent is present in the molecule, such a large upfield shift has not previously been observed. It is of interest that Heyns *et al.* [8,9] have noted that the three-proton singlets of the ortho-acetyl group occur at τ 8.33 and 8.50. We now report on the assignment of the high-field acetoxyl signal for compound **1** and related acetates.

RESULTS AND DISCUSSION

The empirical rule, that the acetoxyl signal at highest field for hexose derivatives is that of the 6-acetoxyl group, has often been used for tentative assignments[10–14].

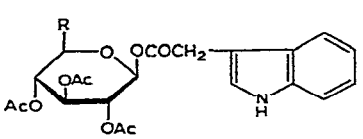
*Supported, in part, by a P. L. 480 grant from the National Institutes of Health, Bethesda, Md., U. S. A.; Agreement No. 719810.

However, recent investigations[15,16] with selectively deuterated compounds have shown that this assumption may not be always valid. In our case, this rule can easily be checked, since the corresponding 1-*O*-acyl derivatives from the glucuronic acid series are available.

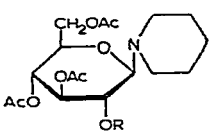
Methyl 2,3,4-tri-*O*-acetyl-1-*O*-(indol-3-ylacetyl)-β-D-glucopyranuronate[17] (**2**) showed three well-resolved acetoxyl signals, one of them at τ 8.42. It therefore appeared that the upfield signal could be assigned to the 2-acetoxyl group, which is close to the bulky indol-3-ylacetyl substituent. To confirm this assumption, the specifically deuterated analogue of **1** was prepared by using *N*-(3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)piperidine[18] (**3**) as the starting compound. Acetylation of **3** with acetic anhydride-d_6 and pyridine afforded *N*-(3,4,6-tri-*O*-acetyl-2-*O*-trideuteroacetyl-β-D-glucopyranosyl)piperidine (**4**), which was hydrolysed to give 3,4,6-tri-*O*-acetyl-2-*O*-trideuteroacetyl-D-glucopyranose (**5**). Condensation of **5** with indol-3-ylacetic acid, in the presence of dicyclohexylcarbodiimide, yielded 3,4,6-tri-*O*-acetyl-2-*O*-trideuteroacetyl-1-*O*-(indol-3-ylacetyl)-D-glucopyranose (**6**). Extensive studies by Horton and Lauterbach[19] showed that no detectable migration of acetoxyl groups takes place under the conditions of the acetylation with deuterated reagents and pyridine.

The n.m.r. spectrum of compound **6** revealed it to be a mixture of anomers, as shown by the presence of an additional, narrow doublet (equatorial H-1) at τ 3.68, $J_{1,2}$ 3.2 Hz. In all other details, the spectrum was identical with that of **1**, except that the acetoxyl signal at highest field was absent. Thus, the signal at τ 8.42 is unequivocally identified as that of the 2-acetoxyl group.

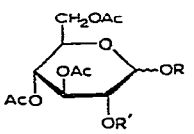
It was then of interest to examine the n.m.r. spectrum of the α-D anomer of **1**. The condensation of tetra-*O*-acetyl-β-D-glucopyranosyl chloride with the silver salt of indol-3-ylacetic acid gave, as the main product, the anomeric mixture of **1** in which



1 R = CH_2OAc
2 R = CO_2Me



3 R = H
4 R = $COCD_3$



5 R = H, R' = $COCD_3$
6 R = (Indol-3-ylacetyl), R' = $COCD_3$
7 R = (Indol-3-ylpropionyl)-β-, R' = Ac
8 R = (Indol-3-ylbutyryl)-β-, R' = Ac

the α-D anomer strongly prevailed. However, the latter compound decomposed on columns of silica gel, and attempts to obtain it in the pure state failed. The n.m.r. spectra of the anomeric mixtures of **1** revealed two high-field acetoxyl signals (at τ 8.23 and 8.42), corresponding together to three protons. The ratio of anomers in a particular sample could be determined from the relative intensities of the two peaks.

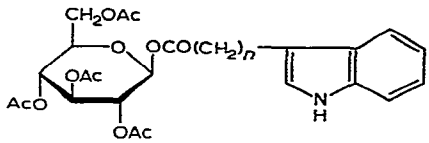
Since the large, upfield shift of the 2-acetoxyl singlet was caused by the neighboring aromatic ring, information about the influence of the relative distances between these groups was sought by examining homologues of **1**. The n.m.r. spectra

of a number of acetylated methyl 1-*O*-acyl-D-glucopyranuronates, synthesized in the course of previous work[17], have also been examined.

2,3,4,6-Tetra-*O*-acetyl-1-*O*-(indol-3-ylpropionyl)-*β*-D-glucopyranose (7) and the *β*-D anomer **8** of the butyryl homologue were prepared by the silver salt method. The n.m.r. spectrum of the propionyl derivative **7** contained the 2-acetoxyl signal at τ 8.18, which, although at distinctly lower field than for **1**, was still outside the region characteristic for the equatorial acetoxyl groups. In the spectrum of the butyryl derivative **8**, all four acetoxyl singlets were within the normal region (Table I).

TABLE I

CHEMICAL SHIFTS (τ VALUES) OF ANOMERIC AND ACETOXYL-METHYL PROTONS IN 2,3,4,6-TETRA-*O*-ACETYL-1-*O*-(INDOL-3-YLACYL)-*β*-D-GLUCOPYRANOSES



Compound	n	*Anomeric proton* ax	eq	*Coupling constant* $J_{1,2}$ (*Hz*)	*Acetoxyl-methyl protons*
1	1	4.28		7.8	7.93, 7.99, 8.03, *8.42*
1[a]	1	4.28	3.68	7.8 3.2	7.93, 7.99, 8.03, *8.23* *8.42*
7	2	4.23		7.2	7.96, 8.00, 8.02, *8.18*
8	3	4.22		7.0	7.93, 7.98, 7.99, 8.02

[a]The mixture of anomers.

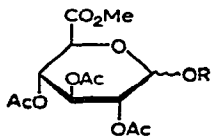
The chemical shifts of the acetoxyl-methyl protons of the acetylated methyl 1-*O*-acyl-D-glucopyranuronates are given in Table II. The anomeric configuration of the glucopyranuronates, which was previously predicted on the basis of optical rotatory data[17,20], has been confirmed by the position and the magnitude of splitting of the anomeric protons[11,21]. It should be emphasized that for the derivatives **2**, **9**, and **15–17** containing the indol-3-ylacetyl or the arylacetyl substituent at C-1 one of the acetoxyl signals appears at high field. Compound **16** shows two high-field signals in the acetoxyl region, together corresponding to three protons. The signal at τ 8.23 was found also in the *β*-D derivative **15**, and it is assigned to the 2-acetoxyl group, whereas the lower-field signal at τ 8.18 is ascribed to the 2-acetoxyl group of the *α*-D anomer. From Table II, it can be seen that in the 1-*O*-aroyl-D-glucopyranuronates (**11–14**), as well as in compound **10**, where the carboxyl group is directly linked to the aromatic ring, no significant shift of the acetoxyl group has been detected.

Hence, the common feature of all acetylated glucose and glucopyranuronate derivatives showing a high-field 2-acetoxyl signal is the presence of an aromatic-ring substituent in the 1-*O*-acetyl grouping. In passing, it should be mentioned that a representative of glucopyranuronates containing a benzyloxy group at C-1, methyl

2,3,4-tri-*O*-acetyl-1-*O*-benzyl-β-D-glucopyranuronate[22,17], does not show any pronounced shift in the region of the acetoxyl signals (found at τ 8.01).

TABLE II

CHEMICAL SHIFTS (τ VALUES) OF ANOMERIC AND ACETOXYL-METHYL PROTONS IN METHYL 2,3,4-TRI-*O*-ACETYL-1-*O*-ACYL-D-GLUCOPYRANURONATES[17,20]



Compound	*R*	*Anomeric proton* ax	eq	*Coupling constant* $J_{1,2}$ (*Hz*)		*Acetoxyl-methyl protons*
2	Indol-3-ylacetyl-β-	4.21		7.8		7.98, 8.03, *8.42*
9	5-Benzyloxyindol-3-ylacetyl-β-	4.24		7.8		7.99, 8.04, *8.43*
10	5-Benzyloxyindol-2-ylcarbonyl-β-	3.93		8.0		7.92, 7.93, 8.08
11	Benzoyl-β-	3.97		7.4		7.95 (×2)[a], 8.02
12	*p*-Methoxybenzoyl-β-	4.01		7.6		7.95 (×2)[a], 8.02
13	Tri-*O*-acetylgalloyl-β-	4.0		7.6		7.97 (×2)[a], 8.01
14	*m*-Nitrobenzoyl-	3.92	3.31	7.6	4.0	7.92 (×2)[a], 8.00
15	Phenylacetyl-β-[20]	4.21		8.0		8.00, 8.02, *8.23*
16	Phenylacetyl-	4.21	3.61	8.0	3.6	7.98, 8.00, *8.18* *8.23*
17	Diphenylacetyl-β-	4.16		8.0		8.02, 8.06, *8.38*
18	Cinnamoyl-β-	4.05		8.0		7.96 (×2)[a], 8.00
19	Cinnamoyl-α-		3.44		4.0	7.95 (×2)[a], 8.00
20	Propionyl-	4.12	3.58	7.8	3.5	7.97 (×2)[a], 8.01

[a]Unresolved.

The effect of an aromatic nucleus in causing upfield shifts[23] in the n.m.r. spectra has been attributed[24] to the magnetic field of the aromatic ring. The statement that the signal due to a proton placed above or below the plane of the aromatic ring is shifted diamagnetically suggested an inspection of Dreiding stereomodels of the pertinent 1-*O*-acyl-D-glucoses and -glucopyranuronates. From these, it is evident

that the presence of the grouping –O–CO–CH_2–Ar at C-1 places the aromatic ring in a position which allows an easy and simultaneous approach of all of the protons of the 2-acetoxyl group to the plane of the ring. However, for a –O–CO–Ar group, it becomes more difficult to have all of the acetoxyl protons in the same position above the ring. On the other hand, by increasing the distance between the carbonyl group and the aromatic substituent at C-1, the aromatic ring is much less restricted in movement, and the possibility that the methyl protons of the 2-acetoxyl group could come directly over the aromatic ring is diminished.

EXPERIMENTAL

Melting points are uncorrected. Specific rotations were measured in chloroform at 20–23° if not stated otherwise. Thin-layer chromatography was conducted on silica gel G (Merck) with the solvent system specified; components were detected with 10% sulfuric acid and heating at 100°. Column chromatography was conducted on silica gel (0.2–0.5 mm, Merck). The i.r. spectra were recorded on a Perkin–Elmer Model 137 instrument. The n.m.r. spectra were obtained for solutions in chloroform-*d* with a Varian A-60*A* spectrometer, and tetramethylsilane was used as an internal standard.

N-(3,4,6-Tri-O-acetyl-2-O-trideuteroacetyl-β-D-glucopyranosyl)piperidine (**4**). — *N*-(3,4,6-Tri-*O*-acetyl-β-D-glucopyranosyl)piperidine[18] (**3**) (1.12 g) was treated with acetic anhydride-d_6 (0.3 ml) and pyridine (8 ml) at room temperature overnight. The solvents were coevaporated with ethanol, and the residue was dried over P_2O_5, triturated with cold ethanol (3 ml), filtered off, and crystallized from absolute ethanol to give the product (750 mg, 60%), which, after recrystallization from absolute ethanol, had m.p. 121–122° and $[\alpha]_D$ −3.2° (*c* 2.5); lit.[18] for *N*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)piperidine, m.p. 123° and $[\alpha]_D$ −3.5° (chloroform). N.m.r. data: τ 7.93, 7.97, and 8.01 (OAc, 9 H).

Anal. Calc. for $C_{19}H_{26}D_3NO_9$: C, 54.53; (H+D), 7.70; N, 3.35. Found: C, 54.67; (H+D), 8.04; N, 3.10.

3,4,6-Tri-O-acetyl-2-O-trideuteroacetyl-1-O-(indol-3-ylacetyl)-D-glucopyranose (**6**). — A mixture of ethanol (2 ml) and 0.5% aqueous formic acid (7 ml) was saturated with carbon dioxide. Compound **4** (163 mg) was added, and the suspension was refluxed for 30 min. Monitoring of the reaction mixture by t.l.c. (ether), with an authentic sample of tetra-*O*-acetyl-D-glucose, showed that the reaction was then complete. From the cooled solution, ethanol was removed by evaporation, and the aqueous solution was extracted with chloroform. The extract was washed successively with 0.2N hydrochloric acid, 5% aqueous sodium hydrogen carbonate, and water, and dried with sodium sulfate. The solvent was evaporated *in vacuo*, leaving acetate **5** as a thick syrup (125 mg, 91%), $[\alpha]_D$ +72.4° (*c* 1.22). The infrared spectrum and chromatographic behavior were indistinguishable from those of 2,3,4,6-tetra-*O*-acetyl-D-glucose.

To a solution of **5** (125 mg) in dichloromethane (1 ml) containing dry pyridine

(0.04 ml) and dicyclohexylcarbodiimide (83 mg), a hot solution of indol-3-ylacetic acid (63 mg) in dichloromethane (20 ml) was added. The mixture was kept, with occasional shaking, at room temperature overnight. Precipitated *N,N'*-dicyclohexylurea (52%) was removed, and the filtrate was concentrated *in vacuo* to a semicrystalline solid which, upon treatment with ethyl acetate–benzene (2 ml), gave *N*-(indol-3-ylacetyl)-*N,N'*-dicyclohexylurea[17] (28 mg). The filtrate was chromatographed on a column of silica gel (15 g) by using ethyl acetate–benzene (4:3). The product (96 mg, 52%) was obtained in the form of a foam, and its chromatographic behavior and i.r. spectrum were indistinguishable from those[1] of compound **1**. N.m.r. data: τ 3.68 ($J_{1,2}$ 3.2 Hz, H-1, α-D anomer); 4.28 ($J_{1,2}$ 7.8 Hz, H-1, β-D anomer); 7.93, 7.99, and 8.03 (OAc, 9 H).

2,3,4,6-Tetra-O-*acetyl-1*-O-(*indol-3-ylpropionyl*)-β-D-*glucopyranose* (**7**). — A suspension of tetra-*O*-acetyl-α-D-glucopyranosyl bromide (1.85 g), silver indol-3-ylpropionate (1.33 g), and Drierite (1 g, dried at 100° and powdered before use) in absolute benzene (40 ml) was shaken in the dark for 48 h at room temperature; the reaction was monitored by t.l.c. in ethyl acetate–benzene (1:1). The insoluble salts were centrifuged off and washed with benzene, and the combined supernatants were concentrated *in vacuo*, leaving a viscous, yellow oil that was chromatographed on a column of silica gel (32 g) with ether–light petroleum (4:1). The fractions containing the product contaminated with indol-3-ylpropionic acid were combined and rechromatographed on a column of silica gel (30 g) with ethyl acetate–benzene (1:1). Chromatographically homogeneous compound **7** was obtained as a thick syrup (922 mg, 40%) which crystallized on standing. Recrystallization from 96% ethanol gave material (586 mg) having m.p. 139–140°, $[\alpha]_D$ −16.5° (*c* 1.0).

Anal. Calc. for $C_{25}H_{29}NO_{11}$: C, 57.80; H, 5.63; N, 2.69. Found: C, 58.06; H, 5.80; N, 2.74.

2,3,4,6-Tetra-O-*acetyl-1*-O-(*indol-3-ylbutyryl*)-β-D-*glucopyranose* (**8**). — A suspension of tetra-*O*-acetyl-α-D-glucopyranosyl bromide (1.85 g), silver indol-3-ylbutyrate (1.40 g), and Drierite (1 g) in absolute benzene (40 ml) was stirred, with exclusion of moisture, for 6 h at 60°. The reaction mixture was worked up, and the residual, brown oil was chromatographed on two columns of silica gel, as described for **7**. Chromatographically homogeneous compound **8** was obtained as a viscous oil (529 mg, 22%) which crystallized from diisopropyl ether; m.p. 97–98°, $[\alpha]_D$ *ca.* 0° (*c* 2.0), $[\alpha]_D$ −3° (*c* 1.28, benzene).

Anal. Calc. for $C_{26}H_{31}NO_{11}$: C, 58.53; H, 5.86; N, 2.62. Found: C, 58.77; H, 5.94; N, 2.86.

Attempted synthesis of 2,3,4,6-tetra-O-*acetyl-1*-O-(*indol-3-ylacetyl*)-α-D-*glucopyranose*. — A suspension of tetra-*O*-acetyl-β-D-glucopyranosyl chloride (2.20 g, $[\alpha]_D$ −5°), silver indol-3-ylacetate (1.69 g), and Drierite (1 g) in absolute benzene (70 ml) was treated in the same way as described for **7**. During column chromatography, a progressive decomposition of the product into tetra-*O*-acetyl-D-glucose and indol-3-ylacetic acid was observed; finally, an oil (264 mg, 19%) was isolated which was indistinguishable (t.l.c.; ethyl acetate–benzene, 1:1) from tetra-*O*-acetyl-1-

O-(indol-3-ylacetyl)-β-D-glucopyranose (**1**). When a solution of the oil in absolute ether was gradually treated with light petroleum at 0°, crystals (80 mg) of the pure β-D anomer **1** were deposited. The mother liquor was evaporated *in vacuo*, leaving a pale-pink foam, $[\alpha]_D$ +52° (*c* 1.0), which turned violet-brown on standing. Inspection of the mixture by t.l.c. and n.m.r. revealed a progressive decomposition of the predominant α-D anomer.

ACKNOWLEDGMENTS

We are indebted to Dr. O. Hadžija for microanalyses and to Miss L. Berc for n.m.r. spectra. We also thank Dr. H. G. Fletcher Jr., of the National Institutes of Health, Bethesda, Md., U. S. A., for stimulating discussions and for the gift of acetic anhydride-d_6.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

SYNTHESIS OF 6-AMINO-6-DEOXY-5-THIO-D-GLUCOPYRANOSE*

ROY L. WHISTLER AND R. E. PYLER
Department of Biochemistry, Purdue University, Lafayette, Indiana 47907 (U. S. A.)
(Received July 24th, 1969)

ABSTRACT

Periodate oxidation of 1,2-*O*-isopropylidene-α-D-glucofuranose (**2**) and condensation of the resulting dialdehyde with nitromethane in anhydrous methanol gives 6-deoxy-1,2-*O*-isopropylidene-6-nitro-α-D-glucofuranose (**4**) in 63% yield. Treatment with acetic anhydride and sodium acetate produces 3-*O*-acetyl-5,6-dideoxy-1,2-*O*-isopropylidene-6-nitro-α-D-*xylo*-hex-5-enofuranose (**5**) in 83% yield. Addition of α-toluenethiol then leads to 5-*S*-benzyl-6-deoxy-1,2-*O*-isopropylidene-6-nitro-5-thio-α-D-glucofuranose (**6**) in 80% yield. Compound **6** is reduced with lithium aluminum hydride to 6-amino-5-*S*-benzyl-6-deoxy-1,2-*O*-isopropylidene-5-thio-α-D-glucofuranose (**8**). Reductive scission of the benzyl group with sodium in liquid ammonia and hydrolysis of the isopropylidene group give 6-amino-6-deoxy-5-thio-D-glucopyranose (**12**). Acetylation of **12** affords crystalline 6-acetamido-1,2,3,4-tetra-*O*-acetyl-6-deoxy-5-thio-α-D-glucopyranose (**13**). Alternatively, the Birch reduction product was acetylated directly to yield 6-acetamido-3-*O*-acetyl-5-*S*-acetyl-6-deoxy-1,2-*O*-isopropylidene-5-thio-α-D-glucofuranose (**14**), which was then hydrolyzed with aqueous acetic acid and the product acetylated to yield **13**. The configuration at C-5 was proved by deamination of **8** with nitrous acid, and acetylation to yield the known 3,6-di-*O*-acetyl-5-*S*-benzyl-1,2-*O*-isopropylidene-5-thio-α-D-glucofuranose (**9**).

INTRODUCTION

There has been recent interest in the synthesis of sugar derivatives containing sulfur or nitrogen as the ring heteroatom[1]. This laboratory has been especially concerned with the synthesis of sugars having sulfur in the ring, and with the preparation from them of nucleosides and other biologically active molecules[2–9].

Compounds containing the 2-mercaptoethylamine grouping are effective in protecting organisms against the effects of ionizing radiation[10,11]. Several interesting carbohydrate structures containing these groups have been synthesized[12–16]. Herein is reported the synthesis of 6-amino-6-deoxy-5-thio-D-glucopyranose.

*Journal Paper No. 3754 of Purdue University Agricultural Experiment Station.

RESULTS AND DISCUSSION

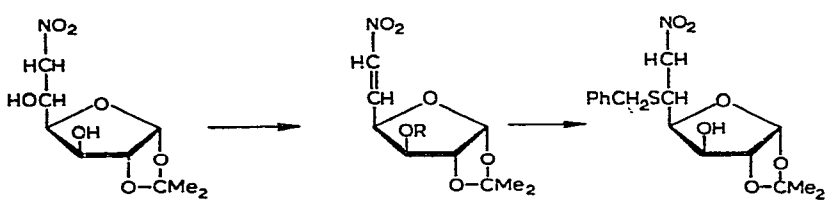
1,2:5,6-Di-*O*-isopropylidene-α-D-glucofuranose[17] (**1**) was hydrolyzed with 75% aqueous acetic acid at 25° to 1,2-*O*-isopropylidene-α-D-glucofuranose (**2**). On treatment with periodate in 50% aqueous ethanol, compound **2** gave, in almost quantitative yield, a mixture of 1,2-*O*-isopropylidene-α-D-*xylo*-pentodialdo-1,4-furanose (**3**) and its dimer[18] which was the major product.

On treatment with nitromethane in anhydrous methanol containing sodium methoxide, this syrupy mixture gave 6-deoxy-1,2-*O*-isopropylidene-6-nitro-α-D-glucofuranose (**4**) in 63% yield within 2 h. Longer reaction times are reported[19,20] to give, in substantial proportion, the L-idose derivative, which is probably produced by epimerization of the *aci*-nitro structure (observed under both aqueous and anhydrous conditions[21,22]).

On treatment with acetic anhydride and sodium acetate, compound **4** produced 3-*O*-acetyl-5,6-dideoxy-1,2-*O*-isopropylidene-6-nitro-α-D-*xylo*-hex-5-enofuranose[22a] (**5**). The reaction gave, directly, an 84% yield of the olefin. In our hands, treatment of compound **4** with acetic anhydride and pyridine also produced the olefin **5**, in contrast to earlier findings[20]. However, in the latter reaction, the olefin was obtained in 37% yield and was isolated only after chromatographic separation on silica gel.

Compound **5** exhibited absorption at 1740 (*O*-acetyl), 1655 (olefin), and 1510 cm^{-1} (nitro-olefin). The n.m.r. spectrum integrated for 15 protons as expected. Two one-proton singlets at τ 2.78 and 2.84 were indicative of olefinic protons having the *trans* configuration[23].

The nitro-olefin **5** reacts with α-toluenethiol in piperidine at 25° to give 5-*S*-benzyl-6-deoxy-1,2-*O*-isopropylidene-6-nitro-5-thio-α-D-glucofuranose (**6**) in 80% yield. The identical product was also obtained in 83% yield by reaction of the nitro-alcohol **4** with neutral aluminum oxide and α-toluenethiol in refluxing toluene[24]. The intermediate nitro-olefin **7** was isolated from a similar reaction from which α-toluenethiol was omitted. The syrupy product (**7**) exhibited strong olefinic and nitro-olefinic absorptions in its i.r. spectrum.



4 5 R=Ac 7 R=H 6

In both of these addition reactions with α-toluenethiol, only one of the members of the epimeric pair (D-*gluco*, L-*ido*) was observed by t.l.c. The melting point did not change on several further recrystallizations.

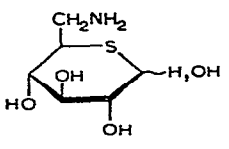
It is interesting that the attack of the α-toluenethiol anion on the olefin **5** proceeds with rather complete stereochemical specificity, to afford a product having

the D-*gluco* configuration. It is, perhaps, rational to expect this stereospecificity, inasmuch as the alkaline conditions of the reaction medium would require that the nitrovinyl group attached to C-4 of compound **5** be forced, by charge effects, to swing away from the partially ionized 3-hydroxyl group and to approach as near as possible to the ring-oxygen atom. It could be assumed, with some logic, that the nitro group occupies a position *trans* to C-4, as its negative character would cause it to remain as far as possible from the non-bonding electrons both of the ring-oxygen atom and of the 3-hydroxyl group, before and during the elimination reactions giving rise to olefin formation. With the nitrovinyl grouping swung rather far away from the oxygen atom of the 3-hydroxyl group, nucleophilic attack on the vinyl carbon atom at C-5 would be expected to come from a direction away from the ring and not from a direction over the ring. The latter approach would be unlikely, both from steric considerations and because the attacking group would also be unlikely to pass over, or close to, the nonbonding electrons of the ring-oxygen atom. Hence, the highly favored attack from a direction away from the ring would result in a product having the D-*gluco* configuration.

Paulsen[20] recently added ammonia to 3-*O*-acetyl-1,2-*O*-cyclohexylidene-5,6-dideoxy-6-nitro-α-D-*xylo*-hex-5-enofuranose to obtain a 2:3 distribution of the D-*gluco* and L-*ido* epimers. This ratio can be explained on the basis of the neutral character of the ammonia molecule, which allows it to attack with some ease by passing over, or near to, an electronic environment; its small size does not subject it extensively to steric factors. Ammonia is, of course, somewhat subject to steric factors, as shown by its attack on 3,4,5,6-tetra-*O*-acetyl-1,2-dideoxy-1-nitro-L-*arabino*-hex-1-enitol to afford mainly a product of the *erythro* configuration at C-2 and C-3. Here, the stereospecificity has been explained as a consequence of steric factors only, the ammonia approaching from the less-hindered side of the carbohydrate derivative, namely, the side flanked by a proton at C-3. Other workers[25,26] have commented on nucleophiles directionally affected by electron-rich regions.

Compound **6** was reduced with lithium aluminum hydride to the 6-amino derivative (**8**), which showed i.r. absorption at 3345 (OH), 3280 and 1595 (NH), and 701 cm^{-1} (*S*-benzyl). The n.m.r. spectrum integrated for the 23 protons expected. Significant signals were a five-proton singlet at τ 2.78 (aromatic protons), a one-proton doublet at τ 4.21 (anomeric proton, $J_{1,2}$ 3.7 Hz), and two three-proton singlets at τ 8.54 and 8.73 (isopropylidene methyl protons).

Acetylation of **8** yielded 6-acetamido-3-*O*-acetyl-5-*S*-benzyl-6-deoxy-1,2-*O*-isopropylidene-5-thio-α-D-glucofuranose (**10**), whose i.r. spectrum exhibited amide absorption bands at 1640 and 1535 cm^{-1}, and whose n.m.r. spectrum was consistent with structure **10**.



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On treatment with sodium in liquid ammonia, compound **8** gave a homogeneous product (**11**) which showed a strong, positive test with 2,3,5-triphenyltetrazolium chloride, indicating the presence of the free thiol. Hydrolysis yielded 6-amino-6-deoxy-5-thio-D-glucopyranose (**12**) which, on acetylation, gave crystalline 6-acetamido-1,2,3,4-tetra-*O*-acetyl-6-deoxy-5-thio-α-D-glucopyranose (**13**). The i.r. and n.m.r. spectra of **13** were consistant with the structure proposed. A small proportion of chloroform remained in the crystalline product, as indicated by a positive flame-test for halogen and the presence of a resonance signal at τ 2.72, integrating for less than one proton, in the n.m.r. spectrum. Elemental analysis indicated the presence of 0.173 molecule of chloroform per molecule of **13**. Benzene of crystallization has been found in crystals of the corresponding L-*ido* isomer[12].

Alternatively, compound **13** could be prepared from **11** by the following steps. Acetylation gave 6-acetamido-3-*O*-acetyl-5-*S*-acetyl-6-deoxy-1,2-*O*-isopropylidene-5-thio-α-D-glucofuranose (**14**), whose i.r. spectrum showed absorption bands at 1740 (*O*-acetyl), 1685 (*S*-acetyl), and 1650 and 1535 cm^{-1} (*N*-acetyl). Hydrolysis of **14** in 70% aqueous acetic acid for 48 h at 90–95°, and acetylation of the product, yielded compound **13**.

The D-*glycero* configuration at C-5 of compounds **6** and **8–14** was established by treating compound **8** with nitrous acid followed by acetylation to yield the known[27] 3,6-di-*O*-acetyl-5-*S*-benzyl-1,2-*O*-isopropylidene-5-thio-α-D-glucofuranose (**9**).

EXPERIMENTAL

General methods. — The purity of products and the course of reactions were determined by t.l.c. on glass plates (5 × 13 cm) coated[28] with Silica Gel G. The irrigants employed were *A*, 9:2 chloroform–acetone; *B*, 9:4 chloroform–acetone; *C*, 6:1 benzene–ethyl acetate; *D*, 10:1 benzene–ethyl acetate; and *E*, 3:1 chloroform–methanol. Solvent ratios are v/v. Components were located by spraying the dried plates with 5% sulfuric acid in ethanol, and heating until permanently charred spots were visible.

Melting points were determined on a Fisher–Johns apparatus and are corrected. N.m.r. spectra were recorded with a Varian Associates A-60 instrument for solutions in chloroform-*d* containing tetramethylsilane as the internal standard. I.r. spectra were recorded with a Perkin–Elmer Model 337 infrared spectrometer. Optical rotations were determined at 25° with a Perkin–Elmer Model 141 polarimeter. Evaporations were conducted under diminished pressure at a bath temperature below 40°. Absorption chromatography was performed on silica gel[28].

Nitromethane (spectrophotometric grade) and α-toluenethiol were obtained from Matheson, Coleman, and Bell*, and were used without further purification. The piperidine used was Fisher Reagent Grade†. 1,2:5,6-Di-*O*-isopropylidene-α-D-glucofuranose (**1**) was prepared according to standard procedures[17]; m.p. 110°.

*Matheson, Coleman, and Bell, Norwood, Ohio, U.S.A.
†Fisher Scientific Co., Fair Lawn, New Jersey, U.S.A..

1,2-*O*-Isopropylidene-α-D-glucofuranose (**2**) was prepared by selective hydrolysis of **1** with 75% aqueous acetic acid[29]; m.p. 160–161°. 1,2-*O*-Isopropylidene-α-D-*xylo*-pentodialdo-1,4-furanose (**3**) was prepared by treating **2** with sodium periodate in 50% aqueous ethanol[20], to yield **3** as a heterogeneous syrup.

6-Deoxy-1,2-O-isopropylidene-6-nitro-α-D-glucofuranose (**4**). — A solution of compound **3** (8.8 g) in absolute methanol (200 ml) was treated with three equivalents of nitromethane and sodium methoxide at 0°. The reaction was essentially complete after 30 min, and, after 2 h, the solution was deionized with methanol-washed[30] Amberlite IR-120 (H^+) ion-exchange resin, and the solution was evaporated under diminished pressure to yield crude **4**. Recrystallization from warm ethyl acetate–hexane gave pure **4** (4.8 g). The mother liquor was evaporated to a syrup, and this was applied to a column of silica gel, which was then eluted with irrigant *E* to yield an additional 2.5 g of **4**. The total yield of **4** was 7.3 g (63%), m.p. 125–126°, $[\alpha]_D^{25}$ −17.3° (*c* 3.7, water); lit.[19] m.p. 126–127°, $[\alpha]_D^{25}$ −16.8° (*c* 4.2, water). Also isolated from the column was a second syrupy component (130 mg) whose i.r. spectrum showed absorption bands characteristic of the nitro group; this component was not further characterized.

3-O-Acetyl-5,6-dideoxy-1,2-O-isopropylidene-6-nitro-α-D-xylo-hex-5-enofuranose (**5**). — *Method A.* To a solution of **4** (6 g) in anhydrous pyridine (18 ml) at 0° was added acetic anhydride (12 ml), with stirring. After 24 h at 0°, the darkened solution was poured into ice–water (200 ml). The mixture was extracted with chloroform (3 × 150 ml), and the extracts were combined, washed successively with water, sodium hydrogen carbonate solution, cold 0.1M hydrochloric acid, and water, dried (sodium sulfate), decolorized with activated carbon, and evaporated to a thick, yellow syrup. This syrup was heterogeneous, as shown by t.l.c. in irrigants *A* and *D*, and consisted of two major components, **5** and **5a**. The i.r. spectrum of the mixture showed no hydroxyl absorption. Compound **5** was isolated by chromatography on silica gel with elution with irrigant *C*, to yield 2.4 g of crystalline **5** (37%), m.p. 113°, $[\alpha]_D^{25}$ +5.9° (*c* 2.8, chloroform).

Method B. Compound **4** (1 g) was dissolved in cold acetic anhydride (25 ml) containing one drop of concentrated sulfuric acid. After 15 min, t.l.c. with irrigant *C* indicated the presence of **4**, a minor proportion of a second component identical with **5a** found in the mixture obtained by *method A*, and a major proportion of a third, stationary component which was not identified.

Method C. A solution of compound **4** (38 g) in acetic anhydride (225 ml) was cooled to 0°; sodium acetate (76 g) was added, and the suspension was stirred for 18 h at 25°. The mixture was then poured slowly into cold sodium hydrogen carbonate (3.5 liters), and the precipitate was removed by filtration. The filtrate was twice extracted with chloroform, and the extracts and precipitate were combined, washed with water (2 × 150 ml) and 150 ml of cold 0.1N hydrochloric acid, dried (sodium sulfate), and evaporated to dryness under diminished pressure, to yield crude **5**; this was recrystallized from hot ether to give 22.5 g of pure **5**. The mother liquor was evaporated to a syrup, which was titrated with hot ether to yield an additional 3.5 g

of **5**. The mother liquor was applied to the top of a column of silica gel which was then eluted with 4:1 benzene–ethyl acetate to yield 9 g of **5**. The total yield of **5** was 35 g (84%), m.p. 113°, $[\alpha]_D^{25}$ +5.9° (*c* 2.0, chloroform).

The i.r. spectrum of **5** in Nujol exhibited absorption bands at 1740 (*O*-acetyl), 1655 (olefin), and 1510 cm^{-1} (nitro-olefin). The n.m.r. spectrum integrated for 15 protons. Significant resonance signals were two one-proton singlets at τ 2.78 and 2.84 (olefinic protons, H-5 and H-6), a three-proton singlet at τ 7.97 (acetyl group protons), a one-proton doublet centered at τ 4.00 (anomeric proton $J_{1,2}$ 3.8 Hz), and two three-proton singlets at τ 8.45 and 8.65 (isopropylidene methyl groups).

Anal. Calc. for $C_{11}H_{15}NO_7$: C, 48.4; H, 5.5; N, 5.1. Found: C, 48.7; H, 5.4; N, 5.0.

5-S-Benzyl-6-deoxy-1,2-O-isopropylidene-6-nitro-5-thio-α-D-glucofuranose (**6**). — *Method A.* Compound **5** (22.5 g) was dissolved in piperidine (75 ml), and the reaction flask was flushed with dry nitrogen. α-Toluenethiol (10.5 ml) was added, and the mixture was stirred for 14 h at 25° under nitrogen. The piperidine and excess of α-toluenethiol were removed under diminished pressure at 40°, and the resulting syrup was applied to the top of a column (80 × 4.5 cm) of silica gel, which was then eluted with 4:1 benzene–ethyl acetate to yield 23.5 g of **6** (80%), m.p. 131–132°, $[\alpha]_D^{25}$ −22.8° (*c* 3.48, chloroform). The i.r. spectrum of **6** in Nujol showed absorption bands at 3340 (OH), 1535 and 1365 (nitro group), and 698 cm^{-1} (*S*-benzyl). The n.m.r. spectrum integrated for 21 protons; five appeared as a singlet at τ 2.68 (aromatic protons), six as two three-proton singlets at τ 8.60 and 8.72 (isopropylidene methyl groups), and one as a doublet centered at τ 4.12 (anomeric proton, $J_{1,2}$ 3.9 Hz).

Method B. Compound **4** (3 g) was dissolved in toluene (reagent grade; 150 ml) and neutral alumina (7.2 g) was added. After the flask had been flushed with nitrogen, α-toluenethiol (3 ml) was added, and the mixture was refluxed for 2 h under nitrogen. The mixture was cooled, toluene and α-toluenethiol were removed under diminished pressure, the residue was extracted with chloroform, and the extract was decolorized with carbon. After removal of the chloroform under diminished pressure, the resulting syrup was applied to the top of a column of silica gel; this was then eluted with 6:1 benzene–ethyl acetate to yield 3.5 g (83%) of **6** which was identical with that obtained by *method A*.

Anal. Calc. for $C_{16}H_{21}NO_6S$: C, 54.1; H, 6.0; N, 4.0; S, 9.0. Found: C, 54.4; H, 6.1; N, 4.0; S, 8.9.

5,6-Dideoxy-1,2-O-isopropylidene-6-nitro-α-D-xylo-hex-5-enofuranose (**7**). — Compound **4** (100 mg) was dissolved in toluene (reagent grade; 15 ml) and neutral alumina (240 mg) was added. The mixture was refluxed, with protection from atmospheric moisture, and the course of the reaction was followed by t.l.c. with irrigant *B*. Compound **4** gradually disappeared, and was replaced by a single, homogeneous component (**7**). After 2 h, the suspension was cooled and filtered, and the filtrate was evaporated to dryness under diminished pressure. The i.r. spectrum of the residue, compound **7**, exhibited strong nitro-olefinic absorption at 1650 and 1525 cm^{-1}.

6-Amino-5-S-benzyl-6-deoxy-1,2-O-isopropylidene-5-thio-α-D-glucofuranose (**8**).

— Lithium aluminum hydride (17 g) was suspended in absolute ether (500 ml) in a 2-liter flask equipped with a reflux condenser and a magnetic stirrer. The suspension was refluxed, with stirring, for 10 min, and then cooled to 25°. A solution of compound **6** (23 g) in absolute ether (500 ml) was now added (by means of a dropping funnel) at a rate sufficient to maintain reflux. The dropping funnel was rinsed with about 50 ml of absolute ether, and the suspension was refluxed for 1 h. The suspension was then cooled, and 95% ethanol was added dropwise to decompose the excess of hydride. Water was added, and the precipitate was removed by filtration. The residue was washed extensively with chloroform, and the filtrate and washings were combined and evaporated to a syrup. This material was dissolved in chloroform, and the solution was washed with water, dried (sodium sulfate), and evaporated to dryness under diminished pressure. The solid residue (21.5 g) was treated with ether, and the insoluble material (11.5 g) was removed by filtration; this was crude **8**. Recrystallization from warm ethyl acetate gave 8.5 g of pure **8**, m.p. 137–138°. The mother liquor was evaporated to a syrup which was dissolved in the minimal volume of chloroform. Ether was added to turbidity, and addition of a seed crystal of **8** resulted in the deposition of an additional 1 g of **8**. The mother liquor and ether-soluble material were combined, and evaporated to a syrup which was applied to the top of a column (75 × 4.5 cm) of silica gel which was eluted with 6:1 chloroform–methanol to yield 3.0 g of **8**. The total yield of **8** was 12.5 g (59%), m.p. 137–138°, $[\alpha]_D^{25}$ −19.3° (*c* 1.03, chloroform).

The i.r. spectrum of **8** in Nujol showed absorption bands at 3345 (OH), 3280 and 1595 (NH), and 701 cm^{-1} (*S*-benzyl). The n.m.r. spectrum integrated for 23 protons. Significant signals were a five-proton singlet at τ 2.78 (aromatic protons), a one-proton doublet centered at τ 4.21 (anomeric proton, $J_{1,2}$ 3.7 Hz), and two three-proton singlets at τ 8.45 and 8.73 (isopropylidene methyl groups).

Anal. Calc. for $C_{16}H_{23}NO_4S$: C, 59.1; H, 7.1; N, 4.3; S, 9.9. Found: C, 59.3; H, 7.1; N, 4.5; S, 9.9.

*6-Acetamido-3-*O*-acetyl-5-*S*-benzyl-6-deoxy-1,2-*O*-isopropylidene-α-*D*-glucofuranose* (**10**). — Compound **8** (50 mg) was acetylated with acetic anhydride (1 ml) in pyridine (2 ml). After 14 h at 25°, the mixture was poured into ice–water, and the mixture was extracted with chloroform; the extract was washed several times with water, dried (sodium sulfate), and evaporated under diminished pressure. The resulting syrup was dissolved in the minimal volume of chloroform, and hexane was added to turbidity; the crystalline material resulting was filtered off, to yield compound **10**, m.p. 130°, $[\alpha]_D^{25}$ −97.7° (*c* 1.07, chloroform). The i.r. spectrum of **10** in Nujol exhibited absorption bands at 3280 (NH), 1730 (*O*-acetyl), 1640 and 1535 (*N*-acetyl), and 704 cm^{-1} (*S*-benzyl). The n.m.r. spectrum integrated for 27 protons. Significant signals were a five-proton singlet at τ 2.77 (aromatic protons), two three-proton singlets at τ 8.53 and 8.72 (isopropylidene methyl groups), two three-proton singlets at τ 8.08 and 8.17 (acetyl protons), and a one-proton doublet centered at τ 4.20 (anomeric proton, $J_{1,2}$ 3.8 Hz).

*6-Amino-6-deoxy-1,2-*O*-isopropylidene-5-thio-*D*-glucofuranose* (**11**). — Com-

pound **8** (8.5 g) was dissolved in liquid ammonia (200 ml), and clean, dry sodium (2 g) was added slowly in small pieces. The blue color persisted after all of the sodium had been added; after 15 min, ammonium chloride (7 g) was carefully added. The excess of ammonia was allowed to evaporate under a stream of nitrogen, and the last traces were removed by adding chloroform and warming the flask in a warm-water bath. The precipitated salts were then removed by filtration, and the filtrate was evaporated under diminished pressure to give **11** as a white solid (6.3 g).

6-Amino-6-deoxy-5-thio-D-*glucopyranose* (**12**). — Compound **11** (6.3 g) was immediately dissolved in 0.05M sulfuric acid (50 ml), and the solution was stirred for 18 h at 25° under nitrogen. The sulfuric acid was neutralized with barium carbonate, the precipitate was removed by filtration, and the filtrate was decolorized with activated carbon, extracted once with chloroform (to remove residual bibenzyl), and evaporated under diminished pressure to give 5 g of **12** as a solid foam which could not be crystallized.

6-Acetamido-3-O-*acetyl-5*-S-*acetyl-6-deoxy-1,2*-O-*isopropylidene-5-thio-α*-D-*glucofuranose* (**14**). — Compound **8** (1 g) was treated with sodium in liquid ammonia as already described. After filtration, and evaporation of the filtrate under diminished pressure, the residue (**11**) was dissolved in pyridine (5 ml) and acetic anhydride (3 ml), and the solution was stirred under nitrogen for 18 h. The mixture was slowly poured into cold sodium hydrogen carbonate solution, and the solution was extracted twice with chloroform. The extracts were combined, washed with water, dried (sodium sulfate), and evaporated under diminished pressure, to give crude **14**. The product was recrystallized from ether, to yield 596 mg of chromatographically pure **14**, m.p. 139–140°, $[\alpha]_D^{25}$ +5.1° (*c* 1, chloroform). The mother liquor was applied to the top of a column of silica gel which was then eluted with 6:1 chloroform–acetone to yield an additional 54 mg of **14**. The total yield of **14** was 650 mg (59%). The i.r. spectrum of **14** showed absorption bands at 3355 (NH), 1740 (*O*-acetyl), 1685 (*S*-acetyl), and 1650 and 1535 cm^{-1} (*N*-acetyl).

Anal. Calc. for $C_{15}H_{23}NO_7S$: C, 49.9; H, 6.4; N, 3.9; S, 8.9. Found: C,50.2; H, 6.3; N, 4.1; S, 8.8.

6-Acetamido-1,2,3,4-tetra-O-*acetyl-6-deoxy-5-thio-α*-D-*glucopyranose* (**13**). — *Method A.* Compound **12** (5 g) was dissolved in glacial acetic acid (50 ml) with gentle heating. Pyridine (30 ml) and acetic anhydride (20 ml) were added, and the solution was stirred at 25° with the exclusion of moisture. After 24 h, presence of **12** was no longer observable by t.l.c. (irrigant *E*). The solution was poured into cold sodium hydrogen carbonate solution with stirring, the precipitate was removed by filtration, and the filtrate was extracted once with chloroform. The precipitate and extract were combined, and the solution was washed twice with water, dried (sodium sulfate), and evaporated to dryness under diminished pressure. The syrupy product was dissolved in the minimal volume of chloroform, hexane was added to turbidity, a few drops of chloroform were added, and the solution was kept at 25° until crystallization had begun, and then at 5° overnight. Compound **13** (2.9 g) was obtained by filtration, m.p. 84°, $[\alpha]_D^{25}$ +238° (*c* 2.24, chloroform).

Method B. Compound **14** (600 mg) was dissolved in glacial acetic acid (21 ml) and water (9 ml) was added. The solution was stirred under nitrogen for 44 h at 90–95°, cooled, and evaporated to dryness under diminished pressure. Pyridine was several times added and evaporated off under diminished pressure to remove the last traces of water, the residues was dissolved in pyridine (5 ml) and acetic anhydride (3 ml), and the solution was stirred for 6 h at 25°. The major product was chromatographically identical with **13** (irrigant *E*). The mixture was poured into cold sodium hydrogen carbonate solution, and the solution was extracted twice with chloroform. The extracts were combined, washed with water, dried (sodium sulfate), and evaporated to dryness under diminished pressure. The resulting syrup was applied to the top of a column of silica gel which was then eluted with 3:1 chloroform–acetone to yield **13**. This material could not be crystallized from methanol or methanol–ether. Crystallization from chloroform–ether yielded 216 mg of **13**; m.p. 84°, $[\alpha]_D^{25}$ +238° (*c* 2.4, chloroform).

The i.r. spectrum of **13** exhibited absorption bands at 3300 (NH), 1735 (*O*-acetyl), and 1650 and 1545 cm^{-1} (*N*-acetyl). No absorption at 1685 cm^{-1}, characteristic of *S*-acetyl groups, was observed. The n.m.r. spectrum integrated for 23 protons. Significant signals were four singlets at τ 7.85, 7.93, 8.00, and 8.03, integrating for 15 protons (*N*- and *O*-acetyl protons), and a one-proton doublet centered at τ 3.92 (anomeric proton, $J_{1,2}$ 2.8 Hz). Also present was a resonance signal at τ 2.72 which integrated for less than 0.5 proton.

Anal. Calc. for $C_{16}H_{23}NO_9S \cdot 0.173\ CHCl_3$: C, 45.5; H, 5.5; N, 3.3; S, 7.5. Found: C, 44.7; H, 5.6; N, 3.2; S, 7.6.

3,6-Di-O-acetyl-5-S-benzyl-1,2-O-isopropylidene-5-thio-α-D-glucofuranose (**9**). — Compound **8** (500 mg) was dissolved in 5:1 (v/v) water–acetic acid (30 ml), the solution was cooled to 0°, and sodium nitrite (500 mg) was added. After 30 min, t.l.c. (irrigant *C*) showed that there was no **8** in the solution; it was made neutral with sodium carbonate, and extracted with chloroform. The extract was washed with water, dried (sodium sulfate), and evaporated to dryness under diminished pressure. The resulting syrup was applied to the top of a column of silica gel, and elution with benzene–ethyl acetate yielded 5-*S*-benzyl-1,2-*O*-isopropylidene-5-thio-α-D-glucofuranose (307 mg). The syrup was acetylated with acetic anhydride (1 ml) in pyridine (2 ml) for 6 h at 25°, the mixture was poured into ice–water, and this mixture was extracted twice with chloroform. The extract was washed with water, dried (sodium sulfate), and evaporated to dryness under diminished pressure. The resulting syrup was stirred with 95% ethanol, to yield **9** (270 mg), m.p. 94°, $[\alpha]_D^{25}$ −52° (*c* 1.52, chloroform); lit.[27] m.p. 94°, $[\alpha]_D^{25}$ −51.7° (*c* 1.2, chloroform).

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Carbohyd. Res., 12 (1970) 201–210

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

METHODE ZUM QUANTITATIVEN NACHWEIS VON HEXOSAMINEN ALS *N*-2,4-DINITROPHENYL-DERIVATE

H. J. HAAS UND A. WEIGERDING
Institut für Physiologische Chemie der Universität des Saarlandes, 665 Homburg/Saar (Deutschland)
(Eingegangen den 12. April, 1969; modifiziert den 25. Juli, 1969)

ABSTRACT

D-Glucosamine hydrochloride treated with 1-fluoro-2,4-dinitrobenzene and sodium hydrogen carbonate gave 2-deoxy-2-(2,4-dinitroanilino)-D-glucose in a yield up to 96%. On the basis of this reaction, which can also be applied to D-galactosamine hydrochloride, it is possible to estimate quantitatively 10 μg of hexosamine hydrochloride at a concentration of at least 25 μg per ml. 2-Deoxy-2-(2,4-dinitroanilino)-D-galactose may be separated from the D-glucose analog by thin-layer chromatography.

ZUSAMMENFASSUNG

Aus D-Glucosamin-hydrochlorid und 1-Fluor-2,4-dinitrobenzol erhält man in Gegenwart von Natriumhydrogencarbonat bis zu 96 Proz. der Theorie 2-Desoxy-2-(2,4-dinitroanilino)-D-glucose (Dnp-D-glucosamin). Die Umsetzung, die auch auf D-Galaktosamin-hydrochlorid übertragbar ist, ermöglicht es, 10 μg Hexosamin-hydrochlorid in einer Konzentration von mindestens 25 μg/ml zu bestimmen. 2-Desoxy-(2,4-dinitroanilino)-D-galaktose (Dnp-D-galaktosamin) läßt sich dünnschichtchromatographisch von Dnp-D-glucosamin unterscheiden.

EINFÜHRUNG

Die Umsetzung von D-Glucosamin und anderen Aminozuckern zu den entsprechenden *N*-2,4-Dinitrophenyl(Dnp)-derivaten ist von verschiedenen Autoren[1-9] zum Nachweis von Hexosaminen angewendet worden.

In der vorliegenden Arbeit beschreiben wir am Beispiel der Umsetzung von α-D-Glucosamin-hydrochlorid mit 1-Fluor-2,4-dinitrobenzol (DNFB) die Bedingungen, unter denen die Reaktion zum quantitativen Nachweis von Aminozuckern geeignet ist und verschiedene Verfahren zur Reinigung und chromatographischen Trennung von Dnp-hexosaminen. Die Anwendung der Methode zur quantitativen Bestimmung von Hexosaminen in Gemischen biologischer Herkunft wird in einer gesonderten Arbeit geschildert.

ERGEBNISSE

Bei der Reaktion eines Amin-hydrochlorids mit DNFB müssen mindestens 2 Äquivalente Base zur Neutralisation der freiwerdenden Säure zugesetzt werden, um eine quantitative Umsetzung zu erzielen. Da die Reaktion durch Art und Menge der zugesetzten Base beeinflußt wird[10], wurde zunächst untersucht (Abb. 1), wie die Ausbeute an Dnp-D-glucosamin von der verwendeten Base abhängt, wobei berücksichtigt werden mußte, daß sowohl D-Glucosamin als auch Dnp-D-glucosamin in alkalischer Lösung instabil ist.

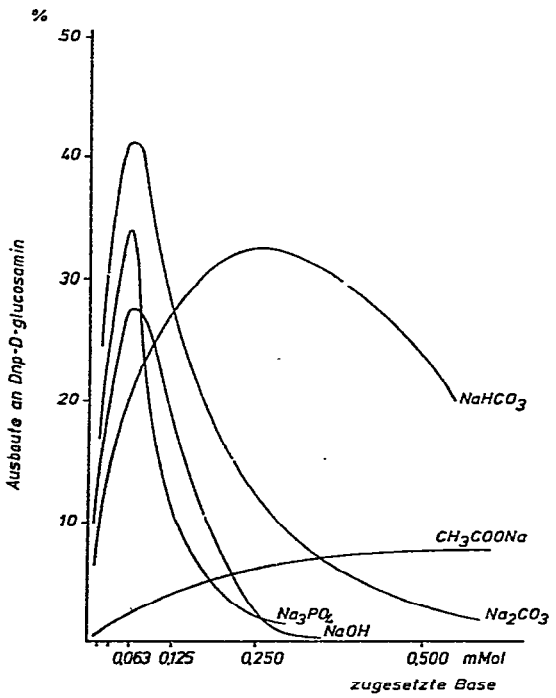

Abb. 1. Abhängigkeit der Ausbeute an Dnp-D-glucosamin von Art und Menge der zugesetzten Base: Eine 0,2M wäßrige Lösung von α-D-Glucosamin-hydrochlorid (0,5 ml) wurde mit einer 0,6M Lösung von DNFB (3 Äquivalente) in Dioxan (0,5 ml) und verschiedenen Mengen Base (0,5—0,016 mMol) versetzt. Nach 12 Stdn. wurden Proben (0,01 ml) dünnschichtchromatographisch an Kieselgel H in Chloroform–Methanol–Eisessig (90:7:3) aufgetrennt. R_F-Werte: Dnp-D-glucosamin 0,29; 2,4-Dinitrophenol 0,98; 3 nicht isolierte Nebenprodukte unbekannter Konstitution 0,23 bzw. 0,36 bzw. 0,41 (vgl. Abb. 2). Das Dnp-D-glucosamin wurde mit Methanol (5 ml) eluiert und bei 355 nm photometriert (Spectronic 20 der Firma Bausch & Lomb).

In wäßriger Lösung ist in Gegenwart von Natriumcarbonat eine relativ hohe Ausbeute an Dnp-D-glucosamin zu erzielen, die jedoch bei geringer Änderung der optimalen Natriumcarbonat-Menge sehr stark sinkt. Auch bei Verwendung zu großer Mengen von Natriumhydroxid oder Trinatriumphosphat sinken die Ausbeuten. In Abb. 2 ist dies anhand der Umsetzung von α-D-Glucosamin-hydrochlorid

mit DNFB in Gegenwart steigender Mengen Natriumcarbonat dargestellt. Der Zusatz von Natriumacetat liefert stets nur geringe Ausbeuten an Dnp-D-glucosamin.

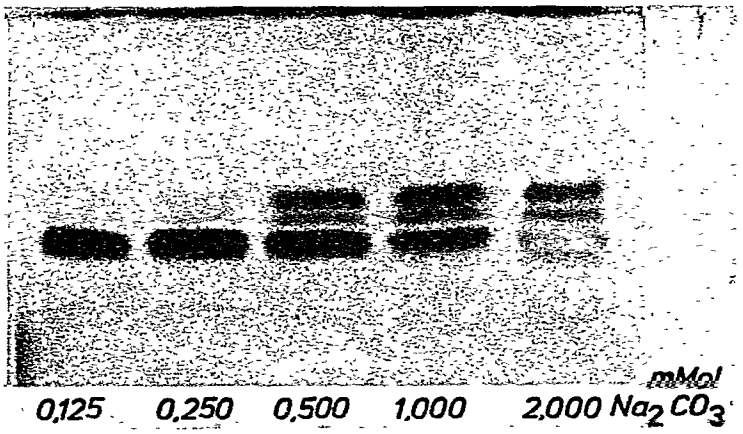

Abb. 2. Dünnschichtchromatographie der Reaktionsansätze nach 1-stündiger Umsetzung von α-D-Glucosamin-hydrochlorid mit 1 Äquivalent DNFB bei Zusatz verschiedener Mengen Natriumcarbonat: je 1 ml einer 0,2M wäßrigen Lösung von α-D-Glucosamin-hydrochlorid wurde mit einer 0,2M Lösung von DNFB in Dioxan (1 ml) und verschiedenen Mengen (0,125–2,0 mMol) Natriumcarbonat versetzt. Nach 1 Std. wurden Proben (0,05 ml) entnommen und wie bei Abb. 1 chromatographiert. Fotografie: das Chromatogramm wurde im UV-Licht (366 nm, Desaga-UVIS der Firma Desaga, Heidelberg) fotografiert. Film: Agfa-Isopan 15/10 DIN. Belichtungszeit: 10 sec. Blende: 2,8.

Beim Arbeiten mit Natriumhydrogencarbonat erhält man zwar geringere Mengen Dnp-D-glucosamin als beim Arbeiten mit Natriumcarbonat, jedoch ist die Ausbeute wesentlich weniger von der zugesetzten Basenmenge abhängig. Wir haben daher alle weiteren Versuche in Gegenwart von Natriumhydrogencarbonat durchgeführt.

Die Geschwindigkeiten bei Umsetzungen von DNFB mit Aminen und die Ausbeuten an Dnp-aminen sind stark vom Lösungsmittel abhängig[11–14]. Daher wurde der Einfluß verschiedener Lösungsmittelzusätze [Dimethylsulfoxid, *N,N*-Dimethylformamid (DMF), 2-Methoxyäthanol, Methanol, Dioxan, Aceton] auf die Reaktion von α-D-Glucosamin-hydrochlorid mit DNFB und Natriumhydrogencarbonat in Gegenwart von Wasser untersucht. Die größten Reaktionsgeschwindigkeiten und die höchsten Ausbeuten an Dnp-D-glucosamin wurden in Anwesenheit von Dimethylsulfoxid oder DMF erreicht. Die Steigerung der Reaktionsgeschwindigkeit bei Zusatz von DMF stimmt mit Erfahrungen bei Umsetzungen von 4-Nitrohalogenbenzolen mit Piperidin überein[11]. Dagegen sinkt bei Umsetzung von Seidenfibroin mit DNFB bei Anwesenheit von DMF die Ausbeute an Dnp-protein[15]. Die weiteren Umsetzungen von α-D-Glucosamin-hydrochlorid mit DNFB erfolgten stets in Anwesenheit von DMF.

Abb. 3 zeigt, daß Dnp-D-glucosamin umso schneller und mit umso größerer Ausbeute entsteht, je mehr DMF anwesend ist. Aus Abb. 4 geht hervor, daß bei

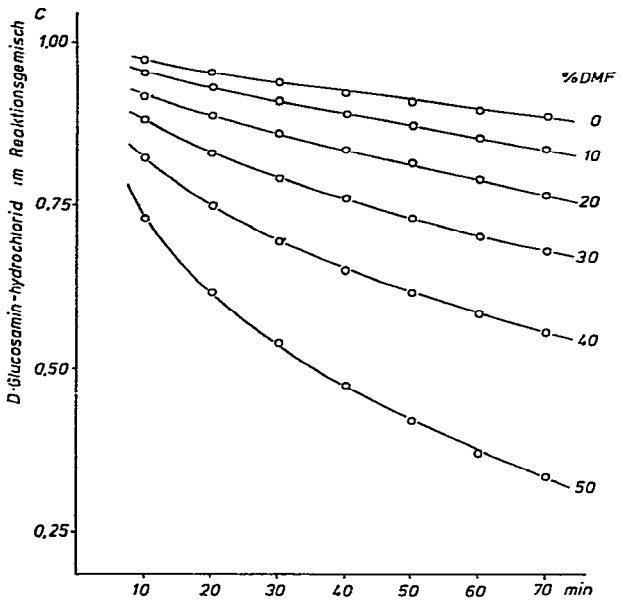

Abb. 3. Abhängigkeit der zeitlichen Abnahme an α-D-Glucosamin-hydrochlorid vom Prozentsatz an DMF im Reaktionsgemisch bei Umsetzung mit 1 Äquivalent DNFB und 2 Äquivalenten Natriumhydrogencarbonat: Eine 0,5M wäßrige Lösung von α-D-Glucosamin-hydrochlorid (1 ml) wurde mit einer 0,5M wäßrigen Lösung von Natriumhydrogencarbonat (2 ml), einer 0,5M Lösung von DNFB in Dioxan (1 ml) und Wasser (6 ml) versetzt. In analogen Versuchen wurde das zugefügte Wasser in steigendem Maße durch DMF ersetzt. Probeentnahmen, Chromatographie und Photometrie wie bei Abb. 1. (Setzt man 3 Äquivalente Natriumhydrogencarbonat ein, so entsteht so viel 2,4-Dinitrophenol aus DNFB, daß dieses zur vollständigen Umsetzung von α-D-Glucosamin-hydrochlorid nicht ausreicht.)

einem DMF-Anteil von 50% die Halbwertszeit der Reaktion bereits so klein ist, daß weiterer Zusatz von DMF ohne praktische Bedeutung ist, zumal dann auch vor allem Natriumhydrogencarbonat zunehmend schwerer löslich wird.

Aus den der Abb. 3 zugrundeliegenden Meßwerten wurden die Reaktionskonstanten berechnet (Tab. I). Die Reaktion von α-D-Glucosamin-hydrochlorid mit DNFB und Natriumhydrogencarbonat verhält sich demnach in Wasser–Dioxan wie eine Reaktion 3. Ordnung, in 50% DMF jedoch annähernd wie eine Reaktion 2. Ordnung. Die Reaktionskonstanten für Umsetzungen in Lösungsmitteln mit DMF-Gehalten zwischen 0 und 50% enthalten Terme für Reaktionen 2. und 3. Ordnung. Mit steigendem Prozentsatz DMF nimmt der Term für die Reaktion 2. Ordnung zu, der für die Reaktion 3. Ordnung ab.

Die Änderung der Reaktionsgeschwindigkeit und der Reaktionsordnung bei Steigerung des DMF-Anteils könnten dadurch bedingt sein, daß bei An- bzw. Abwesenheit von DMF verschiedenartige Formen von Glucosamin reagieren, oder daß die Zwischenstufen bzw. die Geschwindigkeiten der Einzelschritte der nucleophilen Reaktion zwischen Glucosamin und DNFB in den beiden Lösungsmitteln unterschiedlich sind.

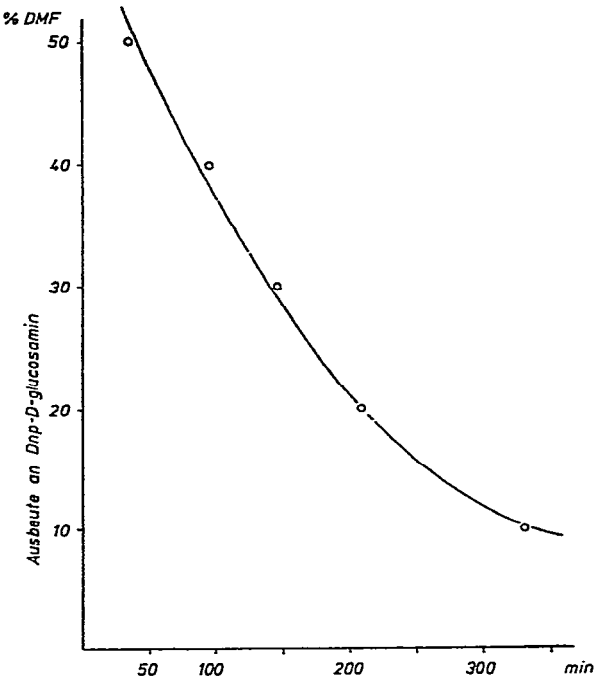

Abb. 4. Halbwertszeiten der Umsetzung von α-D-Glucosamin-hydrochlorid mit 1 Äquivalent DNFB und 3 Äquivalenten Natriumhydrogencarbonat in Abhängigkeit vom prozentualen Anteil an DMF im Lösungsmittel. Experimentelles entsprechend Abb. 3.

β-D-Glucosamin mutarotiert in DMF etwa 70 mal langsamer als in Wasser[16]. Auch die Mutarotation von α-D-Glucosamin-hydrochlorid verläuft in DMF–Wasser (1:1) wesentlich langsamer als in Wasser. Dagegen mutarotiert es bei Zusatz von 2,5 Äquivalenten Natriumhydrogencarbonat in 50% DMF etwas schneller als in Wasser. Während der Mutarotation ist daher in Natriumhydrogencarbonat-haltigem 50% DMF stets mehr β-D-Glucosamin enthalten als in Wasser. Ob dies die Vergrößerung der Reaktionsgeschwindigkeit und der Ausbeute an Dnp-glucosamin verursacht, vermögen wir nicht zu sagen.

Ebenso ist auch der Einfluß des aprotischen, dipolaren DMF bzw. des protischen, polaren Wassers auf die Umsetzung nicht eindeutig interpretierbar, da der Reaktionsmechanismus nicht restlos geklärt ist. Es ist denkbar, daß die Änderung der Reaktionsordnung bei Steigerung des DMF-Anteils damit zusammenhängt, daß der pH-Wert des Reaktionsgemisches sinkt, wenn der DMF-Gehalt steigt[11].

Durch einen Überschuß an DNFB und Natriumhydrogencarbonat beim Arbeiten in 50% DMF ist die Ausbeute an Dnp-D-glucosamin weiter zu erhöhen. Bei Verwendung von je 3 Äquivalenten Natriumhydrogencarbonat und DNFB ist eine fast quantitative Ausbeute an Dnp-D-glucosamin zu erzielen.

Die Umsetzung mit mindestens je 3 Äquivalenten DNFB und Natriumhydrogencarbonat in Wasser–Dioxan–DMF (4:1:5) eignet sich zum quantitativen Nachweis

TABELLE I

REAKTIONSKONSTANTEN DER UMSETZUNG VON α-D-GLUCOSAMIN-HYDROCHLORID MIT 1 ÄQUIVALENT DNFB UND 2 ÄQUIVALENTEN NATRIUMHYDROGENCARBONAT IN WASSER–DIOXAN (9:1) BEI ZUSATZ STEIGENDER MENGEN DMF (20°)

	% DMF							
	0		10	20	30	40	50	
Zeit (min)	C_t[a]	$10^{-4} \cdot k_{0\%}$[b]	C_t[a]	C_t[a]	C_t[a]	C_t[a]	C_t[a]	$10^{-2} \cdot k_{50\%}$[b]
10	5,26	0,277	5,13	4,95	4,75	4,45	3,93	1,01
20	5,16	0,250	5,03	4,80	4,48	4,05	3,32	0,843
30	5,07	0,256	4,90	4,65	4,25	3,75	2,90	0,773
40	4,96	0,250	4,80	4,50	4,10	3,50	2,55	0,755
50	4,91	0,240	4,71	4,40	3,95	3,30	2,25	0,756
60	4,84	0,237	4,60	4,25	3,80	3,15	2,00	0,765
90	4,63	0,266	4,30	3,90	3,40	2,75	1,45	0,816
120	4,38	0,246	4,00	3,60	3,08	2,34	1,21	0,782
180	4,03	0,252	3,50	2,90	2,36	1,56	0,89	0,764
240	3,77	0,249	3,20	2,30	1,72	0,93	0,71	0,742

[a] C_t = Konzentration von D-Glucosamin-hydrochlorid [mg·ml^{-1}] zur Zeit t [min]; C_0 = 5,4 mg·ml^{-1} = Konzentration von α-D-Glucosamin-hydrochlorid zur Zeit t = 0 min. [b] Die Konstante $k_{0\%}$ [ml^2·mg^{-1}·min^{-1}] bei der Umsetzung in Gegenwart von 0% DMF wurde nach Gleichung (1), die Konstante $k_{50\%}$ [ml·mg^{-1}·min^{-1}] bei der Umsetzung in Gegenwart von 50% DMF nach Gleichung (2) berechnet:

$$(1) \qquad \frac{1}{2C_0^2}\left[\left(\frac{C_0}{C_t}\right)^2 - 1\right] = 3kt$$

$$(2) \qquad \frac{1}{C_0}\left(\frac{C_0}{C_t} - 1\right) = 2kt$$

von D-Glucosamin-hydrochlorid und D-Galaktosamin-hydrochlorid. Tab. II zeigt, daß bis zu 96% der eingesetzten Aminozucker erfaßt werden, wenn man zu den Dnp-Derivaten umsetzt, diese dünnschichtchromatographisch abtrennt und anschließend photometriert. Beispielsweise wurde 0,5 mg D-Glucosamin-hydrochlorid in 10 ml umgesetzt; 0,02 ml (entsprechend 10 μg D-Glucosamin-hydrochlorid) wurden zur Dünnschichtchromatographie entnommen, das Dnp-D-glucosamin wurde mit 8 ml Methanol eluiert und in dieser Lösung photometriert. Die Wiederfindungsquote beträgt bei diesen Reaktionsbedingungen 89%. Jedoch ist es durch Verkleinerung des Reaktions- und des Elutionsvolumens sowie durch Vergrößerung der zur Chromatographie entnommenen Probe möglich, bis zu minimal 10 μg in einer Konzentration von etwa 25 μg D-Glucosamin-hydrochlorid/ml mit der angegebenen Genauigkeit nachzuweisen.

D-Galaktosamin-hydrochlorid liefert mit DNFB bei analogen Reaktionsbedingungen Dnp-D-galaktosamin[4] vom Schmp. 180–182°. Dieses ist durch Dünnschichtchromatographie an boratgepuffertem Kieselgel oder Kieselgur von Dnp-D-glucosamin zu trennen[4,17]. Am besten bewährte sich die Dünnschichtchromato-

TABELLE II

QUANTITATIVER NACHWEIS VON HEXOSAMIN-HYDROCHLORIDEN[a]

D-*Glucosamin-hydrochlorid*	D-*Galaktosamin-hydrochlorid*	D-*Glucosamin-hydrochlorid*	D-*Galaktosamin-hydrochlorid*
Einwaage (mg)		*Wiedergefunden (%)*	
43,2		96,5	
30,0		96,0	
21,5		96,0	
13,0		94,0	
4,3		92,0	
0,5		89,0	
30,0	15,3[b]	95,0	96,0
20,5	10,2[b]	96,0	94,5
11,2	5,1[b]	93,0	94,0

[a]Die angegebenen Einwaagen an Aminozucker wurden in je 1 ml Wasser gelöst. Nach Zugabe einer Lösung von DNFB (112 mg) in Dioxan (1 ml) und einer 0,5M wäßrigen Lösung von Natriumhydrogencarbonat (3 ml) wurde mit DMF (5 ml) versetzt. Nach 1 Std. wurden 0,02 ml entnommen und an Kieselgel H (E. Merck) (Schichtdicke: 2 mm) im System Chloroform–Methanol–Eisessig (90:7:3) chromatographiert. Die Dnp-Derivate der Aminozucker wurden mit Methanol (8 ml) eluiert und photometriert. [b]Gemische von D-Glucosamin-hydrochlorid und D-Galaktosamin-hydrochlorid.

graphie an luftgetrocknetem Kieselgel H, gepuffert mit Dinatriumtetraborat (mit Natriumhydroxid auf pH 10,5 eingestellt) im System 1-Butanol–Wasser–Methanol (5:1:1). Die R_F-Werte für Dnp-D-glucosamin bzw. Dnp-D-galaktosamin betragen 0,50 bzw. 0,34.

EXPERIMENTELLER TEIL

Darstellung von 2-Desoxy-2-(2,4-dinitroanilino)-D-glucose (größere Mengen). — Eine Lösung von α-D-Glucosamin-hydrochlorid (0,86 g) in Wasser (10 ml) wurde bei 20° mit 0,5M wäßrigem Natriumhydrogencarbonat (10 ml), einer Lösung von 1-Fluor-2,4-dinitrobenzol (DNFB) (1,5 g) in Dioxan (10 ml) und *N,N*-Dimethylformamid (DMF) (30 ml) versetzt. Es wurde 2 Stdn. bei 20° geschüttelt und dann mit Kieselgel S (120 g) (E. Merck AG, Darmstadt) verrührt. Das Gemisch wurde bei 20° und 12 Torr unter sorgfältigem Ausschluß von Licht getrocknet und dann mit Chloroform extrahiert, bis dieses frei von 2,4-Dinitrophenol, d. h. farblos war. Dann wurde mit Äther–Methanol (1:1) bis zur Entfärbung des Kieselgels gewaschen und bei Lichtausschluß i. Vak. bei möglichst tiefer Temperatur eingedampft (Rohausbeute: 90%). Das Rohprodukt wurde mehrmals mit Chloroform gewaschen, in Methanol–Aceton (1:3) gelöst und zur Kristallisation 24 Stdn. bei 0° gehalten. Nach Absaugen wurde mit Chloroform gewaschen und i. Vak. über Paraffin getrocknet (Ausb. 56%), Schmp. 204–206°; [Lit.: 167–169° (Ref. 1,3), 196 ±2° (Ref. 2), 202–204° (Ref. 4)].

Ber. für $C_{12}H_{15}N_3O_9$: C, 41,7; H, 4,4; N, 12,2. Gef.: C, 42,1; H, 4,3; N, 11,9.

Darstellung von Dnp-hexosaminen (kleine Mengen). — Bei Umsetzung von etwa 40 mg α-D-Glucosamin-hydrochlorid oder D-Galaktosamin-hydrochlorid mit entsprechend verkleinerten Mengen an Natriumhydrogencarbonat und DNFB in entsprechend geringeren Volumina an Wasser, Dioxan und DMF empfiehlt es sich, das entstandene Dnp-hexosamin dünnschichtchromatographisch an Kieselgel H (E. Merck AG, Darmstadt) (Schichtdicke: 2 mm) im System Chloroform–Methanol–Eisessig (90:7:3) abzutrennen. Das Dnp-Derivat wurde mit Äther–Methanol (1:1) eluiert. Nach Trocknung unter Lichtausschluß wurde mit Chloroform gewaschen. Die Dünnschichtchromatographie dauert etwa 30 min und sollte möglichst unter Vermeidung direkten Lichteinfalls durchgeführt werden.

DANK

Wir danken dem Fonds der Chemischen Industrie für eine Sachbeihilfe.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

EINE NEUE SYNTHESE VON METHYL-2,3-DIDESOXY-α-D-*erythro*-HEX-2-ENOPYRANOSID

A. KLEMER UND G. MERSMANN
Organisch-Chemisches Institut der Universität Münster, 44 Münster (Deutschland)
(Eingegangen den 29. Juli, 1969)

ABSTRACT

Treatment of methyl α-D-mannopyranoside 2,3-carbonate with potassium thiocyanate gave, in addition to carbon dioxide, methyl 2,3-dideoxy-α-D-*erythro*-hex-2-enopyranoside, the structure of which was established mainly by n.m.r. spectroscopy. The mechanism of formation is discussed.

ZUSAMMENFASSUNG

Methyl-α-D-mannopyranosid-2,3-carbonat bildet mit Kaliumthiocyanat unter Kohlendioxid-Eliminierung Methyl-2,3-didesoxy-α-D-*erythro*-hex-2-enopyranosid, dessen Struktur hauptsächlich durch n.m.r. Spektroskopie bewiesen wird. Der Mechanismus wird diskutiert.

EINLEITUNG

Cyclische Kohlensäureester von Zuckern sind besonders auf dem Synthesegebiet zur Blockierung bestimmter Hydroxylgruppen von Bedeutung. Da die Carbonatgruppierung ziemlich säurestabil ist, jedoch leicht im alkalischen Gebiet abgespalten werden kann, stellt sie eine wertvolle Ergänzung zur Isopropylidengruppe dar[1].

Wenig erfolgreich waren bisher Versuche, durch Decarboxylierung von Zuckercarbonaten zu Produkten von Wert zu gelangen. Aus der Reihe der gut untersuchten aliphatischen cyclischen Kohlensäureester sind folgende Reaktionen in diesem Zusammenhang von Interesse: pyrolytische Decarboxylierung unter Mitwirkung einer freien Hydroxylgruppe (Nachbargruppeneffekt)[2,3] oder durch Basenkatalyse[4] führt zu cyclischen Äthern mit drei bis zu sechs Ringatomen. Unter dem Einfluß von Kaliumthiocyanat bilden sich unter Kohlendioxid-Eliminierung cyclische Sulfide[5]. In der Zuckerreihe gelang bisher lediglich die Umwandlung von 1,2-*O*-Isopropyliden-α-D-glucofuranose-5,6-carbonat in das entsprechende 3,6-Anhydro-Derivat[6-8].

ERGEBNISSE

Als Ausgangsverbindung für unsere Untersuchungen wählten wir Methyl-α-D-mannopyranosid-2,3-carbonat, das die folgenden sterischen und strukturellen

Voraussetzungen erfüllt: die Hydroxylgruppe am C-6 und die Carbonatgruppierung liegen auf einer Seite des Ringes. Nucleophiler Rückseitenangriff von dieser Stelle auf die Estergruppe ist demgemäß ausgeschlossen. Diese Möglichkeit, die, wie bekannt, grundsätzlich die Reaktion erleichtert, ist aber für die Hydroxylgruppe am C-4 gegeben, die benachbart *trans*-ständig zur Carbonatgruppierung steht.

Für die Darstellung von Methyl-α-D-mannopyranosid-2,3-carbonat (**2**) nach Hough und Priddle[9] entwickelten wir ein vereinfachtes Verfahren, das die zeitraubende Kristallisation des als Zwischenprodukt auftretenden Methyl-4,6-di-*O*-benzyloxycarbonyl-α-D-mannopyranosid-2,3-carbonats (**1**) umgeht.

Methyl-α-D-mannopyranosid-2,3-carbonat (**2**) erwies sich thermisch als ausgesprochen stabil. Erst ab *ca.* 250° trat allmähliche Zersetzung ein. Auch unter dem Einfluß von Natriumchlorid, Kaliumcarbonat oder Bariumchlorid erfolgte nur unspezifische Zersetzung. Decarboxylierung fand jedoch mit molaren Mengen Kaliumthiocyanat in der Schmelze schon ab 120° statt. Aus dem dunkelgefärbten Reaktionsgemisch ließ sich durch Sublimation bei 10^{-2} mm Hg ein Gemisch aus Methyl-2,3-didesoxy-α-D-*erythro*-hex-2-enopyranosid (**3**), zwei noch nicht identifizierten Substanzen und Methyl-α-D-mannopyranosid abtrennen. Letzteres blieb beim Aufnehmen des Gemisches mit Aceton nahezu quantitativ ungelöst und konnte so in kristalliner Form abgetrennt werden. Die drei übrigen Komponenten wurden säulenchromatographisch an Kieselgel mit wassergesättigtem Butanon getrennt, wobei das Hauptprodukt **3** am schnellsten wanderte.

Ein ester informatorischer Hinweis auf die Natur von **3** wurde durch die saure Hydrolyse erhalten. Sie verlief unter erheblicher Zersetzung und führte zu einem Produkt mit einem (im Papierchromatogramm) erheblich höheren R_F-Wert als freie Hexosen. Dieser Befund zeigte, daß bei der Decarboxylierung nicht der in Analogie zum Verhalten aliphatischer Carbonate erwartete Epoxy- bzw. Epithio-zucker entstanden sein konnte. Dieser hätte unter den Hydrolysebedingungen freie Hexosen bzw. ihre Mercapto-Derivate ergeben müssen.

Das i.r.-Spektrum zeigte eine OH-, eine OMe-, eine aliphatische CH- und eine C=C-Bande. Auch durch Entfärben einer acetonischen Kaliumpermanganatlösung ließ sich die Doppelbindung nachweisen. Die Identifizierung von **3** als Methyl-2,3-

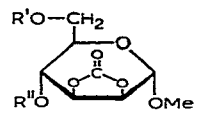
TABELLE I

N.M.R.-DATEN FÜR DAS HAUPTPRODUKT **3** UND DIE VIER ISOMEREN METHYL-4,6-*O*-BENZYLIDEN-2,3-DIDESOXY-D-HEX-2-ENOPYRANOSIDE **5**, **6**, **7** UND **8**[a]

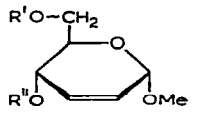
Verbindung	*Verschiebung* (τ)				*Kopplung* (*Hz*)	
	H-1	*H-2*	*H-3*	*H-4*	$J_{1,2}$	$J_{2,3}$
α-*erythro* (**3**)	5,07	4,24	3,99	5,76	2,4	10
α-*erythro* (**5**)	5,05	4,23	3,80	5,70	2,2	10
α-*threo* (**6**)	4,91	3,88	3,86	5,85	2,0	10
β-*erythro* (**7**)	4,66	4,30	3,81	5,66	1,3	10
β-*threo* (**8**)	4,82	3,88	3,86	5,80	0,75	10

[a]Die Vergleichsdaten wurden aus Lemieux *et al.*[10] entnommen.

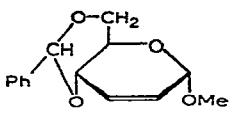
didesoxy-α-D-*erythro*-hex-2-enopyranosid gelang durch Vergleich seiner n.m.r.-Daten mit denen von Methyl-4,6-*O*-benzyliden-2,3-didesoxy-α-D-*erythro*-hex-2-enopyranosid (**5**). Sie stimmen, wie die Tabelle I zeigt, gut überein, während die der zum Vergleich herangezogenen drei Isomeren **6**, **7**, und **8** deutlich abweichen. Außer den in der Tabelle aufgeführten Peaks zeigt das Spektrum noch einen Peak für die OMe-Gruppe bei τ 6,52.



1 R'=R''=CO_2CH_2Ph
2 R'=R''=H



3 R'=R''=H
4 R'=R''=CO-Ph



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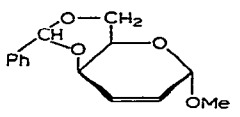

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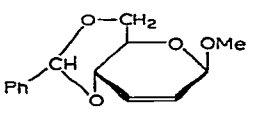

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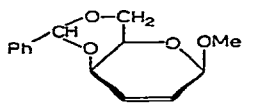


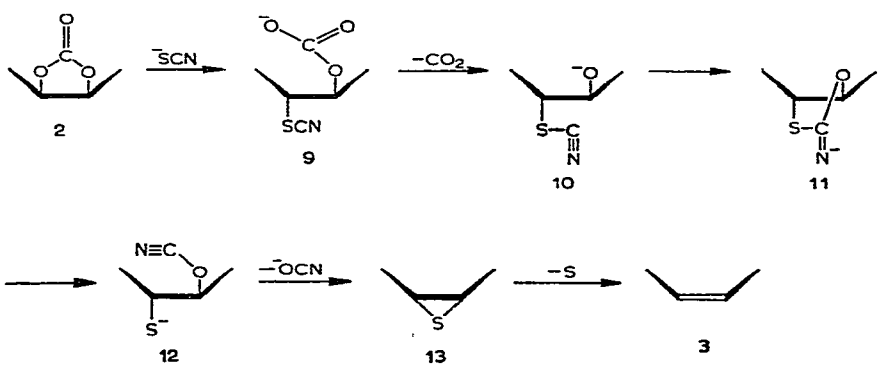
8

Auch Drehwert und Brechungsindex stimmen mit den in der Literatur[11] angegebenen Werten überein. Charakteristisch für diese Stoffklasse ist ferner die beobachtete Säureempfindlichkeit[12]. Die Benzoylierung von **3** führt zu dem bekannten[13] Kristallinen Methyl-4,6-di-*O*-benzoyl-2,3-didesoxy-α-D-*erythro*-hex-2-enopyranosid (**4**).

Die Identifizierung der beiden Nebenprodukte, von denen das eine (mit dem höheren R_F-Wert) kristallin erhalten wurde, ist noch nicht abgeschlossen.

DISKUSSION

Die Synthese eines ungesättigten Zuckers auf dem beschriebenen Weg ist neuartig. Sie dürfte nach folgendem Mechanismus verlaufen: es kommt zunächst durch nucleophilen Angriff des Thiocyanats zur Bildung des Salzes eines Kohlen-



säurehalbesters **9**, das sehr instabil ist und sich durch Austritt von Kohlendioxid zur Gruppierung **10** stabilisiert. Diese steht über das 1,3-Oxathiolan-2-imin-anion **11** im

Gleichgewicht mit der Gruppierung **12**, aus der durch Nachbargruppenangriff des negativ geladenen Schwefels und Eliminierung von Cyanat die Epithioverbindung **13** entsteht, die dann in eine ungesättigte Verbindung übergeht.

Der Mechanismus wird durch folgende Befunde gestützt: die Instabilität von Salzen eines Kohlensäure-kohlenhydrat-halbesters beschrieben Siegfried und Howwjanz[14]. Der Übergang **10**→**13** verläuft analog der von van Tamelen[15] untersuchten Reaktion eines Epoxids mit Kaliumthiocyanat zu einer Epithioverbindung. Diese gehen, wie Lightner und Djerassi[16] fanden, schon durch bloßes Erhitzen in ein ungesättigtes System über.

EXPERIMENTELLER TEIL

Das Einengen von Lösungen erfolgte im Rotationsverdampfer bei 40–50°. Schmelzpunkte wurden mit einem Thermopan-Heiztischmikroskop der Firma C. Reichert, Wien, bestimmt, Drehwerte mit einem Perkin-Elmer Polarimeter 141 mit einer 1 dm-Küvette. Die Aufnahme von i.r.-Spektren erfolgte mit einem Perkin-Elmer Natriumchlorid-Spektrophotometer 157; Feststoffe wurden als Kaliumbromid-Preßlinge, Flüssigkeiten und sirupöse Stoffe zwischen Natriumchloridfenstern in den Strahlengang gebracht. N.m.r.-Spektren wurden bei 100 MHz mit einem Varian HA-100 Spektrometer aufgenommen. Es wurde eine *ca.* 10-proz. Lösung in Aceton-d_6 mit Tetramethylsilan (τ 10,00) als innerem Standart verwandt; der Acetonpeak (τ 7,83) diente als Bezugspunkt. Dünnschichtchromatogramme wurden auf 0,25 mm starken Schichten von Kieselgel G, <0,08 mm (E. Merck, Darmstadt), gemacht. Die Indikation erfolgte durch Besprühen mit konz. Schwefelsäure und anschließendes Erhitzen auf 110°. Als Laufmittel dienten: A, Benzol–Äthanol–Wasser–konz. Ammoniak (200:47:15:1); B, Butanon, mit Wasser gesättigt. Für die Säulenchromatographie wurde Kieselgel, <0.08 mm, der Firma E. Merck, Darmstadt, in einem Gewichtsverhältnis von ca. 200:1 zur zu trennenden Substanz verwandt.

Methyl-α-D-mannopyranosid-2,3-carbonat (**2**) (cf. *Ref. 9*). — Methyl-α-D-mannopyranosid (4,85 g) wird in Wasser (13 ml) gelöst, dazu wird Chlorameisensäurebenzylester (25 g), der kurz vorher mit wenig festem Natriumhydrogencarbonat neutralisiert worden ist, gegeben. Nach Zufügen von Dioxan (25 ml) wird die Reaktionsmischung bei 0° heftig gerührt, während 3M Natriumhydroxid (50 ml) zutropft. Nach einer weiteren Stunde wird soviel Wasser zugegeben, bis etwa ausgefallenes Natriumsalz aufgelöst ist. Die Lösung wird mit Chloroform (4×40 ml) extrahiert, mit Natriumsulfat getrocknet und zu einem dünnen Sirup eingeengt. Die in diesem noch enthaltenen aromatischen Verunreinigungen werden bei 10^{-3} mm Hg und bis zu 210° Badtemperatur abdestilliert. Es hinterbleibt ein dunkelbrauner, in der Kälte fast glasharter Rückstand (10,56 g).

Ein Teil hiervon (3,5 g) wird in abs. Dioxan (150 ml) gelöst, mit Palladium-Aktivkohle (1 g, 10 proz., E. Merck, Darmstadt) versetzt und bei Normaldruck im Wasserstoffstrom hydriert. Das Ende der Reaktion wird dünnschichtchromatographisch

(Laufmittel A) bestimmt (Verschwinden der Ausgangsprodukte). Die Umsetzung ist im allgemeinen nach 2 Stdn. beendet.

Nach Entfernung des Katalysators wird mit Natriumsulfat getrocknet und die Lösung *in vacuo* zur Trockne eingeengt. Der Rückstand wird im Vakuumexsikkator über Schwefelsäure vollständig getrocknet und darauf mit trockenem Butanon (40 ml) 2 Stdn. lang gerührt. Dabei bleibt kristallines Methyl-α-D-mannopyranosid, das bei der Hydrierung als Nebenprodukt entsteht, weitgehend ungelöst. Es wird abfiltriert und das Filtrat bei ganz schwachem Vakuum langsam durch eine mit Butanon eingeschlämmte Schicht von Kieselgel (Dicke: 2 cm, Durchmesser: 5 cm) gesaugt und mit Butanon (150 ml) nachgewaschen. Auf diese Weise wird auch der letzte Rest Methyl-α-D-mannopyranosid abgetrennt. Das Filtrat wird *in vacuo* eingeengt bis die Abscheidung des Carbonats gerade beginnt. Sie wird bei −10° in 10 Stdn. vervollständigt. Es wird abfiltriert und mit Äther–Aceton (5:1) gewaschen; Ausb. 560 mg (31% bezogen auf Methyl-α-D-mannopyranosid). Nach nochmaliger Umkristallisation aus Äther–Aceton (3:1) stimmen Schmp., Drehwert und i.r.-Spektrum gut mit den von Hough und Priddle[9] angegebenen Werten überein.

Methyl-2,3-didesoxy-α-D-erythro-hex-2-enopyranosid (**3**). — Methyl-α-D-mannopyranosid-2,3-carbonat (**2**, 5,00 g) wird mit der molaren Menge an feingepulvertem, bei 160° *in vacuo* über Phosphorpentoxid getrocknetem Kaliumthiocyanat (2,2 g) vermischt und in einem Kolben mit aufgesetztem Kühlfinger zunächst im Heizbad für 10 Min. auf 120° erhitzt. Es setzt Gasentwicklung (Kohlendioxid) und eine Braunfärbung der Mischung ein. Die Temperatur wird im Verlauf von 30 Min. auf 160° gesteigert. Wenn die Gasentwicklung nachläßt, wird Vakuum angelegt. Es scheidet sich am Kühlfinger ein gelber, übelriechender Sirup ab. Die Sublimation wird nach Beendigung der Gasentwicklung bei 180° Badtemperatur vervollständigt. Der Sirup wird mit Aceton vom Kühlfinger gelöst, wobei Methyl-α-D-mannopyranosid (*ca.* 100 mg) auskristallisiert, das abfiltriert wird.

Das Filtrat wird zu einem zähen Sirup eingeengt (1,67 g). Das Dünnschichtchromatogramm (Laufmittel B) zeigt, daß er aus **3** als ganz überwiegendem Hauptprodukt, das sich beim Besprühen mit konz. Schwefelsäure sofort und schon in der Kälte schwarz färbt, zwei Nebenprodukten sowie einer Spur noch nicht entfernten Methyl-α-D-mannopyranosids besteht. Das Gemisch wird in wenig Butanon gelöst und an einer Kieselgelsäule mit wassergesättigtem Butanon getrennt. Isoliert werden **3** (440 mg, 12%) und die beiden Nebenprodukte (220 bzw. 225 mg). Verbindung **3** fällt als schwach gelblicher Sirup an und kristallisiert auch nach nochmaliger Reinigung durch Sublimation *in vacuo* nicht; $[\alpha]_D^{22}$ +106,8° (*c* 1, Wasser); n_D^{22} 1,4902 [Lit.[11]: $[\alpha]_D^{21}$ +102,3–102,6° (Wasser); n_D^{21} 1,4913–1,4616]; i.r.-Spektrum: 3250–3550 (OH), 2920 (CH, aliphatisch), 2870 (OMe), 1665 cm^{-1} (HC = CH, *cis*); n.m.r.-Spektrum: τ 6,52 (s, OMe), τ 5,07 (d, $J_{1,2}$ 2,4 Hz, H-1), τ 4,24 (q, $J_{2,1}$ 2,4 Hz und $J_{2,3}$ 10 Hz, H-2); τ 3,99 (d, $J_{3,2}$ 10 Hz, H-3), τ 5,76 (s, H-4). Verbindung **3** entfärbt eine acetonische Kaliumpermanganatlösung.

Anal. Ber. für $C_7H_{12}O_4$: C, 52,49; H, 7,55. Gef.: C, 50,63; H, 7,43.

Die Benzoylierung von **3** mit Benzoylchlorid in Pyridin ergibt Methyl-4,6-di-

O-benzoyl-2,3-didesoxy-α-D-*erythro*-hex-2-enopyranosid (4) in kristalliner Form (aus Äthanol). Schmp. 72–73°; $[\alpha]_D^{22}$ +187° (*c* 0,3, Chloroform). [Lit.[13]: Schmp. 74–75°; $[\alpha]_D$ +190° (*c* 1, Chloroform)].

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

SYNTHESIS OF 2-AMINO-2-DEOXY-D-MANNURONIC ACID AND SOME DERIVATIVES THEREOF*

NITYA G. KUNDU, JOHN F. CRAWFORD**, BRONISLAW PRAJSNAR†, ELIZABETH J. REED, AND SPENCER ROSENTHAL

Department of Biochemistry, Woman's Medical College of Pennsylvania, Philadelphia, Pa. 19129 (U. S. A.)

(Received May 21st, 1969; in revised form, July 30th, 1969)

ABSTRACT

2-Amino-2-deoxy-D-mannose hydrochloride was converted into the *N*-benzyloxycarbonyl derivative (**2**) by treatment with benzyl chloroformate in ethanol–water at pH 7.0. Benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-α-D-mannoside, obtained from **2** by treatment with benzyl alcohol containing 2% of hydrogen chloride, was oxidized with oxygen in the presence of a platinum catalyst to benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-α-D-mannopyranosiduronic acid (**4**). Periodate oxidation showed that **4** had a pyranoid structure. Hydrogenolysis of the uronic acid **4** in the presence of a palladium–carbon catalyst gave 2-amino-2-deoxy-D-mannuronic acid (**5**), which differed from its 2-epimer (2-amino-2-deoxy-D-glucuronic acid) in its optical rotation, and paper chromatographic and electrophoretic behavior.

INTRODUCTION

In 1954, Webster *et al.*[1] isolated, as a hydrolytic product of the Vi antigen that is a major immunogenic component of *Salmonella typhosa*, an aminohexuronic acid later shown to be 2-amino-2-deoxy-D-galacturonic acid[2]. Williamson and Zamenhof[3] reported the occurrence of 2-amino-2-deoxy-D-glucuronic acid in *Hemophilus influenzae*, Type d. Smith[4] has isolated uridine 5′-(2-acetamido-2-deoxy-D-glucosyluronic acid pyrophosphate) from *Achromobacter georgiopolitanum*, an organism containing a polysaccharide rich in 2-amino-2-deoxy-D-glucuronic acid. 2-Acetamido-2-deoxy-D-mannuronic acid (**5**) was first reported[5] to occur as one of the constituents of the D-glucose-containing polymer isolated from the cell walls of *Micrococcus lysodeikticus*. In 1964, Rosenthal and Sharon[6] isolated, from extracts of *M. lysodeikticus*, a UDP-sugar which appeared to be an *N*-acetylated amino-

*Supported by Grant AI 07211 and Career Development Award AI 13278 from the National Institutes of Health, as well as by Grant GB 6778 from the National Science Foundation to S. R. A preliminary account of part of this work was given[22].

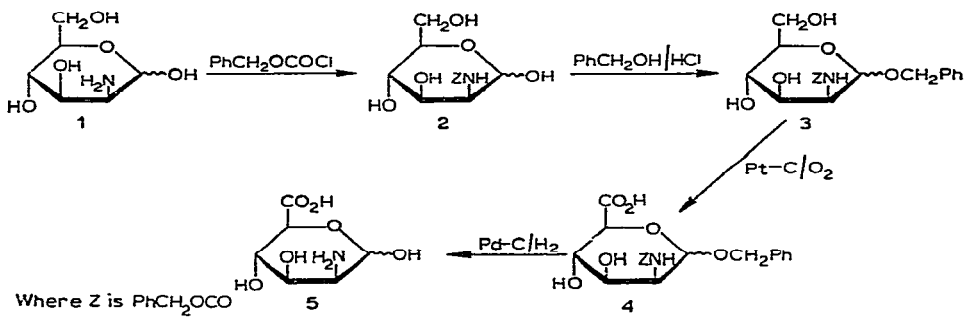
**Portions of this work will constitute fulfilment of the requirements for the degree of Master of Science.

†Present address: Politechnical Institute, Gliwicie, Poland.

hexuronic acid. In view of the likelihood that this UDP-sugar is an immediate precursor of the polysaccharide from the cell wall of *M. lysodeikticus*, it was hypothesized that the *N*-acetylated aminohexuronic acid was 2-acetamido-2-deoxy-D-mannuronic acid. Because of the possibility of isomerization of the D-*manno* acetamido derivatives into the corresponding D-*gluco* derivatives, it therefore became desirable to synthesize 2-amino-2-deoxy-D-mannuronic acid (**5**) and its derivatives, and to study their properties. Mayer[7] has obtained evidence for the occurrence of acid **5** in an antigenic polysaccharide of *Escherichia coli* K-7; this finding suggests that the occurrence of **5** in natural products may be more common than had previously been supposed.

RESULTS AND DISCUSSION

2-Amino-2-deoxy-D-glucuronic acid[8] and 2 amino-2-deoxy-D-galacturonic acid[9] have already been synthesized; synthesis of 2-amino-2-deoxy-D-mannuronic acid (**5**) from 2-amino-2-deoxy-D-mannose (**1**) is now reported.



Because of the susceptibility of the amino group in **1** to oxidation, it was protected by means of a benzyloxycarbonyl group. On attempting to synthesize 2-(benzyloxycarbonyl)amino-2-deoxy-D-mannose (**2**) from the hydrochloride of **1**, we found that **2** was formed rapidly, but that it underwent epimerization [to 2-(benzyloxycarbonyl)amino-2-deoxy-D-glucose], as demonstrated by paper chromatography. In a series of comparative experiments, we observed that: in water at room temperature, the formation of **2** proceeded slowly; in water at 55–60°, the reaction proceeded slowly (with the formation of as-yet-unidentified side-products); in aqueous *p*-dioxane, no reaction occurred; and in aqueous pyridine, larger proportions of side-products were formed. The highest yields of **2** were obtained with an excess of benzyl chloroformate in aqueous alcohol.

Compound **2** was converted into the benzyl 2-(benzyloxycarbonyl)amino-2 deoxy-D-mannosides (**3**) on heating with benzyl alcohol containing hydrogen chloride; the maximum optical rotation was observed after 2.5 h at 60°. The mixture of glycosides was not resolved by column chromatography on cellulose, but data from periodate oxidation of the transformation product **4** showed that **3** was a pyranoside, probably an anomeric mixture rich in the α-D form (compare refs. 8, 10, 11).

Compound **3** in aqueous *p*-dioxane was oxidized with oxygen in the presence of Adams' platinum catalyst at about 80°, the pH of the solution being kept at 7–8. On acidification of the alkaline solution, the acid **4** was precipitated as a solid that was recrystallized from aqueous ethanol. Acid **4** differed from its 2-epimer [benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-α-D-glucosiduronic acid] in melting point and optical rotation (See Table I). Indication that compound **4** was a D-*manno* derivative was obtained by reduction with sodium borohydride–boron trifluoride etherate[12] and subsequent hydrogenation of the product to give an aminodeoxyhexose which, on acetylation with acetic anhydride at pH 7, afforded a syrup identical in paper-chromatographic behavior with a sample of 2-acetamido-2-deoxy-D-mannose.

TABLE I

SPECIFIC ROTATIONS OF TWO 2-AMINO-2-DEOXY-D-HEXURONIC ACIDS AND DERIVATIVES THEREOF

Acid	$[\alpha]_D^{25}$	$[\alpha]_{587}^{25}$	$[\alpha]_{546}^{25}$	$[\alpha]_{436}^{25}$	$[\alpha]_{365}^{25}$
			(*degrees*)		
Benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-α-D-glucosiduronic[a]	+124.8	+130.4	+148.2	+252.2	+395.8
Benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-D-mannosiduronic[b] (4)	+45.65	+47.72	+54.50	+93.67	+151.5
2-Amino-2-deoxy-D-glucuronic[c,d]	+18.62	+19.32	+21.55	+34.48	+38.80
2-Amino-2-deoxy-D-mannuronic[e] (5)	− 9.93	−10.28	−11.99		

[a]In *p*-dioxane (*c* 0.3205). [b]In *p*-dioxane (*c* 0.3395). [c]In water (*c* 0.5800). [d]The sample was slightly impure. [e]In water (*c* 0.584).

The pyranoid structure of acid **4** was established by periodate oxidation at pH 3.5, the reaction being followed spectrophotometrically[13] at 224 nm (where the substrates and the products do not have significant absorption). The results of titrimetric determination agreed well with those obtained spectrophotometrically. From Fig. 1, it may be seen that the substituted 2-amino-2-deoxy-D-mannuronic and -galacturonic acids have almost the same rate of oxidation, whereas that for the corresponding D-glucuronic acid derivative seems to be much lower. A rate of oxidation of methyl α-D-galactopyranosiduronamide comparable to that of methyl α-D-mannopyranosiduronamide was reported by Edington, Hirst, and Percival[14]. The results can best be explained on the basis of a pyranoid structure for the benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-α-D-hexosiduronic acids, as the furanoid forms of these compounds would not be expected to react at appreciable rates with periodate. For the D-*gluco* derivatives, we found rates higher than those reported by Heyns and Paulsen[8], probably because of effects of temperature and concentration. The oxidation of these uronic acid derivatives did not cease after the uptake of one mole of periodate per mole of the compound oxidized. Extensive "over-oxidation" occurred, particularly with the D-*manno* and D-*galacto* derivatives, presumably by the well-known[15] malonaldehyde-type of route, as shown in (**4**→**6**→**7**). It may be concluded that the *N*-benzyloxycarbonyl group in the uronic acid derivatives does not undergo oxidation

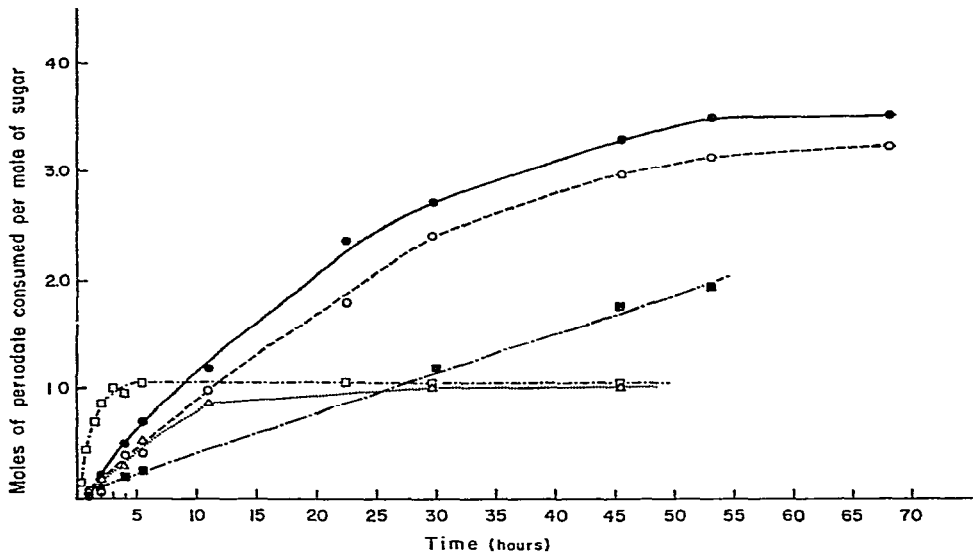
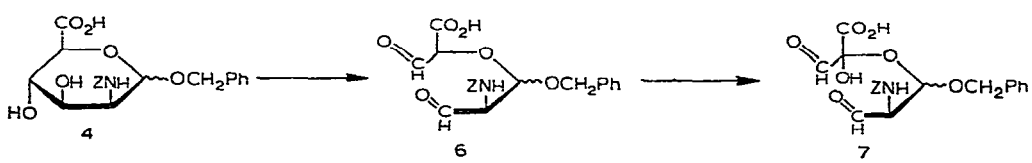


Fig. 1. Consumption of periodate, with time, by benzyl 2-(benzyloxycarbonyl) derivatives of various amino sugars. Reaction conditions, and calculations of uptake of periodate by each sugar, are described in the text. Rates of oxidation are shown for the following derivatives: □, benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-α-D-galactoside; △, benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-α-D-glucoside; ■, benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-α-D-glucosiduronic acid; ○, benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-α-D-galactosiduronic acid; and ●, benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-D-mannosiduronic acid.

by periodate, because Clamp and Hough[16] have observed that the presence of an electron-withdrawing substituent on the amino group prevents its participation in periodate oxidation; this is also apparent from the oxidation of benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-α-D-glucoside and -galactoside, which consumed only one mole of periodate per mole of the glycoside. Similar "over-oxidation" of uronic glycosides has been observed by Edington, Hirst, and Percival[14].



On hydrogenolysis in the presence of palladium–carbon, compound **4** was converted into **5**. On evaporating the aqueous solution of acid **5** at 25° and drying the residue overnight in a desiccator, an analytically pure, crystalline monohydrate of acid **5** was obtained; it gave a characteristic, brownish color with the ninhydrin reagent. The i.r. spectrum of **5** is very similar to that of 2-amino-2-deoxy-D-glucuronic acid, but the two acids are readily differentiated by their optical rotations (see Table I). On electrophoresis at pH 5.0 (molybdate buffer[17]), acid **5** exhibits a mobility of 1.20 (relative to that of 2-amino-2-deoxy-D-glucuronic acid as unity). The two aminohexuronic acids may also be separated by paper chromatography in 7:7:6

1-pentanol–pyridine–water. With 2-acetamido-2-deoxy-D-glucose as the standard, a ratio for reducing-sugar value[18] to a Morgan–Elson assay[19] of the *N*-acetylated sugar was found to be 1.73 for acid **5** in contrast to 1.04 for 2-amino-2-deoxy-D-glucuronic acid. Both aminohexuronic acids were found to be acid-labile. On treatment with hydrochloric acid, acid **5** was readily converted into a mixture of three products (as shown by paperchromatography); these may be its lactone and lactam, and the free uronic acid.

EXPERIMENTAL

General. — 2-Amino-2-deoxy-D-mannose hydrochloride was obtained from Pfanstiehl Laboratories, Inc., Waukegan, Illinois, and benzyl 2-(benzyloxycarbonyl)-amino-2-deoxy-α-D-glucosiduronic acid from Sigma Chemical Company. Benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-α-D-galactoside and benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-α-D-galactosiduronic acid were synthesized according to Heyns and Beck[9], and benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-α-D-glucoside according to Heyns and Paulsen[8]. The optical rotations were measured with a Perkin–Elmer Model 141 polarimeter by use of a quartz cell 1 dm long. All chromatography was conducted by the descending method. I.r. spectra were recorded with a Beckman IR-8 spectrometer. Low-voltage electrophoresis was performed on Whatman No. 1 paper in a Misco Model 7605 electrophoresis apparatus (Microchemical Specialties Co., Berkeley, California). Melting points are uncorrected.

2-(Benzyloxycarbonyl)amino-2-deoxy-D-mannose (**2**). — The hydrochloride of **1** had m.p. 172–173°, $[\alpha]_D^{27}$ −5.9 ±0.1° (*c* 0.76, water). To a solution of **1** (8.5 g) in water (40 ml) was slowly added during 20 min, with magnetic stirring, a solution of benzyl chloroformate (8.5 ml) in 80 ml of absolute ethanol. The pH of the mixture was monitored with a Corning pH meter, and adjusted to 7 by the dropwise addition of an aqueous solution (~19%) of sodium hydrogen carbonate. After ~30 min, the pH began to rise, and sufficient 2M hydrochloric acid was added dropwise during the next 90 min to keep the pH at ~7. The pH was then brought to 2, and the solution was evaporated to dryness *in vacuo* at 28°. The resulting, orange-yellow syrup was extracted twice with ether, and was then dried by adding and distilling off toluene at 28°, to yield 17.7 g of **2** mixed with salts. Preliminary attempts to purify **2** gave evidence (paper chromatography) for its 2-epimerization; hence, pure **2** was not isolated. When chromatographed on Whatman No. 1 paper saturated with borate[20], with 7:7:6 1-pentanol–pyridine–water for 16 h, compound **2** had a mobility of 0.79 relative to that of 2-(benzyloxycarbonyl)amino-2-deoxy-D-glucose.

2-(Benzyloxycarbonyl)amino-2-deoxy-D-glucose from the hydrochloride of **1**. — To a solution of the hydrochloride (0.5 g) of **1** in water (5 ml) were added sodium hydrogen carbonate (0.5 g), ethanol (5 ml), and benzyl chloroformate (0.5 ml), and the solution was heated at 60°. After 2 h, sodium hydrogen carbonate (0.2 g) and benzyl chloroformate (0.2 ml) were added. After a further 4 h, the almost clear solution was allowed to evaporate overnight in the air, giving a yellow resin (1.42 g) which was successively washed with chloroform (7 ml) and water (6.5 ml); the

suspension was filtered, and the colorless solid was dried to yield 0.196 g of material having m.p. 207–208°. On recrystallization from 30% methanol, it gave 2-(benzyloxycarbonyl)amino-2-deoxy-D-glucose as fine needles, m.p. 214–215°; the mixed m.p. with an authentic sample was undepressed. Its chromatographic properties on Whatman No. 1 paper saturated with borate, with 7:7:6 1-pentanol–pyridine–water, were identical with those of the D-*gluco* derivative[21]. The specific rotation of the product agreed well with that of the authentic D-*gluco* derivative.

*Benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-*D-*mannoside* (3). — Crude compound **2** (17.7 g) was added to a freshly prepared solution (2%) of hydrogen chloride in benzyl alcohol (115 ml), and the solution was stirred at 60° until the maximum optical rotation was reached (3 h). The acid was neutralized with lead carbonate, the solids were filtered off, and the filtrate was evaporated to dryness at 100° (bath)/5 torr. The syrupy residue was washed twice with 1:1 ether–petroleum ether, the resulting dark gum (7.7 g) was dissolved in hot ethyl acetate (75 ml), and the suspension was filtered. The filtrate was evaporated to dryness, and the product was kept in a vacuum desiccator for 24 h, to give 4.28 g (29.7%) of a hard, dark-brown, hygroscopic substance. Paper chromatography in 7:7:6 1-pentanol–pyridine–25mM borate showed it to be free from **1**. Compound **3** had $[\alpha]_D^{25}$ +38.7 ±0.2° (*c* 0.64, *p*-dioxane), whereas the corresponding D-*gluco* derivative has $[\alpha]_D^{25}$ +137.8 ±0.2° (*c* 0.64, *p*-dioxane).

Anal. Calc. for $C_{21}H_{25}NO_7$: C, 62.54; H, 6.20; N, 3.47. Found: C, 62.61; H, 6.25; N, 3.30.

Compound **3** (2.6 g) was chromatographed on a column (2 × 75 cm) of Whatman cellulose (60 g, 200 mesh, ashless) with 500:50:1 benzene–ethanol–water, and fractions (~2 ml each) were collected. The product was present in fractions 59 to 100. The specific rotations of various fractions were all $[\alpha]_D^{25}$ ~+29° (*c* ~0.1, absolute methanol).

*Benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-*D-*mannosiduronic acid* (4). — To a solution of compound **3** (4.65 g) in *p*-dioxane (50 ml) was added water (630 ml); platinum catalyst (2.4 g), prepared according to the procedure of Heyns and Beck[9], was added, and the pH was brought to 7 by addition of 9% aqueous sodium hydrogen carbonate. The suspension was heated to 80–85°, and oxygen was then blown into it through a sintered-glass bubbler, with vigorous mechanical stirring. During the course of the reaction, the pH was kept at 7 to 8 by occasional addition of sodium hydrogen carbonate solution (9%). After 10 h, the suspension was filtered, and the clear, yellow filtrate was concentrated to 25 ml. It was cooled, and acidified with 2M hydrochloric acid; this produced a yellow solid which was filtered off, and dried to give 3.44 g (71.5%) of **4**, m.p. 85–90°. On recrystallization from aqueous ethanol, it afforded fine, colorless needles, m.p. 160–161°, ν_{max}^{KBr} 1700 cm^{-1} (C=O). The corresponding D-*gluco* derivative melts at 188–190°. The melting point of a mixture of the two was 157–175°. The optical rotations are given in Table I.

Anal. Calc. for $C_{21}H_{23}NO_8$: C, 60.42; H, 5.55; N, 3.36. Found: C, 60.12; H, 5.39; N, 3.15.

*2-Amino-2-deoxy-*D-*mannuronic acid* (5). — Powdered compound **4** (500 mg)

was suspended in doubly distilled water (50 ml), and to the suspension was added 10% palladium-on-carbon (500 mg). The compound was hydrogenolyzed in a slow stream of hydrogen, the odor of toluene being apparent for the first 2 h. The reaction was continued for 12 h, and the flask was then flushed with nitrogen, and the mixture kept overnight at 4°. The suspension was repeatedly filtered, finally through a Millipore filter, giving a clear filtrate which was evaporated to dryness under diminished pressure below 28°, affording a gum which, on being dried in a vacuum desiccator, yielded an analytically pure, crystalline material (200 mg; 79%), m.p. 92–94° (dec.); ν_{max}^{KBr} 1600 cm^{-1} (C=O). For analysis, a sample was dried over phosphorus pentoxide under vacuum for 24 h at 25°.

Anal. Calc. for $C_6H_{11}NO_6 \cdot H_2O$: C, 34.13; H, 6.21; N, 6.63. Found: C, 34.10; H, 6.21; N, 6.33.

On electrophoresis at pH 1.85 (formic acid–acetic acid–water), the acid showed a single, positively charged spot; when stained with 0.2% ninhydrin, this gave a brownish color characteristic of an aminohexuronic acid. Electrophoresis in a sodium molybdate buffer (25 mM, pH 5.0) separated the acid **5** from 2-amino-2-deoxy-D-glucuronic acid. The mobility of acid **5** relative to that of the latter acid was 1.20. Paper chromatography for 16 h in 7:7:6 1-pentanol–pyridine–water also separated the two acids; the mobility of acid **5** relative to that of 2-amino-2-deoxy-D-glucuronic acid was 1.10. The optical rotations of the two acids are given in Table I.

Periodate oxidation. — The rates of oxidation of benzyl 2-(benzyloxycarbonyl)-amino-2-deoxy-α-D-glucosiduronic acid (m.p. 187–188°), acid **4** (m.p. 160–161°), benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-α-D-galactosiduronic acid (m.p. 225–228°), benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-α-D-galactoside (m.p. 198–199°), and benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-α-D-glucoside (m.p. 171–172°) with sodium metaperiodate were measured. The glycosides or the uronic acids (5–6.5 mg, 13–16 μmoles) were carefully weighed out and dissolved in absolute methanol (5 ml), with the exception of benzyl 2-(benzyloxycarbonyl)amino-2-deoxy-α-D-glucoside which was dissolved in 96.2% methanol (5.2 ml)owing to its low solubility in absolute methanol. The periodate solution was prepared by dissolving 800 mg (3.74 mmoles) of sodium metaperiodate and 214 μl (3.74 mmoles) of acetic acid in water, and making to 100 ml in a volumetric flask. The sugar solution (1 vol) was mixed with water (0.5 vol) and periodate solution (0.5 vol), giving a substrate concentration of 1.4–1.7 μmoles/ml, a periodate concentration of 9.4 μmoles/ml, and a methanol concentration of ~50%. The reactions were conducted in screw-capped, Pyrex test-tubes at 24° in the dark. At the elapse of various intervals of time, an aliquot of each reaction solution was taken and diluted 100-fold with 1:1 water–methanol. Absorbances of the dilutions were read at 224 nm against a blank of 1:1 water–methanol, on a Gilford spectrophotometer Model 220. An absorbance of A_{224} 0.873 corresponds to the presence of 0.1 μmole of periodate per ml of solution. The amounts of periodate consumed with time are shown in Fig. 1. A periodate blank consisting of 2:1:1 (v/v) absolute methanol–water–periodate was also kept at 24° throughout the reaction period, and its A_{224} was checked periodically. The maximum change

in A_{224} of the periodate blank was less than 10%. In the calculation of uptake of periodate by each sugar, it was therefore assumed that the error contributed by decomposition of periodate due to factors other than periodate–substrate reactions was less than 10%.

Determination of the ratio of the reducing-sugar value to that given by the Morgan–Elson assay. — The synthetic acid **5** and 2-amino-2-deoxy-D-glucuronic acid were isolated by preparative, high-voltage electrophoresis at pH 1.85 (formic acid–acetic acid buffer) on a Gilson High Voltage Electrophorator Model D, with a constant current of 170–180 mA and an applied potential of 2,500 V. The ninhydrin-positive material (brown spot) was collected by elution, and the eluates were assayed. Reducing sugar was determined by the Park–Johnson[18] method, and the Morgan–Elson[19] assay was performed on the sugars after *N*-acetylation. 2-Acetamido-2 deoxy-D-glucose was used as the standard for both of the assays. The ratio of concentration of sugar as determined by the Park–Johnson method to that determined by the Morgan–Elson assay was 1.04 for 2-amino-2-deoxy-D-glucuronic acid, and 1.73 for acid **5**.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

THE ISOLATION OF D-*manno*-HEPTULOSE, PERSEITOL, D-*glycero*-D-*manno*-OCTULOSE, AND OTHER COMPOUNDS FROM PICHI TOPS (*Fabiana imbricata* RUIZ & PAV.)

NELSON K. RICHTMYER

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014 (U. S. A.)

(Received July 30th, 1969)

ABSTRACT

An aqueous extract of Pichi tops (the dried herbage of *Fabiana imbricata*) has yielded D-*manno*-heptulose, the closely related perseitol (D-*glycero*-D-*galacto*-heptitol), and D-*glycero*-D-*manno*-octulose; D-arabinitol, D-mannitol, galactitol, *myo*-inositol, D-xylose, D-galactose, and primeverose (6-*O*-β-D-xylopyranosyl-D-glucose) were also obtained. A search for D-*erythro*-D-*galacto*-octitol in the extract was unsuccessful, as also it had earlier been in *Sedum spectabile* extracts.

INTRODUCTION

Several papers from this laboratory have, in the past dozen years, described the isolation of a number of higher-carbon sugars and polyhydric alcohols from plant sources. Most of this work, together with related studies from other laboratories, was summarized in 1966 by Begbie and Richtmyer[1] or later by the latter author[2,3].

RESULTS AND DISCUSSION

The present paper describes an investigation of the higher-carbon sugars and polyhydric alcohols of Pichi tops (the dried herbage of *Fabiana imbricata* Ruiz & Pav.; family Solanaceae). The finely ground, air-dried material was extracted with water; the extract was deproteinized, deionized, fermented with bakers' yeast, and concentrated; and a portion of the final syrup was fractionated on a cellulose column by elution with aqueous butyl alcohol. Crystalline D-*manno*-heptulose and the closely related perseitol (D-*glycero*-D-*galacto*-heptitol) were isolated in crystalline form; these occur together in the avocado, also. Syrupy D-*glycero*-D-*manno*-octulose was isolated, and characterized further through its known phenylosazone and new phenylosotriazole. The presence of other seven-, eight-, and nine-carbon ketoses seemed likely, but the tedious work that would be involved in their further separation and identification did not seem warranted in view of the earlier careful studies in this laboratory[1,4-8].

In addition to the higher-carbon sugars and polyhydric alcohols just mentioned,

the following carbohydrates were isolated during the fractionation of the Pichi extract, and identified conclusively: D-arabinitol (for the second time from a higher plant[2]), D-mannitol, galactitol, *myo*-inositol, D-xylose, D-galactose, and primeverose (6-*O*-*β*-D-xylopyranosyl-D-glucose). The disaccharide may well have been present as a result of the enzymic degradation of a primeveroside, several of which are known to occur in plants.

Because D-*glycero*-D-*manno*-octulose is accompanied by the closely related D-*erythro*-D-*galacto*-octitol in the avocado[5], a special search was made for that octitol in the Pichi extract. The search was unsuccessful, as it was, also, in *Sedum spectabile* extracts[3].

EXPERIMENTAL

General methods. — Paper chromatography was performed on Whatman No. 1 filter paper by the descending method at room temperature. The solvent system was usually 6:4:3 butyl alcohol–pyridine–water. The spray reagents used were *A*, aniline hydrogen phthalate for aldoses; *B*, orcinol–hydrochloric acid for ketoses; and *C*, ammoniacal silver nitrate for alditols and other polyhydroxy compounds in general. Melting points were determined in capillary tubes. Evaporations were conducted *in vacuo* at 45° (bath) with a rotary evaporator. I.r. spectra were determined on Nujol mulls unless otherwise specified.

Preparation of the Pichi extract. — A quantity (15.2 kg) of Pichi tops* was finely ground and divided into 200-g batches. Each batch was extracted with distilled water for 8 h on a steam bath, in the presence of calcium carbonate to prevent the development of any acidity. The mixture was allowed to cool to room temperature, mixed with Celite No. 535 (filter aid), and filtered on a large Büchner funnel precoated with Celite No. 535. The filter cake was washed with distilled water, and reextracted similarly.

The extracts were deproteinized by adding zinc sulfate, and then saturated aqueous barium hydroxide, with stirring, until the mixture was almost alkaline to phenolphthalein test-paper. Decolorizing carbon was added and, after further stirring for 3 h, the mixture was filtered. The filtrate was deionized by passing it through columns of Amberlite IR-120 and Duolite A-4 ion-exchange resins. The combined eluate and washings were evaporated, and the residue was dissolved in methanol. The methanol solutions from the various batches were subsequently combined into three portions, each of which represented 5.1 kg of the original Pichi tops. By evaporating 1 ml of the 430 ml of solution to dryness at 110° *in vacuo*, the syrup content of one portion was determined to be 284 g. From one of these portions, which had been stored for several months at +5°, was obtained 9.8 g of crude

*This material was obtained from S. B. Penick & Company, 100 Church Street, New York, New York 10007, about 1940, and was kept in storage until the writer began a study of it in 1961. Confirmatory proof of its identity was furnished at the latter time through the courtesy of Mr. Hans R. Schmidt of that Company.

crystals that, after recrystallization from aqueous methanol, were identified by m.p. and mixed m.p., as well as by paper chromatography, as *myo*-inositol.

Fermentation and fractionation of the Pichi extract. — The syrup from one of the three portions of extract, after removal of the methanol by evaporation, was dissolved in water and treated with bakers' yeast for 5 days at 37°, to remove fermentable sugars. The mixture was filtered, and the filtrate was deproteinized, deionized, and evaporated. The resulting syrup was put on the top of a column (110 × 10 cm) of cellulose, and eluted with quarter-saturated aqueous butyl alcohol. The eluate was collected in 500-ml fractions, a total of 552 fractions being collected. The eluting solvent was changed to half-saturated aqueous butyl alcohol at fraction 315. Each fraction was evaporated, and the residue was dissolved in methanol (or aqueous methanol, if necessary to effect dissolution), the solution was transferred to a test tube (100 × 25 mm), and the solvent was usually evaporated slowly at room temperature. The crystals that formed were separated with the aid of methanol, and, on the basis of their behavior on paper chromatograms, the crystals and mother liquors were separately combined. The crystals that were identified were as follows.

D-*Xylose.* Fractions 34–36 yielded 0.12 g of relatively large prisms; these were recrystallized from methanol to give small, chunky prisms having m.p., and mixed m.p. with authentic D-xylose, of 146–147°. The identification was confirmed by paper chromatography, and by comparison of i.r. spectra.

D-*Mannitol.* Fractions 52–58 yielded 32 mg of elongated prisms; upon recrystallization from methanol, the product separated as matted, small needles (23 mg) that appeared to be D-mannitol from paper-chromatographic comparison and from m.p. and mixed m.p. of 166–167°. However, the i.r. spectrum was not identical with that of the prisms of authentic D-mannitol. Accordingly, a sample of authentic, prismatic D-mannitol was dissolved in methanol, concentrated, and seeded with the recrystallized material from fractions 52–58. Matted, small needles separated, and the i.r. spectra of the two samples were identical. The dimorphous nature of D-mannitol was first reported by von Zepharovich[9] in 1888.

Galactitol. Fractions 70–80 contained a compound that, on paper chromatograms sprayed with reagent *B*, gave a greenish-blue color typical of *manno*-heptulose. The fractions were combined (methanol) and concentrated; small prisms separated spontaneously. This product was identified as galactitol by paper chromatography and by its m.p. of 184–186°, and mixed m.p. with authentic galactitol (m.p. 187–188°) of 186–187°.

D-manno-*Heptulose.* The mother liquor from the galactitol, on further concentration and standing, deposited 26 mg of chunky prisms. Paper chromatography and i.r. spectra indicated that the compound was D-*manno*-heptulose, as did also its rotation of $[\alpha]_D^{20}$ +28.4 ±1.0° (*c* 1, water; accepted value, +29.2°). The m.p. was 146–148°; mixed m.p. with authentic material (m.p. 148–149°) 147–149°.

D-*Galactose.* Fractions 81–107 showed, on paper chromatograms, the probable presence of galactose, and yielded 0.41 g of crystalline material. This material was dissolved in water, and evaporated to a syrup that was dissolved in methanol. Two

crops of unidentified crystals were filtered off, and, finally, 0.15 g of clusters of small prisms was removed. The m.p. of 166–167° and mixed m.p. of 165–167° identified this last compound as D-galactose (having m.p. 164–165°), but the i.r. spectrum was not identical with that of the authentic sugar*. Some authentic D-galactose was therefore dissolved in water, the solution was equilibrated overnight and evaporated, the syrup was dissolved in methanol, and the solution was nucleated with a specimen of the recrystallized sugar isolated from fractions 81–107. The nuclei grew, and the crystals of D-galactose thus obtained had $[\alpha]_D^{20}$ +79.4 ±0.8° (*c* 1, water; equilibrated overnight), and an i.r. spectrum identical with that of the crystalline sugar obtained by fractionation of the Pichi extract.

Primeverose (*6-O-β-D-xylopyranosyl-D-glucose*). Fractions 350–432 yielded 19.9 g (plus an additional 1.5 g from the mother liquor) of a crystalline compound that was identified as primeverose by paper chromatography: the compound had the same mobility as authentic material[1] and gave the same pinkish color (due to the presence of D-xylose in the disaccharide) as the authentic material when its paper chromatogram was sprayed with reagent *A*. A melting point of 194–197° (dec.) was recorded, with a mixed m.p. of 193–197° (dec.), and m.p. of 191–195° (dec.) for authentic primeverose[1]. When heated more rapidly, the sample melted at ~208° (dec.). The i.r. spectrum was identical with that of authentic primeverose.

myo-*Inositol.* Fractions 433–520 yielded 5.9 g of *myo*-inositol, identified readily through its crystalline form and by its m.p. 223–224° and mixed m.p. 224–225°; the authentic material had m.p. 225–226°.

Further fractionation of the "heptulose fraction"; isolation of D-*arabinitol.* — Fractions 36–119 were combined, after removal of any crystalline material, into a fraction called the "heptulose fraction" because on paper chromatograms it gave spots that showed characteristic blue or greenish-blue spots when sprayed with reagent *B*. Aldoses in this fraction (20.6 g) were first decomposed by oxidation with bromine water in the presence of barium carbonate, and 12.0 g of ketoses and polyhydric alcohols remained. This material was put on top of a column (100 × 6 cm) of cellulose, and eluted with quarter-saturated aqueous butyl alcohol in 500-ml portions. Fractions 18–19 yielded D-arabinitol (20 mg) which gave clusters of prismatic needles when recrystallized from methanol. The compound melted at 100–101°; a mixture with authentic D-arabinitol (m.p. 102–103°) melted at 101–102°; a mixture with L-arabinitol (m.p. 101–102°) melted at 80–85°; and a mixture with ribitol (m.p. 102–103°) melted at 84–88°. On paper chromatograms, the compound migrated at the same rate as D-arabinitol, but more slowly than ribitol.

Additional small quantities of galactitol, D-mannitol, and D-*manno*-heptulose were isolated from later fractions.

Fractions 18–28 contained a heptulose that migrated faster than D-*manno*-heptulose. An attempt to isolate this material was made by refractionating it on a

*[Note added in proof]. A comparison of the i.r. spectrum of the authentic sugar with the i.r. data published by R. S. Tipson and H. S. Isbell [*J. Res. Nat. Bur. Stand.*, 66A (1962) 31] indicated that our "authentic" α-D-galactose contained a small proportion of the β anomer.

column (93 × 4 cm) of Dowex 50-W X8 (Ba^{2+}) ion-exchange resin by elution with water. This new fractionation was not entirely successful, but some impure material was obtained that had the same mobility as D-*allo*-heptulose on paper chromatograms developed in three different solvent systems (6:4:3 butyl alcohol–pyridine–water, 40:11:19 butyl alcohol–ethyl alcohol–water, and 18:3:1:4 ethyl acetate–acetic acid–formic acid–water). The three solvent systems clearly differentiated the substance from sedoheptulose and sedoheptulosan, but the possibility that it was D-*talo*-heptulose was not excluded. However, since both D-*talo*-heptulose[5] and D-*allo*-heptulose[1] had already been isolated from plant extracts, further efforts to identify this second heptulose from Pichi tops were halted.

Further fractionation of the first and second "octulose fractions"; isolation of perseitol. — Fractions 120–215 (8.8 g) and 216–300 (17.3 g) from the original, cellulose-column fractionation were combined separately into two "octulose fractions", so named because on paper chromatograms they gave spots that showed characteristic red-turning-to-gray colors when sprayed with reagent *B* and then heated. These two fractions were chromatographed separately on columns (93 × 4 cm) containing Dowex 50W-X8 (Ba^{2+}) ion-exchange resin by elution with water, but separation into the expected octuloses and nonuloses was quite unsatisfactory, and the fractions were eventually recombined.

From about the middle of the refractionation of the "second octulose fraction" was obtained 30 mg of perseitol; it was identified conclusively by paper chromatography, m.p. and mixed m.p., and comparison of its i.r. spectrum with that of authentic perseitol.

Further fractionation of the recombined "octulose fractions"; isolation of D-glycero-D-manno-*octulose.* — To aid in the further study of these combined fractions, the aldoses in them were first oxidized with bromine water in the presence of calcium carbonate in the usual way. The resulting syrup (7.8 g), containing ketoses and polyhydric alcohols, was put on the top of a column (95 × 5 cm) of cellulose, and eluted with half-saturated aqueous butyl alcohol, 15-ml fractions being collected. Fractions 481–820 were combined, because they appeared to contain an octulose having the mobility of D-*glycero*-D-*manno*-octulose on paper chromatograms. This octulose fraction was refractionated on a cellulose column (95 × 5 cm) by elution with quarter-saturated aqueous butyl alcohol, 15-ml fractions being collected. Paper chromatograms sprayed with reagent *B* showed that fractions 1031–1370 contained D-*glycero*-D-*manno*-octulose as the only ketose. An i.r. spectrum made from a film of combined fractions 1051–1071 (which contained only a trace of impurities as determined by a paper chromatogram sprayed with reagent *C*) showed bands at 3340 (broad), 2930, 1645, 1417 (broad), 1098 (sh), 1053, 899, and 847 cm^{-1}. These values are in excellent agreement with those of the bands obtained similarly for D-*glycero*-D-*manno*-octulose isolated from the avocado and from *Sedum* species[5]. The specific rotation, $[\alpha]_D^{20}$ +12.7° (*c* 1, methanol), was observed on combined fractions 1071–1091 (which contained some impurities, as determined by a paper chromatogram sprayed with reagent *C*); this is of the same sign as, but of lesser

magnitude than, the values +20° and +26.5° previously reported[5,6]. G.l.c. on a column containing 2,2-dimethyl-1,3-propanediol (neopentyl glycol) succinate polyester on Gas-Chrom A, and also on a column containing neopentyl glycol sebacate polyester on Chromosorb W, confirmed the identity of the per(trimethylsilyl) derivative of this octulose from Pichi with that of authentic D-*glycero*-D-*manno*-octulose.

D-glycero-D-manno-*Octulose phenylosazone.* — This compound was described by Charlson and Richtmyer[5] as melting at 188–189° (dec.). A preparation of the phenylosazone (227 mg) from combined fractions 1101–1300 (284 mg) showed that the m.p. is close to 220° (dec.) when the capillary m.p. tube is put into the bath at 200° and heated quite rapidly (75 sec). A new examination of the three old samples confirmed this fact. The i.r. spectra (potassium bromide disk) of the octulose phenylosazones from Pichi and from *Sedum* were identical. The rotation of the new phenylosazone preparation was $[\alpha]_D^{20}$ +109.1 (20 min) → +53.8° (constant, 7 days; *c* 1, pyridine). The sign of the initial rotation, and the direction of mutarotation, are in accord with the phenylosazone rules of Mester[10].

D-glycero-D-manno-*Octulose phenylosotriazole.* — A suspension of 443 mg (1.06 mmoles) of the phenylosazone from Pichi octulose in a solution of 292 mg (1.17 mmoles) of copper(II) sulfate pentahydrate was boiled for 2 h. The mixture was processed as described for primeverulose phenylosotriazole[11], and crystals (0.153 g; 44%) separated readily as the aqueous solution was concentrated. The product was recrystallized from hot water: colorless, small needles having m.p. 189–190° and $[\alpha]_D^{20}$ −21.2° (*c* 1, pyridine). The sign of rotation is in accord with the phenylosotriazole rule of El Khadem[12].

Anal. Calc. for $C_{14}H_{19}N_3O_6$: C, 51.69; H, 5.89; N, 12.92. Found: C, 51.98; H, 5.86; N, 12.85.

A search for D-erythro-D-galacto-*octitol in the Pichi extract.* — A second portion of the Pichi extract, equivalent to 5.1 kg of Pichi tops, was heated for several days with an excess of aqueous barium hydroxide solution in a stainless-steel pot on a hot plate, to decompose all reducing sugars. The solution was made neutral to phenolphthalein with carbon dioxide, treated with Darco G-60 decolorizing carbon until the solution was practically colorless, the suspension was filtered, and the filtrate was deionized completely by passage through columns of Amberlite IR-120, Duolite A-4, and, finally, Amberlite IRA-400 (OH^-) (to remove any resistant lactones). Upon evaporation, the solution yielded 16.3 g of large, prismatic crystals that were readily identified as *myo*-inositol by m.p., mixed m.p., and paper-chromatographic comparison with authentic material.

On paper-chromatographic examination, the mother liquor showed nothing when sprayed with reagents *A* or *B*; with reagent *C*, nine or ten spots were visible, but none of these spots indicated the presence of any D-*erythro*-D-*galacto*-octitol.

ACKNOWLEDGMENTS

The author thanks Mr. Edward W. Tracy of this Laboratory of Chemistry for his assistance in grinding and extracting the Pichi tops, in deproteinizing and

concentrating the extract, in fractionating one third of that extract on a large cellulose column, and in making many of the paper-chromatographic examinations. The author thanks Dr. William C. Alford and his associates of the Section on Microanalytical Services and Instrumentation of this Laboratory for obtaining the elemental analyses, the i.r. spectral comparisons, and some of the optical rotations. The author also thanks Dr. Ingvar Johansson of this Laboratory for performing the gas–liquid chromatography.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

SYNTHESIS OF DERIVATIVES OF 2,3-DIAMINO-2,3-DIDEOXY-α-D-GLUCOSE AND 2,3,4-TRIAMINO-2,3,4-TRIDEOXY-α-D-GLUCOSE*†

HANS H. BAER AND FAROUK RAJABALEE
Department of Chemistry, University of Ottawa, Ottawa (Canada)

(Received June 9th, 1969; in revised form, July 30th, 1969)

ABSTRACT

This work describes the synthesis of 2,3-diamino-2,3-dideoxy- and 2,3,4-triamino-2,3,4-trideoxy-D-glucose derivatives from α-D-glycosidic 3-deoxy-3-nitro sugars. The reaction of ammonia with methyl 4,6-*O*-benzylidene-2,3-dideoxy-3-nitro-α-D-*erythro*-hex-2-enopyranoside (**1**) or with methyl 2-*O*-acetyl-4,6-*O*-benzylidene-3-deoxy-3-nitro-α-D-glucopyranoside (**2**) furnished methyl 2-amino-4,6-*O*-benzylidene-2,3-dideoxy-3-nitro-α-D-glucopyranoside (**3**) in high yield. From **2**, a small proportion of an isomeric amine, possibly having the D-*manno* configuration, arose as a by-product. The *N*-acetyl derivative **4** of **3** was debenzylidenated to give methyl 2-acetamido-2,3-dideoxy-3-nitro-α-D-glucopyranoside (**8**). Compound **8** was converted by catalytic hydrogenation and subsequent acetylation and hydrolysis into 2,3-diamino-2,3-dideoxy-α-D-glucose dihydrochloride (**13**) and the α-D-glycosidic derivatives **10**, **11**, and **12**. The 4,6-diacetate **9** of **8** reacted with ammonia at room temperature to give methyl 2,4-diacetamido-2,3,4-trideoxy-3-nitro-α-D-glucopyranoside (**15**) in 57% yield. Acetylation of **15** gave its 6-acetate **16**, whereas catalytic hydrogenation furnished methyl 2,4-diacetamido-3-amino-2,3,4-trideoxy-α-D-glucopyranoside hydrochloride (**17**), from which the corresponding 2,3,4-triacetamido compound **18** and its 6-acetate **19** were prepared.

INTRODUCTION

Some diamino sugars have been found to be components of antibiotics[2] and bacterial cell-walls[3], and a large number have been synthesized. In addition to the structural types enumerated earlier[4], glycosidic derivatives of 3,6-diamino-3,6-dideoxy-L-talose[5] and 2,3-diamino-2,3-dideoxy-D-galactose[6], and also the free 2,3-diamino-2,3-dideoxy-D-mannose dihydrochloride[7], have been prepared more recently. By contrast, triamino sugars have not been encountered in Nature to date. However, the chemical synthesis of such compounds deserves attention, in view of their potential biological activities and in anticipation of their eventual discovery. Substituted 2,3,6-triamino derivatives of β-D-allopyranose[8] and α-D-glucopyranose[9]

*Reactions of Nitro Sugars XVI. For parts XV and XIV see ref. 1 and ref. 7, respectively.
†Part of the Ph. D. thesis of Farouk Rajabalee, University of Ottawa, 1969.

and substituted 2,3,5-triamino derivatives of β-D-arabinofuranose[10] and β-D-xylofuranose[10], were synthesized in reaction sequences involving nucleophilic displacements by azide ion and subsequent hydrogenation of the azido sugars. 2,3,4-Triamino-1,6-anhydro-2,3,4-trideoxy-β-D-idopyranose was obtained[11] from a nitromethane cyclization[12] of periodate-oxidized 1,6-anhydro-β-D-glucopyranose that was performed in the presence of benzylamine and was followed by catalytic hydrogenation.

The activating effect of the nitro group in certain aliphatic nitro compounds permits a facile introduction of nucleophiles at neighboring carbon atoms[12]. This method has been widely utilized for aminations of alditols[13] and glycosides[1,4,6,7,14–18] possessing an α-nitroalkene or a β-acetoxynitro grouping. Recently, it was shown[19] that, in β,β′-nitrodiol diacetates, the two acetoxy groups flanking the nitro group may be replaced with amino groups by the action of ammonia, in a single preparative step. This method of diamination was applied[20] to the synthesis of methyl 2,3,4-triacetamido-2,3,4-trideoxy-β-D-glucopyranoside*. We now report a sequence of reactions that includes the *stepwise* introduction of two amino groups into a nitro sugar. In the α-glycosidic series, the sequence parallels, in part, the synthesis of 2,3-diamino-2,3-dideoxy-D-glucose that has already been accomplished[4] in the β-anomeric series, and in addition, it has led to derivatives of methyl 2,3,4-triamino-2,3,4-trideoxy-α-D-glucopyranoside.

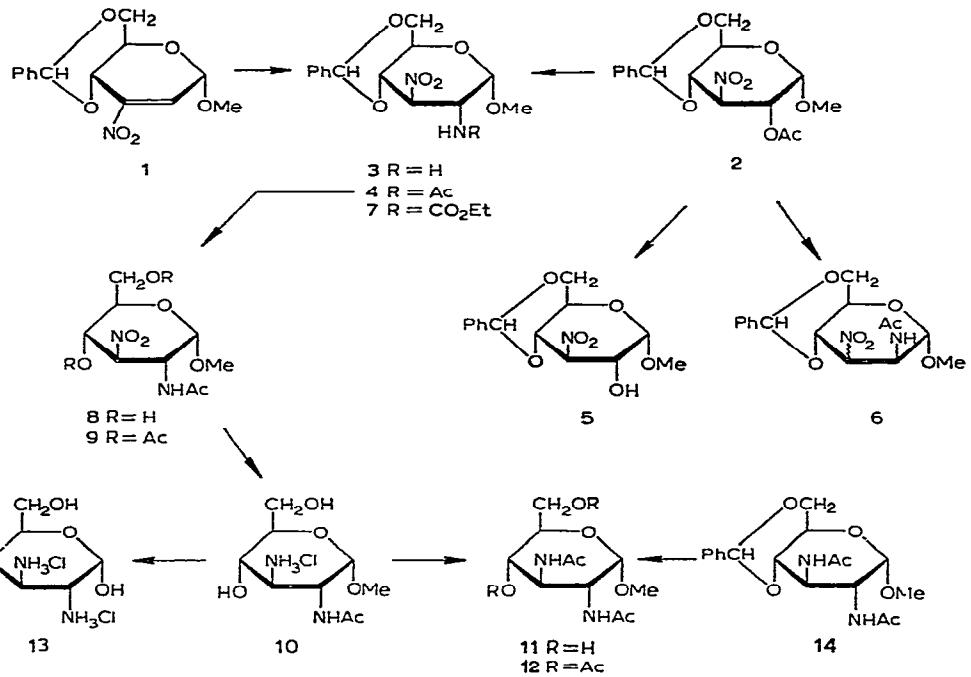
RESULTS

Methyl 4,6-*O*-benzylidene-2,3-dideoxy-3-nitro-α-D-*erythro*-hex-2-enopyranoside (**1**) readily added ammonia to furnish methyl 2-amino-4,6-*O*-benzylidene-2,3-dideoxy-3-nitro-α-D-glucopyranoside (**3**) in yields of 81–95% (Scheme I). The amine **3** was also produced, in equally good yield, from methyl 2-*O*-acetyl-4,6-*O*-benzylidene-3-deoxy-3-nitro-α-D-glucopyranoside (**2**), the more easily accessible, preparative precursor of **1**. However, the crude amine produced from **2** contained small amounts of two by-products. The removal of these was possible by column chromatography but was not essential at that stage since a satisfactory separation could be achieved following *N*-acetylation of the crude mixture. Upon *N*-acetylation, pure **3** gave methyl 2-acetamido-4,6-*O*-benzylidene-3-deoxy-3-nitro-α-D-glucopyranoside (**4**) in 90% yield, whereas the mixture obtained from **2** gave **4** in 76% yield. At this point, one of the by-products was isolated in 3% yield and identified as the known methyl 4,6-*O*-benzylidene-3-deoxy-3-nitro-α-D-glucopyranoside (**5**). The other by-product was obtained in 5% yield and proved to be an acetamido glycoside (**6**) isomeric with **4**. The configuration of **6** has not been completely established. The n.m.r. spectrum in chloroform-*d* showed an H-1 doublet having a very small splitting (*ca.* 1 Hz), in line with the 1,2-diequatorial orientation of protons, and it exhibited a 3-proton singlet at τ 7.96, indicative[22–25] of an axial acetamido group. These spectral features would agree with **6**

*Monoaminations[15] and diaminations[21] of nitro compounds possessing *unprotected*, vicinal hydroxyl groups have also been performed, by use of a number of primary and secondary amines, but not with ammonia.

having the α-D-*manno* configuration. The minor isomer found[4] in the analogous reaction in the β-series has recently been shown[7] to possess the β-D-*manno* configuration.

Scheme I



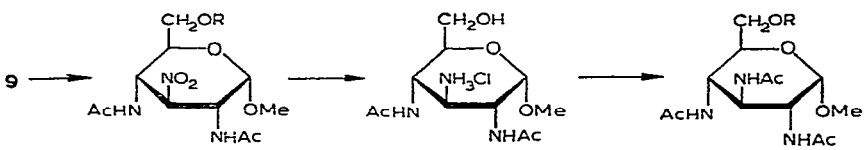
For the formation of the nitro alcohol **5**, two explanations could be considered: (*a*) ammonolysis of the 2-*O*-acetyl group in **2**, competing with β-elimination; and (*b*) hydration, in competition with amination, of intermediate **1** that arises from **2**. The fact that **5** was not observed as a product of the amination of **1** supports the former explanation. In analogous work[4], with the β-anomer of **2**, no product of such an ammonolysis was found. It appears possible, therefore, that in the α-D-glycoside (**2**) the first step required for elimination of acetate, namely axial abstraction of a proton from C-3, is slightly retarded relative to the β-D-glycoside, allowing competitive *O*-acyl fission to take place to a noticeable (yet small) extent.

The subsequent reaction steps depicted in Scheme I were performed by standard procedures and followed the examples elaborated[4] in the β-series. The new α-D-glycosides (**7**–**12**) were obtained crystalline and in good yields. Hydrolysis of compound **10** with hydrochloric acid furnished the known[4,7,8], crystalline 2,3-diamino-2,3-dideoxy-α-D-glucose dihydrochloride (**13**) in 30% yield. Although this hydrolysis proceeds less satisfactorily than the similar hydrolysis in the β-series[4], and although the starting compound **2** is prepared with some difficulty as compared to its anomer, the synthesis of **13** described here has the economic advantage of being based, ultimately, on inexpensive methyl α-D-glucopyranoside, which may more than compensate for a lower over-all yield.

An independent proof for the structure and configuration of compounds **3**, **4** and **7–13** was forthcoming when the diacetamido glycoside **11** was found to be identical with a sample produced by acid-catalyzed debenzylidenation of methyl 2,3-diacetamido-4,6-*O*-benzylidene-2,3-dideoxy-α-D-glucopyranoside (**14**). The acetal **14** had been synthesized by an independent route by Ali and Richardson[26], to whom we are obliged for kindly providing a sample. Compound **11** also proved identical with a product obtained[1] in a sequence of reactions that commenced with the addition of anthranilic acid to **1**, and it thus constituted a link with, and served to establish the configuration of, a series of compounds synthesized previously[1].

The starting point for the introduction of a third nitrogen function was methyl 2-acetamido-4,6-di-*O*-acetyl-2,3-dideoxy-3-nitro-α-D-glucopyranoside (**9**) (Scheme II). Treatment of **9** with ammonia in tetrahydrofuran at room temperature gave methyl 2,4-diacetamido-2,3,4-trideoxy-3-nitro-α-D-glucopyranoside (**15**) in 57% yield. Evidently the amino group that entered at C-4 by an elimination–addition process was immediately acetylated by way of acyl migration from the primary, 6-acetoxy group. Many analogous acyl migrations have been observed previously in aminations of acetates of nitro sugars[12]; however, intermolecular transacylation may be a contributing factor. Compound **15** gave its 6-acetate **16** by boron trifluoride-catalyzed acetylation, whereas platinum-catalyzed hydrogenation in the presence of hydrochloric acid afforded methyl 2,4-diacetamido-3-amino-2,3,4-trideoxy-α-D-glucopyranoside hydrochloride (**17**) in 94% yield. Selective *N*-acetylation and full acetylation, respectively, of **17** provided methyl 2,3,4-triacetamido-2,3,4-trideoxy-α-D-glucopyranoside (**18**) and its 6-acetate **19**.

Scheme II



15 R=H
16 R=Ac

17

18 R=H
19 R=Ac

It could be assumed as fairly probable that the reaction between ammonia and **9** would lead to a product having at C-3 and C-4 the more stable, equatorial disposition of substituents (the D-*gluco* configuration). This configuration was ascertained by n.m.r. spectroscopy (100 MHz) of the diacetamidonitro (**15**) and triacetamido-6-*O*-acetyl (**19**) derivatives. The acetamido methyl protons of **15** in methyl sulfoxide-d_6 resonated at τ 8.22 and 8.23, within the range (τ 8.20–8.30) reported for equatorial orientation in a large number of acetylated inosamines[23b] and amino pyranosides[23c] in that solvent. (The range recorded[22b,c] for axial acetamido groups in Me_2SO-d_6 solution is τ 8.07–8.14). While most of the ring-proton signals of **15** were difficult to analyze, that which occurred at lowest field (τ 5.07) was a clearly resolved, symmetrical triplet assignable to H-3. Its splitting ($J_{2,3} = J_{3,4} =$

11.3 Hz) required axial arrangement of H-2, H-3, and H-4, and thus confirmed the *gluco* configuration. The anomeric proton gave a doublet at τ 5.30 ($J_{1,2}$ = 3.5 Hz). Compound **19** in methyl sulfoxide-d_6 exhibited a six-proton singlet at τ 8.27 and a three-proton singlet at τ 8.20 for the three acetamido groups, and a three-proton singlet at τ 8.00 for the acetoxy group. The ring-proton signal at lowest field was the anomeric doublet at τ 5.34 ($J_{1,2}$ = 3 Hz). In methanol solution the acetoxy group of **19** resonated at τ 7.97, and the acetamido groups resonated at τ 8.08 (3H) and 8.12 (6H), in the range generally accepted[22-25] for equatorial orientation in chloroform solution*.

EXPERIMENTAL

General methods. — Melting points were determined in capillaries in an electrically heated, aluminum block equipped with a calibrated thermometer. Optical rotations were measured at room temperature in a Perkin–Elmer Model 141, automatic polarimeter. Unless otherwise specified, i.r. spectra were obtained from Nujol mulls with a Beckman IR-8 spectrophotometer. The i.r. spectra of all new compounds were consistent with the structures assigned; individual bands are listed only where it is deemed especially helpful for purposes of identification or differentiation. Evaporations were performed *in vacuo* at 35–40°. Petroleum ether refers to the fraction of boiling range 30–60°. T.l.c. was performed on silica gel G (E. Merck AG, Darmstadt, Germany). Paper chromatography was performed by the descending technique on Whatman No. 1 paper with the solvent system described by Fischer and Dörfel[27]. R_{GN} means mobility relative to 2-amino-2-deoxy-D-glucose hydrochloride. Spots were indicated by ninhydrin.

Methyl 2-amino-4,6-O-benzylidene-2,3-dideoxy-3-nitro-α-D-glucopyranoside (**3**). — *A. From nitroolefin* **1**. Aqueous 15M ammonia (40 ml) was added to a solution of the nitroolefin[28,1] **1** (765 mg) in benzene (40 ml), and the mixture was gently refluxed for 1 h with magnetic stirring. Inspection by t.l.c. with 1:1 chloroform–ethyl acetate showed that the spot of **1** (R_F 0.9) was replaced by a new spot (R_F 0.4). The mixture was then evaporated to dryness, and the white solid was triturated with water which was then evaporated off. After washing with cold water and drying, the crude product (771 mg, 95%) melted at 168–170°. Recrystallization from ethanol gave **3** as shiny needles (697 mg), m.p. 170–171°, $[\alpha]_D$ +93.3° (*c* 0.9, *N*,*N*-dimethylformamide); i.r. data, 3420, 3350, and 1600 (all weak; NH), 1550 (strong, NO_2), 762 and 702 cm^{-1} (C_6H_5).

Anal. Calc. for $C_{14}H_{18}N_2O_6$ (310.3): C, 54.19; H, 5.85; N, 9.03. Found: C, 54.05; H, 6.08; N, 9.17.

When the reaction was performed at 23°, the yield of **3** was 81%.

B. From acetate **2**. The 2-*O*-acetyl derivative[28] **2** (1.0 g) in tetrahydrofuran

*According to detailed studies by Hasegawa and Sable[24], and in our own limited experience[7,19], methanol and chloroform appear to be comparable solvents as far as chemical shifts of acetyl resonances are concerned.

(30 ml) and aqueous 15M ammonia (30 ml) was briskly stirred for 1 h at 25°. T.l.c. with 3:2 benzene–ethyl acetate indicated the conversion of **2** (R_F 0.9) into **3** (R_F 0.25, chief spot) but also revealed the formation of additional, minor products. Evaporation of the solvents and thorough washing of the residue with water gave crude **3** (803 mg), which was not chromatographically homogeneous. Purification by recrystallization from various solvents was unsatisfactory; in one experiment, pure **3** crystallized in about 50% yield from hot ethanol after seeding and slow cooling, but in a similar run this procedure failed. Eventually, purification of a typical, crude mixture (815 mg) was achieved by passage through a column (2 × 45 cm) of silicic acid (Mallinckrodt, 100 mesh) and elution with benzene–ethyl acetate mixtures. The combined fractions of pure **3** (700 mg) were crystallized from ethanol, and the material was found to be identical with that from procedure *A*.

Methyl 2-acetamido-4,6-O-benzylidene-2,3-dideoxy-3-nitro-α-D-glucopyranoside (**4**). — *A. From pure* **3**. To an ice-cooled solution of **3** (100 mg) in tetrahydrofuran (10 ml) containing a few drops of water was added acetic anhydride (5 ml). The mixture was kept for 2 h at room temperature and was then partially evaporated, whereby **4** began to precipitate. The precipitation was completed by stirring with ice–water. The white solid was filtered off, washed well with water, and dried. The material (102 mg, 90%), which was homogeneous by t.l.c., was recrystallized from ethyl acetate to give beautiful needles, m.p. 277–278° (with partial sublimation from 240°), $[\alpha]_D$ +88° (*c* 0.8, *N*,*N*-dimethylformamide); i.r. data, 3300 (strong, NH), 1660 (amide I), 1555–1540 (amide II, NO_2), 753 and 700 cm^{-1} (C_6H_5).

Anal. Calc. for $C_{16}H_{20}N_2O_7$ (352.3): C, 54.54; H, 5.72; N, 7.95. Found: C, 54.72; H, 5.88; N, 7.85.

B. From crude **3**. Crude **3** as obtained from the 2-*O*-acetyl derivative (**2**) (800 mg) was acetylated as just described for the pure compound. On t.l.c. in 2:3 chloroform–ethyl acetate, the acetylated material showed a main spot corresponding to **4** (R_F 0.35) and minor spots having R_F 0.9 and 0.25. Recrystallization from ethyl acetate afforded pure **4** (557 mg) in 3 crops, and another small crop (48 mg) was obtained on subsequent chromatography of the mother liquors of the recrystallization (see *C*). The total yield of **4**, based on **2**, was 76%. The product was identical with that from *A* according to t.l.c., i.r. spectrum, $[\alpha]_D$-value, and mixed melting-point.

C. Isolation of by-products **5** *and* **6**. The material contained in the ethyl acetate mother liquors (see *B*) was chromatographed on silicic acid (Mallinckrodt, 100 mesh) with benzene–ethyl acetate mixtures as eluants. The fractions were inspected by t.l.c., appropriately pooled, and evaporated. The fast-moving substance (R_F 0.9) crystallized from chloroform–pentane as oblong platelets (22 mg), m.p. 154–155°, $[\alpha]_D$ +95.3° (*c* 0.7, chloroform). The i.r. spectrum suggested identity of the product with the known **5**. Although reported[29] data were m.p. 167° (for crystals from aqueous ethanol) and $[\alpha]_D$ +87.2° (*c* 1, ethanol), a newly prepared sample, which was crystallized from chloroform–pentane, melted at 154–155° (undepressed in admixture with the above by-product) and showed $[\alpha]_D$ +95.5° (*c* 1, chloroform).

The fractions containing the slow-moving substance (R_F 0.25) yielded a syrup

that crystallized from chloroform-pentane as hexagonal plates (40 mg), m.p. 123–125° (with foaming), $[\alpha]_D$ +16° (*c* 0.8, chloroform). The product was the isomeric methyl 2-acetamido-4,6-*O*-benzylidene-2,3-dideoxy-3-nitro-α-D-hexopyranoside (**6**). The i.r. spectrum resembled that of **4** but could be differentiated by three moderately intense peaks at 3315, 3260, and 3200 cm^{-1} as well as by moderate peaks at 1075 and 900 cm^{-1} that were absent from the spectrum of **4**, and by broadening of the 755 cm^{-1} peak. The crystals contained chloroform of crystallization, which was not removed on drying *in vacuo* at 25°. This was revealed by low carbon and hydrogen values (Found: C, 50.60; H, 5.26) and by a mass spectrum showing $CHCl_2^+$ fragments at *m/e* 83, 85, and 87 in an intensity ratio 9:6:1. The chloroform was removed only when a sample was heated for 5 days at 100° and 1 torr. The sample then melted at 186–187° and was free from Cl (mass spectrum).

Anal. Calc. for $C_{16}H_{20}N_2O_7$ (352.3): C, 54.54; H, 5.72; N, 7.95. Found: C, 54.73; H, 5.50; N, 8.13.

Methyl 4,6-O-benzylidene-2,3-dideoxy-2-ethoxycarbonamido-3-nitro-α-D-glucopyranoside (**7**). — The amino derivative **3** (650 mg) was dissolved in pyridine (20 ml), and ethyl chloroformate (7 ml) was added dropwise at 0°. The reaction mixture, which gradually turned purple and then yellow, was kept overnight at room temperature and was then stirred with excess ice–water. A copious, white precipitate formed and was isolated, washed thoroughly with water, and dried (647 mg, 81%). The product (m.p. 209–211°) was recrystallized from ethanol to give fine needles (576 mg), m.p. 213–214°, $[\alpha]_D$ +83.5° (*c* 1.5, *N,N*-dimethylformamide); i.r. data, 3325 (NH), 1695 (C=O), 1550–1540 (NO_2 and amide II), 755 and 703 cm^{-1} (C_6H_5).

Anal. Calc. for $C_{17}H_{22}N_2O_8$ (382.4): C, 53.40; H, 5.80; N, 7.33. Found: C, 53.21; H, 5.90; N, 7.31.

Methyl 2-acetamido-2,3-dideoxy-3-nitro-α-D-glucopyranoside (**8**). — Compound **4** (273 mg) was debenzylidenated with 70% acetic acid (30 ml) on a steam bath (45 min). After work-up[1,7], which included treatment of an ethyl acetate solution of the yellowish, syrupy product with charcoal, the acetamido derivative (**8**) was obtained as colorless prisms (175 mg, 86%) from ethyl acetate–petroleum ether, m.p. 214° (dec.), $[\alpha]_D$ +115° (*c* 2.0, water); i.r. data, 3500–3200 (broad, with ν_{max} at 3420 and 3320, OH, NH), 1650 (amide I), 1555–1530 cm^{-1} (NO_2, amide II).

Debenzylidenation with 90% trifluoroacetic acid for 40 min at room temperature[30] also gave **8** in good yield.

Anal. Calc. for $C_9H_{16}N_2O_7$ (264.2): C, 40.91; H, 6.10; N, 10.60. Found: C, 41.04; H, 6.05; N, 10.46.

Methyl 2-acetamido-4,6-di-O-acetyl-2,3-dideoxy-3-nitro-α-D-glucopyranoside (**9**). — The compound **8** (740 mg) was suspended in acetic anhydride (11 ml), and boron trifluoride etherate (5 drops) was added cautiously. Dissolution of **8** began quickly and was completed by gentle warming. After 1 h at room temperature, the mixture was poured into, and evaporated from, a large excess of ethanol. The amber residue was evaporated 4 times with added ethanol, decolorized in ethyl acetate solution with activated charcoal, and finally recrystallized from that solvent and petroleum ether;

yield 779 mg (80%) of colorless needles (**9**), m.p. 177–178°, $[\alpha]_D$ +101.4° (*c* 1, chloroform); i.r. data, 3340 (NH), 1735–1745 (ester C=O), 1650 (amide I), 1552 (NO_2), and 1530 cm^{-1} (amide II).

Anal. Calc. for $C_{13}H_{20}N_2O_9$ (348.3): C, 44.83; H, 5.79; N, 8.05. Found: C, 44.68; H, 5.74; N, 8.20.

*Methyl 2-acetamido-3-amino-2,3-dideoxy-α-*D*-glucopyranoside hydrochloride* (**10**). — The nitro compound **8** (893 mg) in water (75 ml) was hydrogenated at ambient temperature and pressure with a platinum catalyst (350 mg of PtO_2, prehydrogenated in 35 ml of 0.1M HCl). Although nearly 3 moles/mole of H_2 had been consumed within 10 h, the hydrogenation was continued for another 12 h. The product **10** crystallized readily from ethyl acetate–petroleum ether as colorless needles (880 mg, 96%), m.p. 257–258° (dec.), $[\alpha]_D$ +101.2° (*c* 1.1, methanol), R_{GN} 1.50; i.r. data, 3420, 3320, and 3230 (OH, NH), 2800–2400 (weak), and 1585, 1520 (moderate, $NH_3^+Cl^-$), 1658 and 1542 cm^{-1} (amide I and II).

Anal. Calc. for $C_9H_{19}ClN_2O_5$ (270.7): C, 39.93; H, 7.07; Cl, 13.10. Found: C, 40.03; H, 7.26; Cl, 13.02.

*Methyl 2,3-diacetamido-2,3-dideoxy-α-*D*-glucopyranoside* (**11**). — The hydrochloride **10** (310 mg) was *N*-acetylated (2.5 h at 0°) with acetic anhydride (0.7 ml) in water (15 ml) containing methanol (1 ml), in the presence of Dowex-1 X2 (CO^{2-}_3) (6 ml). The product (**11**), which crystallized from a small amount of 95% ethanol upon careful addition of excess ethyl acetate, formed microscopic needles (260 mg, 82%), m.p. 245–246° (dec.), $[\alpha]_D$ +60.0° (*c* 0.8, methanol).

Anal. Calc. for $C_{11}H_{20}N_2O_6$ (276.3): C, 47.82; H, 7.30; N, 10.14. Found: C, 47.64; H, 7.46; N, 10.02.

A sample was recrystallized from hot, absolute ethanol and an approximately equal amount of ethyl acetate. It then decomposed at 265–267° with partial sublimation near 260°. On reheating, the sublimate melted without decomposition at 266–267°. Prominent i.r. maxima were at 3460, 3425, 3290, 3120, 1645, and 1560–1545 cm^{-1} as well as in the fingerprint region.

A few milligrams of the benzylidene derivative[26] **14** was dissolved in 0.5 ml of 90% trifluoroacetic acid[30]. After 30 min the solution was evaporated with several additions of water and then with ethanol, and the residue obtained was recrystallized from absolute ethanol–ethyl acetate. The colorless crystals gave an i.r. spectrum superposable on that of **11**, and they behaved alike on melting, alone or in admixture with **11**. The two samples were indistinguishable by t.l.c.

*Methyl 2,3-diacetamido-4,6-di-*O*-acetyl-2,3-dideoxy-α-*D*-glucopyranoside* (**12**). — The hydrochloride **10** (79 mg) and anhydrous sodium acetate were heated for 30 min in refluxing acetic anhydride (5 ml). The anhydride was decomposed with ice–water and the sugar acetate was extracted into chloroform. The dried (Na_2SO_4) extract was evaporated to give a residue from which added ethanol was evaporated 4 times. Compound **12** was obtained as a white solid (95 mg, 90%), m.p. 275–276° (dec.), $[\alpha]_D$ +73.5° (*c* 0.9, chloroform); i.r. data, 3315 (NH), 1743 (ester C=O), 1650 (amide I), and 1548 cm^{-1} (amide II). The substance was difficult to recrystallize because it

tended to form gels with a number of solvents tried, but it nevertheless showed correct analytical data.

Anal. Calc. for $C_{15}H_{24}N_2O_8$ (360.4): C, 49.99; H, 6.71; N, 7.77. Found: C, 50.05; H, 6.57; N, 7.63.

2,3-Diamino-2,3-dideoxy-α-D-glucose dihydrochloride (**13**). — The glycoside hydrochloride **10** (222 mg) was hydrolyzed by gentle refluxing in half-concentrated hydrochloric acid (30 ml) for 18 h. The acid was removed by coevaporation (10 times) with added water. The brown syrup obtained was decolorized in water solution with activated charcoal that had been carefully freed from acid-soluble, mineral contaminants, and the dihydrochloride **13** was then crystallized from water and acetic acid as described[4]. The yield was 62 mg (30%) of rhombohedral crystals that were dried *in vacuo* over potassium hydroxide. The compound decomposed gradually between 180 and 188° and exhibited mutarotation, $[\alpha]_D$ +62 (initial, extrapolated) → +55.0 (13 min) → +49.7° (2 h, final) (*c* 1.1, water), in excellent agreement with reported[4] values. Comparison of i.r. spectra and chromatographic mobility (R_{GN} 0.83) of **13** and an authentic sample* confirmed the identity.

Methyl 2,4-diacetamido-2,3,4-trideoxy-3-nitro-α-D-glucopyranoside (**15**). — A solution of compound **9** (2.0 g) in tetrahydrofuran (65 ml) was stirred briskly at room temperature with aqueous, 15M ammonium hydroxide (39 ml). The reaction was complete after 25 min (as indicated, in a pilot run, by t.l.c. with 1:1 chloroform–ethyl acetate, showing the disappearance of (migrating) **9** and the formation of non-migrating product). The reaction mixture was evaporated to give a yellow, semi-solid residue. The latter was triturated with absolute ethanol whereby white, crystalline **15** (805 mg) could be isolated. The ethanolic mother liquor, upon concentration and storage for 2 days at 5°, deposited a second crop (200 mg) of **15** (total yield, 57%). The crude material melted at 285–286° (dec.) and gave a single spot on t.l.c. with 1:1 chloroform–methanol. In a similar experiment, acetic anhydride (2 ml) was added to the ethanol used for work-up, and the total yield of **15** was then 65%. An analytical sample was recrystallized from ethanol; it had m.p. 289–290° (dec.), $[\alpha]_D$ +102° (*c* 0.8, methanol); i.r. data, broad absorption in the OH and NH stretching region, ν_{max} at 3410, 3345, and 3315, 1660 (amide I), 1552 (NO_2), and 1550–1530 cm^{-1} (amide II).

Anal. Calc. for $C_{11}H_{19}N_3O_7$ (305.3): C, 43.27; H, 6.27; N, 13.76. Found: C, 43.44; H, 6.17; N, 13.92.

T.l.c. of the mother liquor showed **15** and a second major spot (which moved more slowly), plus a minor, very slow-moving spot (in 1:1 chloroform–methanol).

Methyl 2,4-diacetamido-6-O-acetyl-2,3,4-trideoxy-3-nitro-α-D-glucopyranoside (**16**). — To a suspension of **15** (600 mg) in acetic anhydride (9 ml) was added boron trifluoride etherate (5 drops), with moderate cooling. The resulting solution was kept for 1 h at 25°, poured into excess, cold ethanol, and evaporated, with several

*The compound had first been synthesized by Dr. W. Meyer zu Reckendorf[8], who kindly provided us with a sample. His substance and the product of our own previous synthesis[4] had already proved identical.

subsequent additions of ethanol. The almost colorless syrup crystallized from absolute ethanol upon dropwise addition of petroleum ether. The yield of **16** was 545 mg (80%), m.p. 248–249° (dec.), $[\alpha]_D$ +90.6° (*c* 0.6, methanol); i.r. data, 3345 and 3310 (NH), 1735 (ester C=O), 1670–1650 (amide I), 1555 (NO_2), and 1530 cm^{-1} (amide II).

Anal. Calc. for $C_{13}H_{21}N_3O_8$ (347.3): C, 44.95; H, 6.09; N, 12.10. Found: C, 44.90; H, 6.07; N, 11.93.

*Methyl 2,4-diacetamido-3-amino-2,3,4-trideoxy-α-*D*-glucopyranoside hydrochloride* (**17**). — A solution of **15** (470 mg) in water (40 ml) was acidified with M HCl (2.6 ml) and hydrogenated at ordinary temperature and pressure with platinum catalyst (300 mg PtO_2, prehydrogenated). The uptake of H_2 was slow but steady, 48 h being required for 3 moles/mole. The filtrate was evaporated, and water was added thrice to the residue and evaporated again. Dissolution of the colorless, glassy material in boiling absolute ethanol and cautious addition of ethyl acetate produced **17** (450 mg, 94%) as beautiful needles, m.p. 234–235° (dec.), $[\alpha]_D$ +101.5° (*c* 1.1, water), R_{GN} 1.35 (single spot); i.r. data, 3500–3200, with ν_{max} at 3280 (OH, NH), 3080, 2700–2500, 1588 and 1510 (all weak; $NH_3^+Cl^-$), 1658 (amide I), 1550 cm^{-1} (amide II). In addition, the spectrum showed a weak band at 1740 cm^{-1} presumably due to a trace of ethyl acetate. The latter could not be removed by drying the sample *in vacuo* (72 h at 100°) but it disappeared by repeated evaporation with water.

Anal. Calc. for $C_{11}H_{22}ClN_3O_5$ (311.8): C, 42.36; H, 7.11; Cl, 11.37. Found: C, 42.57; H, 6.97; Cl, 11.60.

*Methyl 2,3,4-triacetamido-2,3,4-trideoxy-α-*D*-glucopyranoside* (**18**). — The hydrochloride **17** (223 mg) was *N*-acetylated (3 h at 0°) with acetic anhydride (5 ml) in water (10 ml) and methanol (2 ml), in the presence of Dowex-1 X2 (CO_3^{2-}) (4 ml). The filtrate from the resin was treated with a small amount of cation-exchange resin, the mixture was filtered, and evaporated, to give a white solid. Recrystallization was difficult because of gel formation, but it succeeded from absolute ethanol–ethyl acetate when the flask was continually warmed and the inside scratched. The microscopic needles of **18** (114 mg, 50%) began to darken above 310° and largely decomposed at 350–365°. The product had $[\alpha]_D$ +135.2° (*c* 0.7, water); i.r. data, 3470, 3330, and 3300 (with shoulders at 3350 and 3240 cm^{-1}; OH, NH), 1643 (with shoulder at 1620 cm^{-1}; amide I), 1545 cm^{-1} (with shoulder at 1560 cm^{-1}; amide II).

Anal. Calc. for $C_{13}H_{23}N_3O_6$ (317.3): C, 49.20; H, 7.31; N, 13.24. Found: C, 49.08; H, 7.17; N, 13.13.

*Methyl 2,3,4-triacetamido-6-*O*-acetyl-2,3,4-trideoxy-α-*D*-glucopyranoside* (**19**). — The hydrochloride **17** (300 mg) was acetylated (13 h at 25°, with stirring) with acetic anhydride (5 ml) and pyridine (10 ml). The reaction mixture was coevaporated twice with excess methanol and five times with toluene. The resulting, yellowish solid was dissolved in methanol, and the solution was stirred at 0° with Dowex-1 X2 (CO_3^{2-}) in order to remove chloride ion from pyridine hydrochloride. The solvent and liberated pyridine were evaporated with four consecutive additions of toluene. The crude **19** so obtained (208 mg, 60%) melted at 335–340° (dec., with partial sublimation above 275°). Recrystallization was troublesome because of gel formation, but it succeeded

from hot, absolute ethanol when the solution was allowed to cool very slowly. The recrystallized product formed glittering, white needles (136 mg), m.p. 339–340° (dec., with partial sublimation from 290°), $[\alpha]_D$ +109.2° (*c* 0.65, methanol); i.r. data, 3300 (with shoulders at 3340 and 3230 cm^{-1}; NH), 1735 (ester C=O), 1640 (amide I), and 1533 cm^{-1} (amide II).

Anal. Calc. for $C_{15}H_{25}N_3O_7$ (359.4): C, 50.13; H, 7.01, N, 11.69. Found: C, 49.90; H, 6.87; N, 11.68.

ACKNOWLEDGMENTS

Financial assistance received from the Life Insurance Medical Research Fund, Rosemont, Pennsylvania, is gratefully acknowledged. Farouk Rajabalee thanks the National Research Council of Canada for a postgraduate fellowship.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

REACTIONS OF CONNECTIVE TISSUE AND RELATED POLYANIONS WITH HYDRATED ELECTRONS AND HYDROXYL RADICALS

J. S. MOORE, G. O. PHILLIPS,

Department of Chemistry and Applied Chemistry, University of Salford, Salford (Great Britain)

J. V. DAVIES,

Christie Hospital and Holt Radium Institute, Manchester (Great Britain)

AND K. S. DODGSON

Department of Biochemistry, University College of South Wales and Monmouthshire, Cardiff (Great Britain)

(Received October 25th, 1968; in revised form, July 24th, 1969)

ABSTRACT

The effects of ionizing radiations on aqueous solutions of connective tissue and other polyanions have been examined by using pulse radiolysis. Rate constants have been measured for the reactions of hydrated electrons and OH radicals with chondroitin 4-sulphate, chondroitin 6-sulphate, *N*-desulphated heparin, sulphated hyaluronic acid, keratan sulphate, and sodium dextran sulphate, and the results are related to previous measurements on heparin and hyaluronic acid. The results indicate that it is the presence of the hexosamine moiety which is responsible for the enhanced reactivity of certain polyanions towards hydrated electrons, compared with unsubstituted carbohydrates. However, although there is significant reaction with hydrated electrons, it is the OH radicals which exert the main contribution towards the chemical changes induced by irradiation in aqueous solution. Consideration of the nature of the spectra of the transient species produced after pulsed irradiation support this conclusion.

INTRODUCTION

The reactivities of hyaluronic acid[1], heparin, and certain synthetic polyanions[2] towards hydrated electrons (e_{aq}^-) and hydroxyl (OH) radicals have been studied in an effort to understand the susceptibility of glycosaminoglycans (mucopolysaccharides) to the action of ionizing radiations *in vivo* and *in vitro*[3–4]. Moreover, these products of water radiolysis provide unique, model, reducing (e_{aq}^-) and oxidizing (OH radicals) species for evaluating the reactivity of connective tissue polyanions towards such reagents. Oxidation–reducing systems are known to initiate depolymerisation of glycosaminoglycans[3], and clear indications have been obtained about the free-radical character of the active species[5]. As part of a programme to understand the mechanisms of radiation interaction with connective tissue, we utilise here pulse radiolysis to measure the rate constants for attack of e_{aq}^- and of OH radicals on a series of acidic glycosaminoglycans and allied polyanions.

METHODS AND MATERIALS

Chondroitin 4-sulphate I and chondroitin 6-sulphate I were separated[6] from bovine, tracheal cartilage. These glycosaminoglycans were also extracted[7] from horse-rib cartilage (chondroitin 4-sulphate II) and human nucleus pulposus (chondroitin 6-sulphate II). Keratan sulphate was obtained from bovine cornea[6]. *N*-Desulphated heparin was prepared from commercial heparin (Boots Pure Drug Co. Ltd.) by mild hydrolysis with 0.005N HCl, followed by dialysis and precipitation with potassium acetate–ethanol. Sulphated hyaluronic acid was prepared by the method of Lloyd[8]. Sodium dextran sulphate (mol.wt. 2×10^6, 51% SO_4^{2-}) was supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. 2-Deoxy-2-sulphoamino-D-glucose was prepared by the method of Lloyd *et al.*[9]. 2-Acetamido-2-deoxy-D-glucose was supplied by Koch–Light Laboratories Ltd., and 2-acetamido-2-deoxy-D-galactose and 2-amino-2-deoxy-D-galactose hydrochloride by Sigma Chemicals Ltd. The samples were recrystallised before use.

The pulse-radiolysis methods, techniques, and equipment were those at the Christie Hospital, Manchester, and full details have been described[1-10]. The absorption cell had an optical path-length of 25 mm. Concentrations of polyanions are given here, for the purpose of expressing rate constants, as mole equivalents per l. An equivalent is defined as an average molecular weight of a hexose repeating-unit for the carbohydrate polymers.

RESULTS

Reaction rates with e_{aq}^-. — For the measurement of reaction rates with e_{aq}^-, a 0.2 μsec pulse of 4 MeV electrons was used to deliver a dose of *ca.* 200 rads to the irradiated volume (1.5 ml). From the rate of disappearance of e_{aq}^- at 650 nm, the first-order rate constants for reaction with e_{aq}^- can be obtained from the linear plot of log (absorbance) against time after the pulse. Typical semi-log plots are shown in Fig. 1. Second-order constants for the reaction e_{aq}^- + polyanion can be obtained, as previously described[2]. Table I summarises the results for sodium dextran sulphate, chondroitin 6-sulphate, chondroitin 4-sulphate, keratan sulphate, sulphated hyaluronic acid, *N*-desulphated heparin, 2-deoxy-2-sulphoamino-D-glucose, 2-amino-2-deoxy-D-galactose hydrochloride, and 2-acetamido-2-deoxy-D-galactose.

Reaction rates with OH radicals. — As described previously[2], the thiocyanate competition method[11] was used to determine the rate constants for reactions of OH radicals with the polyanions. Typical competition plots, which were used to calculate the results shown in Table I, are illustrated in Fig. 2. The results shown in Table I are based on a rate constant of 6.6×10^9 mole^{-1} sec^{-1} for the reaction of $OH+CNS^-$.

Transient absorption spectra. — The absorption spectra of transient species produced immediately after the pulse and the decay characteristics of the species were examined[1]. 2-Deoxy-2-sulphoamino-D-glucose is used to illustrate the behaviour for a typical monomer molecule, and Fig. 3 shows the absorption spectrum of the

TABLE I

SECOND-ORDER RATE CONSTANTS FOR THE REACTIONS OF e^-_{aq} AND OH RADICALS WITH POLYANIONS AND ASSOCIATED MONOMERS

Polyanion[a]	*Monomer*	e^-_{aq} $10^8 k_2$ ($mole^{-1}$ sec^{-1})	*OH* $10^8 k_2$ ($mole^{-1}$ sec^{-1})
Chondroitin 4-sulphate I		0.26	4.8
Chondroitin 4-sulphate II		0.13	—
Chondroitin 6-sulphate I		0.42	4.1
Chondroitin 6-sulphate II		0.11	—
Keratan sulphate		0.33	—
Heparin[3]		0.22	2.2
Hyaluronic acid[1,2]		1.4	4
Sulphated hyaluronic acid		0.17	3.6
N-Desulphated heparin		0.10	4.8
Dextran sulphate		$\leqslant$0.07	—
	2-Deoxy-2-sulphoamino-D-glucose	0.17	12.7
	2-Amino-2-deoxy-D-galactose hydrochloride	0.84	7.0
	Sodium D-glucuronate[17]	0.02	18.5
	2-Acetamido-2-deoxy-D-glucose	—	18.4
	2-Acetamido-2-deoxy-D-galactose	0.11	9.7

[a]For calculating rate constants of polyanions, concentrations are expressed as mole equivalents per l, where an equivalent represents the average molecular weight of a hexose unit.

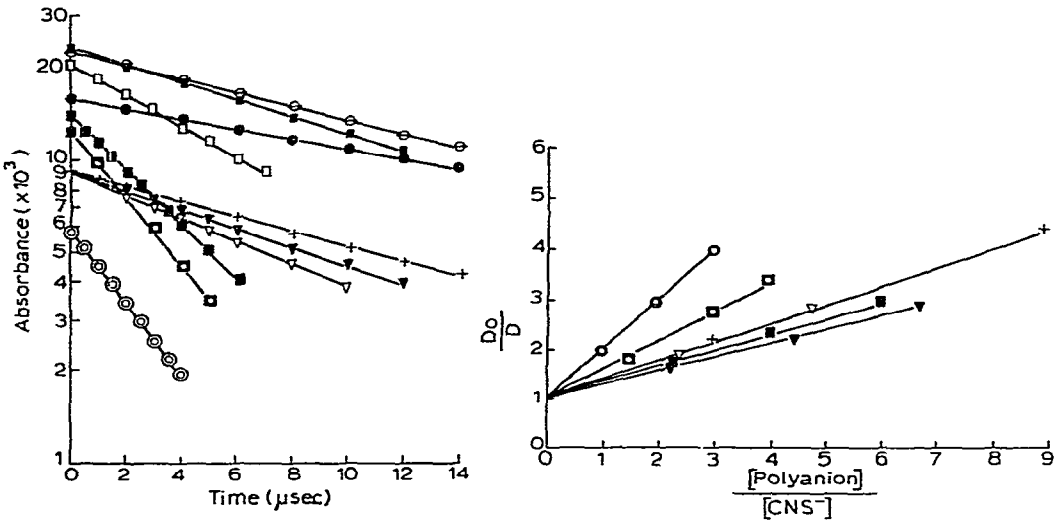

Fig. 1. First-order plot for the decay of e^-_{aq} in aqueous polyanion solutions. ⊖ Chondroitin 6-sulphate I (5 mM), ■ chondroitin 4-sulphate II (5 mM), ● dextran sulphate (5.45 mM), □ keratan sulphate (5 mM), ▩ chondroitin 6-sulphate I (5 mM), ◘ 2-amino-2-deoxy-D-glucose hydrochloride (2.8 mM), + chondroitin 4-sulphate I (4.2 mM), ▼ sulphated hyaluronic acid (4.3 mM), ▽ *N*-desulphated heparin (8.19 mM), ◎ 2-deoxy-2-sulphoamino-D-glucose (16 mM).

Fig. 2. Measurement of the rate of reaction of OH radicals with polyanions. CNS^- = 0.1 mM. Symbols as in Fig. 1.

intermediate produced immediately after pulsed irradiation (dose ~7000 rads) in argon and N_2O. The decay of the intermediate with absorption maximum at 235 nm in argon obeys strict second-order kinetics to greater than 55% decay. In N_2O, the same kinetics are obeyed at 280 nm (Fig. 4). In Fig. 5, the behaviour of chondroitin 4-sulphate I (A) and keratan sulphate (B) are illustrated. Transient species are produced, and the effect of N_2O on the absorption spectra is shown. The lifetime of these species is very long and can still be observed milliseconds after the pulse. Decay is second order over ~1800 μsec, but this represents only 10% of the total disappearance of the radical concentration. Details of the transient spectra obtained from other compounds studied are shown in Table II. The extinction coefficient

TABLE II

TRANSIENT ABSORPTION SPECTRA MEASURED IMMEDIATELY FOLLOWING A PULSE OF 4 MeV ELECTRONS

Compound	λ_{max} (*nm*)	ε (*mole*$^{-1}$ *cm*$^{-1}$)
2-Deoxy-2-sulphoamino-D-glucose	235	180
2-Acetamido-2-deoxy-D-galactose	240	—
2-Acetamido-2-deoxy-D-glucose	240	—
Keratan sulphate	245	160
Chondroitin 4-sulphate I	240	425
Sodium dextran sulphate	245	375

was calculated on the assumption that the absorption at ~240 nm is produced by OH radical abstraction (G_{OH} = 2.9).

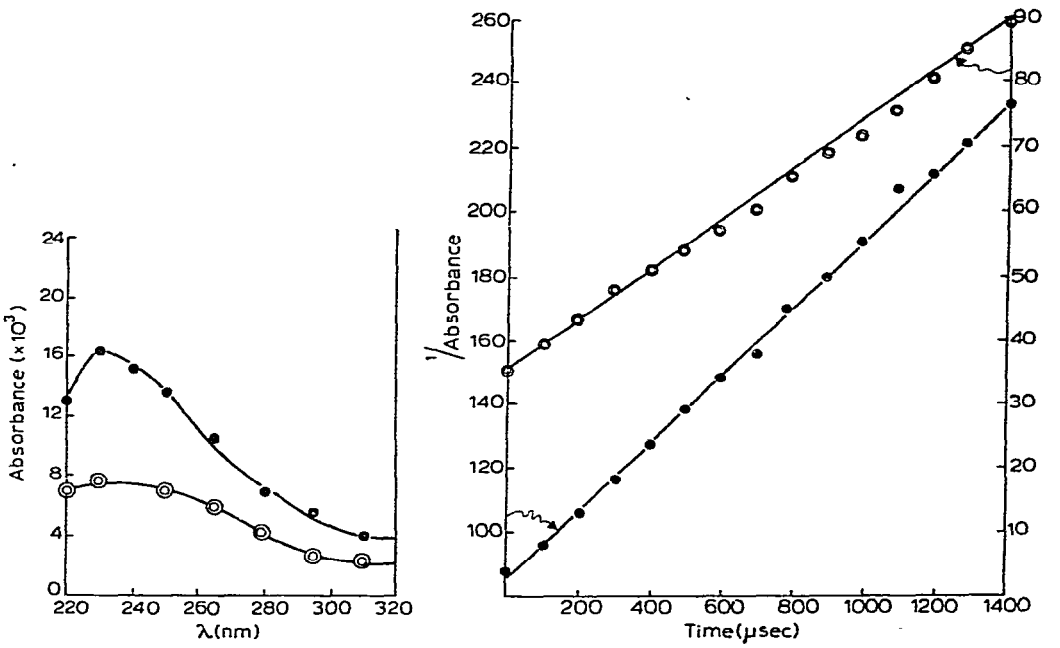

Fig. 3. Transient absorption spectra of pulsed, aqueous solutions of 2-deoxy-2-sulphoamino-D-glucose (0.1 mM). Dose ~7000 rads. ⊙ Equilibrated with argon. ● Equilibrated with nitrous oxide.

Fig. 4. Second-order decay of transient produced after pulsed irradiation of aqueous solutions of 2-deoxy-2-sulphoamino-D-glucose (0.1 mM). ● Equilibrated with nitrous oxide: λ = 280 nm (left-hand ordinate). ⊙ Equilibrated with argon: λ = 235 nm (right-hand ordinate).

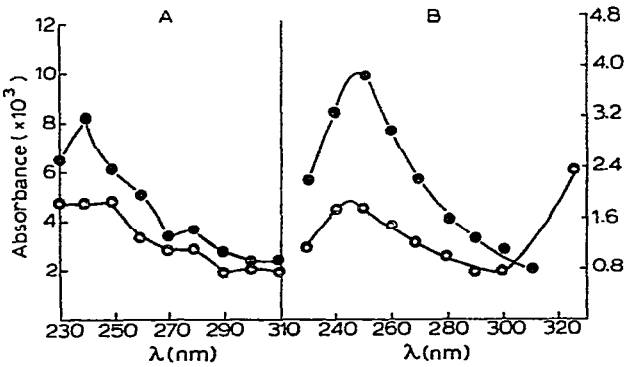

Fig. 5. Transient absorption spectra of pulsed aqueous solutions of (A) chondroitin 4-sulphate I (0.1 mM) and (B) keratan sulphate (0.1 mM). ● Equilibrated with nitrous oxide. ⊙ Equilibrated with argon.

DISCUSSION

The pattern of reactivity of e^-_{aq} and OH radicals towards the acidic glycosaminoglycans previously established[1,2] is further clarified by the results reported

here. Whereas neutral carbohydrates[12] show no significant reaction with e_{aq}^-, the connective-tissue glycosaminoglycans have a significant and measurable reactivity, with k_2 varying from $1\text{–}4\times10^7$ mole^{-1} sec^{-1}. Apart from hyaluronic acid, the variations in reactivity of individual glycosaminoglycans towards e_{aq}^- are small and cannot be considered significant. Indeed, a variation within the range $1\text{–}4\times10^7$ mole^{-1} sec^{-1} is found for samples of chondroitin 4-sulphate and chondroitin 6-sulphate of different biological origin. It is more probable that it is the presence of the hexosamine moiety rather than *O*-sulphate groups which confers the measurable reaction of the glycosaminoglycans with e_{aq}^-. Dextran sulphate ($k_2 \leqslant 7\times10^6$ mole^{-1} sec^{-1}) behaves like an unsubstituted polysaccharide and k_2 is at the lower limit for establishing whether reaction occurs or not. The substituted and unsubstituted *N*-acetyl and *N*-sulphated hexosamines, on the other hand, have comparable reactivity ($k_2 = 1\text{–}8\times10^7$ mole^{-1} sec^{-1}) with the glycosaminoglycans.

The higher reactivity ($k_2 = 14\times10^7$ mole^{-1} sec^{-1}) of hyaluronic acid is consistent with its previously reported, extreme sensitivity to the action of ionizing radiations in aqueous solution[3,4], and factors which might account for this behaviour have been discussed. It is significant that the sulphation treatment removes the reactivity of hyaluronic acid (for sulphated hyaluronic acid, $k_2 = 1.7\times10^7$ mole^{-1} sec^{-1}), which provides a further indication that some reactive, yet labile, grouping or cationic protein[13] is present in native hyaluronic acid, which is responsible for the faster reaction with e_{aq}^-.

The validity of the thiocyanate competitive method[11], which we[2] and many other workers have used to measure rates of OH radicals with many solutes, has recently been called to question. The transient from thiocyanate was presumed to be the CNS radical formed according to

$$OH^\bullet + CNS^- \rightarrow CNS^\bullet + OH^- \qquad (1)$$

It has subsequently been demonstrated[14] that the absorption is due entirely to the species $(CNS)_2^-$ which is in equilibrium with CNS:

$$CNS^\bullet + CNS^- \underset{k_b}{\overset{k_a}{\rightleftharpoons}} (CNS)_2^- \qquad (2)$$

The previously measured, bimolecular rate-constant of 6.6×10^9 mole^{-1} sec^{-1}, which was assigned to reaction 1, refers in fact to k_a in reaction 2. Since $k_a/k_b \sim 3$, the previously measured OH radical rate-constants by this method are too low. For reaction 1, k_2 has been recalculated to be 2.8×10^{10} mole^{-1} sec^{-1}. The equilibrium constant, $K = k_a/k_b \sim 3\times10^5$ mole^{-1} is high and, therefore, under the normal experimental conditions [$(CNS^-) \sim 10^{-4}$M] used, the equilibrium is well over to $(CNS)_2^-$. An additional complication could be due to reaction of $CNS^\bullet$ with the solute. However, deviations would then be apparent at the varying concentrations of thiocyanate which are used. Thus, we and other workers[15] consider that the thiocyanate competitive method is still a useful and valid method for measuring

OH radical rates. Absolute OH-radical rates can be found from the relative rates shown in Table I by using the factor 28/6.6. We retain the value for reaction 1 of $k_2 = 6.6 \times 10^9$ mole^{-1} sec^{-1} here to obtain a direct comparison with previously reported rate-constants.

The reactivity of the glycosaminoglycans with OH radicals is considerably higher than with e_{aq}^-. Since second-order rate constants have been expressed on the basis of average hexose unit, in the absence of any particular steric influence due to an unfavourable polymer configuration, the OH-radical rate should approach the mean k_2 value of the free monomer units. This, however, is not the situation found in practice. Consistently, we have found that the k_2 values for free monomer (or a unit of comparable reactivity) are larger than for the polymer. Whereas the monomeric units have k_2 values of $7–18 \times 10^8$ mole^{-1} sec^{-1}, the polymeric systems have k_2 values of $2–5 \times 10^8$ mole^{-1} sec^{-1}. The indications are that there is substantial steric inhibition during OH radical attack with a proportion of the monomeric units, and our results here fully support our previous conclusions[2].

Information about the transient species formed during pulse radiolysis further demonstrates that OH radicals are responsible for the major portion of the chemical change. The transient having absorption maximum at ~230 nm during pulsed irradiation of 2-deoxy-2-sulphoamino-D-glucose can be attributed almost entirely to the action of OH radicals, since the concentration is doubled in N_2O when all e_{aq}^- are converted into OH radicals by the reaction[16] $e_{aq}^- + N_2O \rightarrow N_2 + OH + OH^-$ which would, therefore, double the yield of OH radicals. It is probable that OH abstraction leads to a radical, which, from Fig. 4, can be seen to decay by strict second-order kinetics to more than 50% reaction. From the effect of N_2O on the transient spectrum produced from chondroitin 4-sulphate I, it is apparent that OH radicals account for the major, although not all the absorption. The radical species associated with the glycosaminoglycans have a considerably greater stability than the radical from 2-deoxy-2-sulphoamino-D-glucose and can still be detected milliseconds after the pulse.

Thus, for the polyanions we have examined, reactions with e_{aq}^- and OH radicals can be observed, although OH radicals clearly exert the main contribution towards the chemical changes induced by irradiation in aqueous solution.

ACKNOWLEDGMENT

The authors thank Tenovus, who have generously supported this investigation.

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Carbohyd. Res., 12 (1970) 253–260

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

THE SYNTHESIS OF 2,3,4,6,7-PENTA-*O*-METHYL-D-*glycero*-L-*manno*-HEPTOSE AND 2,4,6,7-TETRA-*O*-METHYL-D-*glycero*-L-*manno*-HEPTOSE*

PREM PAL SINGH† AND G. A. ADAMS
Biochemistry Laboratory, National Research Council of Canada, Ottawa (Canada)

(Received June 23rd, 1969; in revised form, August 7th, 1969)

ABSTRACT

2,3,4,6,7-Penta-*O*-methyl-D-*glycero*-L-*manno*-heptose was synthesized by methylation of D-*glycero*-L-*manno*-heptose followed by hydrolysis. 2,4,6,7-Tetra-*O*-methyl-D-*glycero*-L-*manno*-heptose was synthesized by using the following sequence of reactions: 2-*O*-benzyl-D-galactose (**7**) → 3-*O*-benzyl-1-deoxy-1-nitro-D-*glycero*-L-*manno*-heptitol (**8**) → 3-*O*-benzyl-D-*glycero*-L-*manno*-heptose (**9**) → methyl 3-*O*-benzyl-2,4,6,7-tetra-*O*-methyl-D-*glycero*-L-*manno*-heptoside (**10**) → methyl 2,4,6,7-tetra-*O*-methyl-D-*glycero*-L-*manno*-heptoside (**11**) → 2,4,6,7-tetra-*O*-methyl-D-*glycero*-L-*manno*-heptose (**12**).

INTRODUCTION

Studies in this laboratory on the core structure of Gram-negative lipopolysaccharides by the methylation technique required 2,3,4,6,7-penta-*O*-methyl-D-*glycero*-L-*manno*-heptose and 2,4,6,7-tetra-*O*-methyl-D-*glycero*-L-*manno*-heptose as reference compounds. Earlier studies by Risse *et al.*[1] on a partial hydrolysis product from the lipopolysaccharides of *Salmonella minnesota* indicated a heptosyl→heptosyl →3-deoxyoctulosonate structure, although the methylated heptoses could not be characterized positively. Periodate oxidation of the heptose residues in the lipopolysaccharide with cleavage between C-6 and C-7, followed by reduction, yielded residues of a mannose; methylation of the periodate-oxidized and reduced structure established[2] the linkage between the sugar residues as (1→3).

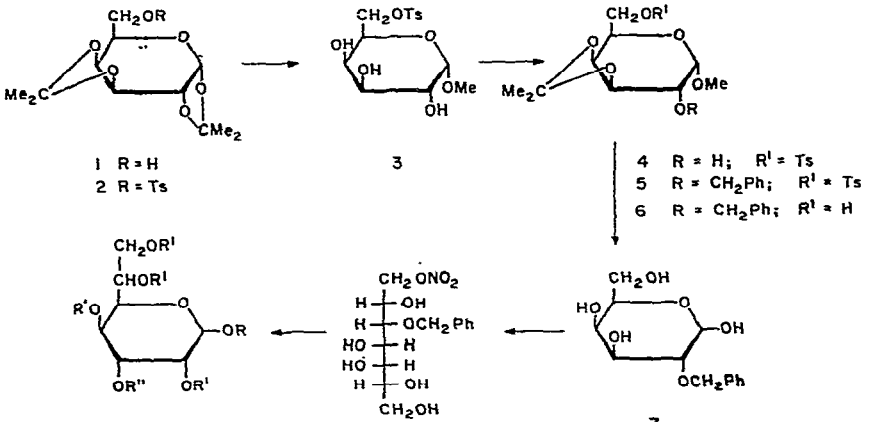
Methylation studies in this laboratory[3] on the lipopolysaccharide of *Neisseria perflava* also established that the heptose residues were (1→3)-linked. It seemed desirable, however, to be able to identify the original methylated heptose residues in these lipopolysaccharides by comparison with authentic 2,3,4,6,7-penta-*O*-methyl-D-*glycero*-L-*manno*-heptose and 2,4,6,7-tetra-*O*-methyl-D-*glycero*-L-*manno*-heptose. With this objective in mind these methyl ethers have been synthesized and their properties are described.

*Issued as NRCC No. 11071.
†National Research Council Postdoctorate Fellow 1967–1969.

Although L-*glycero*-D-*manno*-heptose is the naturally occurring heptose in lipopolysaccharides, it was more expedient in the present work to prepare the enantiomorph, D-*glycero*-L-*manno*-heptose[4]. During the progress of this work a synthesis of L-*glycero*-D-*manno*-heptose has been described[5].

RESULTS AND DISCUSSION

2,3,4,6,7-Penta-*O*-methyl-D-*glycero*-L-*manno*-heptose was obtained by methylation of D-*glycero*-L-*manno*-heptose followed by hydrolysis. The methylated sugar yielded a crystalline aniline derivative. 2,4,6,7-Tetra-*O*-methyl-D-*glycero*-L-*manno*-heptose (**12**) was obtained by methylation of 3-*O*-benzyl-D-*glycero*-L-*manno*-heptose (**9**) followed by debenzylation and hydrolysis. The complete sequence of reactions leading to 3-*O*-benzyl-D-*glycero*-L-*manno*-heptose is shown in the following scheme.



Application of the nitromethane synthesis to 2-*O*-benzyl-D-galactose (**7**) produced, surprisingly, only 3-*O*-benzyl-1-deoxy-1-nitro-D-*glycero*-L-*manno*-heptitol. This result was probably the outcome of steric hindrance caused by the bulky benzyl group at C-2. The low yield of 3-*O*-benzyl-1-deoxy-1-nitro-D-*glycero*-L-*manno*-heptitol could not be explained. The D-*glycero*-L-*manno* configuration of the heptose sugar was shown by debenzylation to D-*glycero*-L-*manno*-heptose; the latter was identified as its phenylhydrazone and by its conversion into D-*glycero*-L-*manno*-heptitol hepta-acetate. Neither 2,4,6,7-tetra-*O*-methyl-D-*glycero*-L-*manno*-heptose nor its aniline or the di-*O*-*p*-nitrobenzoyl derivatives could be crystallized.

The methyl glycosides of 2,3,4,6,7-penta-*O*-methyl-D-*glycero*-L-*manno*-heptose and 2,4,6,7-tetra-*O*-methyl-D-*glycero*-L-*manno*-heptose had retention times on g.l.c. identical with those of the two methylated heptosides obtained by methylation and methanolysis of a heptose oligosaccharide[1] that had been prepared from a lipopoly-

saccharide of *S. minnesota* and kindly supplied by Dr. Otto Lüderitz. The retention time of the synthetic methyl 2,4,6,7-tetra-*O*-methyl-D-*glycero*-L-*manno*-heptoside was also the same as that of the partially methylated heptoside isolated from a methylated lipopolysaccharide of *N. perflava* prepared in this laboratory[3].

EXPERIMENTAL

General methods. — All evaporations were carried out under diminished pressure below 40°. Melting points were determined on a Fisher–Johns hot stage and are corrected. Optical rotations, determined on a Perkin–Elmer 141 polarimeter, are equilibrium values, unless otherwise stated. Methoxyl determinations were made according to Steyermark[6]. Paper chromatography was carried out on Whatman No. 1 paper in the following solvent systems: Solvent *A*: butanone saturated with water, Solvent *B*: pyridine–ethyl acetate–water (2:5:5, v/v). R_F, R_G, and R_{Gal} values are with reference to solvent front, 2,3,4,6-tetra-*O*-methyl-D-glucose and D-galactose, respectively. G.l.c. was carried out on columns (120 × 0.5 cm) having the following packings: (*A*) 10% (w/w) neopentyl glycol sebacate on Chromosorb W (100–120 mesh) at 193°, (*B*) 0.3% (w/w) ECNSS-M on Chromosorb Q (100–120 mesh) at 203°. The gas pressure was 30 lb.in^{-2}. T_G and T_{Gal} are retention times with reference to methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucoside and D-glucitol hexaacetate, respectively. N.m.r. spectra were taken in chloroform-*d* with tetramethylsilane as the internal reference with a Varian A-60A n.m.r. spectrometer. I.r. spectra were recorded with a Perkin–Elmer Infracord spectrometer. Hydrolysis was carried out with 0.5M sulfuric acid for 4 h in a boiling water-bath. The hydrolyzate was neutralized by passing over Duolite A-4 (OH^-) resin and the eluates were concentrated.

2,3,4,6,7-Penta-O-*methyl*-D-glycero-L-manno-*heptose.* — D-*glycero*-L-*manno*-Heptose[4] (1 g) was methylated by Srivastava's method[7] with methyl sulfoxide, silver oxide, and methyl iodide, and then by one Purdie methylation[8]. The glassy syrup obtained (1.1 g) was completely methylated, as shown by the absence of OH absorption in its i.r. spectrum. [OMe: found, 62.42; calc. 63.26]. G.l.c. on column *A* showed two peaks having T_G values of 2.71 (major) and 3.21 (minor). Acid hydrolysis of the fully methylated D-*glycero*-L-*manno*-heptose (0.75 g) yielded 2,3,4,6,7-penta-*O*-methyl-D-*glycero*-L-*manno*-heptose (0.62 g), [OMe: found, 54.9; calc. 55.35], $[\alpha]_D$ −35.4° (*c* 1.3, methanol). The sugar could not be crystallized, but on paper chromatography it migrated as a single spot having an R_F value of 0.84 and an R_G value of 1.04 (solvent *A*).

The syrup (0.22 g) was refluxed for 6 h with aniline (0.085 g) in absolute ethanol (10 ml). The solvent was then removed by evaporation and the aniline derivative was crystallized from ethyl acetate. Recrystallization gave 2,3,4,6,7-penta-*O*-methyl-*N*-phenyl-D-*glycero*-L-*manno*-heptosylamine, m.p. 150–151°, $[\alpha]_D$ +142.3° (*c* 0.2, acetone).

Anal. Calc. for $C_{18}H_{29}NO_6$: C, 60.67; H, 8.41; N, 3.94. Found: C, 60.67; H, 8.61; N, 3.83.

1,2:3,4-Di-O-isopropylidene-6-O-p-tolylsulfonyl-α-D-galactose (**2**). — D-Galactose (120 g) was converted into 1,2:3,4-di-*O*-isopropylidene-α-D-galactose (**1**) by treatment with zinc chloride and concentrated sulfuric acid in acetone[9]. Compound **1** (155.5 g) was *p*-toluenesulfonated with *p*-toluenesulfonyl chloride in an acetone–pyridine mixture[9]. The 1,2:3,4-di-*O*-isopropylidene-6-*O*-*p*-tolylsulfonyl-α-D-galactose (**2**) (150 g) had m.p. 88–89° (lit.[10] m.p. 89–91°).

Methyl 6-O-p-tolylsulfonyl-α-D-galactopyranoside (**3**). — Compound **2** (145.0 g) was refluxed with 2% methanolic hydrogen chloride for 30 min[11]. The solution on cooling gave crystals of methyl 6-*O*-*p*-tolylsulfonyl-α-D-galactopyranoside (**3**). The mother liquor on further concentration gave an additional yield of crystals. The two crops on recrystallization gave pure **3** (65.0 g), m.p. 169–170°, $[\alpha]_D$ +104° (*c* 1, pyridine) (lit.[11] m.p. 170°, $[\alpha]_D$ +103.5°).

Methyl 3,4-O-isopropylidene-6-O-p-tolylsulfonyl-α-D-galactopyranoside (**4**). — Methyl 6-*O*-*p*-tolylsulfonyl-α-D-galactopyranoside (**2**, 62.0 g) was suspended in acetone (3 liters) and was shaken for 48 h with anhydrous copper(II) sulfate (320 g) and concentrated sulfuric acid (8.0 ml). The suspension was filtered and the filtrate was neutralized with ammonia, and the suspension was filtered again. The filtrate was concentrated to half its volume and poured into water. The crystalline product that formed was filtered off and dissolved in ether, and the insoluble material was removed by filtration. The filtrate on concentration yielded pure **4** (31.0 g) having m.p. 129–130°, $[\alpha]_D$ +76.7° (*c* 1, methanol); [lit.[12] m.p. 129–130°, $[\alpha]_D$ +73.7° (methanol)].

Methyl 2-O-benzyl-3,4-O-isopropylidene-6-O-p-tolylsulfonyl-α-D-galactopyranoside (**5**). — Compound **4** (30.0 g) was dissolved in dry *N,N*-dimethylformamide (10 liters). The solution was cooled in ice and to it was added silver oxide (250 g) and benzyl bromide (100 g). The mixture after being stirred overnight was poured into chloroform (2 liters). The precipitated silver salts were removed by filtration and the filtrate was concentrated to a thick syrup. The syrup was dissolved in hot ether and the solution on cooling afforded crystals which on recrystallization gave **5** (35.2 g), m.p. 118–119°, $[\alpha]_D$ +78.5° (*c* 1, methanol). The i.r. spectrum showed absence of OH absorption. The n.m.r. spectrum was consistent with the assigned structure.

Anal. Calc. for $C_{24}H_{30}O_8S$: C, 60.25; H, 6.27; S, 6.69. Found: C, 60.17; H, 6.35; S, 6.62.

Methyl 2-O-benzyl-3,4-O-isopropylidene-α-D-galactopyranoside (**6**). — Compound **5** (32.0 g) was dissolved in 80% aqueous methanol and treated with 4% sodium amalgam[13]. After stirring for 14 h, the mixture was neutralized with carbon dioxide. The solution was decanted from the mercury and concentrated to dryness. The resulting solid was extracted twice with hot absolute ether and the extracts, after filtration, were combined and concentrated. Crystallization took place to give **6** (23.0 g), m.p. 105–106°, $[\alpha]_D$ +96.0° (*c* 1, chloroform). The n.m.r. spectrum was consistent with the assigned structure.

Anal. Calc. for $C_{17}H_{24}O_6$: C, 62.96; H, 7.40. Found: C, 63.08; H, 7.46.

2-O-Benzyl-D-galactose (**7**). — Compound **6** (20.0 g) on acid hydrolysis and concentration gave crystals which, on recrystallization from water, yielded pure 2-*O*-benzyl-D-galactose (**7**, 13.5 g), m.p. 70°, $[\alpha]_D$ +72.5 (5 min) → +58.9° (*c* 1, water). Paper chromatography in solvent *B* showed only one spot, R_F 0.77; R_{Gal} 3.43.

3-O-Benzyl-1-deoxy-1-nitro-D-glycero-L-manno-*heptitol* (**8**). — Compound **7** (7.5 g) was suspended in a mixture of methanol (20 ml) and nitromethane (25 ml) and to the suspension was added a cold solution of sodium (2.0 g) in methanol (60 ml). The reaction mixture was stirred overnight and then cooled to −20°. The precipitate was filtered off, washed with cold methanol, dissolved in water, and passed over Rexyn-101 (H^+) resin to remove sodium ions. The effluent on concentration gave crystals which, on recrystallization from water, gave compound **8** (0.51 g); m.p. 160–16[illegible] $[\alpha]_D$ +9.6° (*c* 0.5, water).

An[illegible]. Calc. for $C_{14}H_{21}NO_8$: N, 4.23. Found: N, 4.30.

3-O-Benzyl-D-glycero-L-manno-*heptose* (**9**). — Compound **8** (0.5 g) in M sodium [illegible]ydroxide (2.5 ml) was added dropwise to a solution of sulfuric acid (0.3 ml) in wate[illegible] 0.4 ml)[14]. After stirring for 15 min, the solution was deionized by passing over Re[illegible]yn-101 (H^+) and Duolite A-4 (OH^-) resins. The effluent was evaporated to drynes[illegible]o give 3-*O*-benzyl-D-*glycero*-L-*manno*-heptose (**9**, 0.35 g); $[\alpha]_D$ −5.34° (*c* 1.8, meth[illegible]ol). The sugar moved as one spot on paper in solvent *B*, having R_F and R_{Gal} valu[illegible] of 0.7 and 3.05, respectively

The 3-*O*-[illegible]-heptose (**9**, 0.03 g) was refluxed with 2.5% methanolic hydrogen ch[illegible] [illegible] ml) for 4 h and the acid was neutralized with Ag_2CO_3 and the precipitate was removed by filtration. The filtrate was taken to dryness and debenzylated[15] with sodium (0.03 g) and absolute ethanol for 4 h at 70°. The reaction mixture was then dissolved in water and deonized by passage through Rexyn-101 (H^+) resin. The effluent was concentrated and hydrolyzed with acid. The hydrolyzate moved on paper chromatography in solvent *B* at the same rate as authentic D-*glycero*-L-*manno*-heptose and yielded a crystalline phenylhydrazone, m.p. 190–191°, undepressed when mixed with an authentic sample.

The hydrazone was decomposed with benzaldehyde[16] and the free sugar was reduced with sodium borohydride. The product was acetylated[17] with acetic anhydride for 3 h at 121°. The acetate showed a single peak (T_{Gal} 2.36) on g.l.c. (column *B*), having the same retention time as the authentic D-*glycero*-L-*manno*-heptitol acetate and was distinguishable from the D-*glycero*-L-*gluco*-heptitol acetate, which had a retention time of T_{Gal} 2.7.

2,4,6,7-Tetra-O-*methyl*-D-glycero-L-manno-*heptose* (**10**). — Compound **9** (0.30 g) was methylated by the methods described earlier. The i.r. spectrum of the methylated product (**10**, 0.31 g) showed complete absence of OH absorption. (*Anal.* Found: OMe, 41.52; calc. 41.89); $[\alpha]_D$ +21.3° (*c* 1, methanol). Product **10** was debenzylated[15] to give methyl 2,4,6,7-tetra-*O*-methyl-D-*glycero*-L-*manno*-heptoside (**11**, 0.22 g) (Found: OMe, 55.0; calc. 55.35). G.l.c. on column *A* showed one major peak (T_G 4.4) and a minor peak (T_G 5.0). Hydrolysis followed by reglycosidation showed essentially a single peak at T_G 4.4.

Compound **11** was hydrolyzed to give 2,4,6,7-tetra-*O*-methyl-D-*glycero*-L-*manno*-heptose (**12**, 0.19 g), $[\alpha]_D$ −28.3° (*c* 1.5, methanol). Paper-chromatographic examination in solvent *A* showed one spot having R_F 0.67 and R_G 0.82. Attempts to crystallize the syrup were unsuccessful. Neither the aniline nor the di-*p*-nitrobenzoyl derivatives could be crystallized.

ACKNOWLEDGMENTS

The authors thank Mr. A. E. Castagne for the microanalyses.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
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Note

Ion-exchange chromatographic separation of the methyl ethers of 2-amino-2-deoxy-D-glucopyranose*

G. A. ADAMS, M. YAGUCHI, AND M. B. PERRY

The Biochemistry Laboratory and The Division of Biology, National Research Council of Canada, Ottawa 2 (Canada)

(Received July 2nd, 1969)

The identification of the methyl ethers of 2-amino-2-deoxy-D-glucose obtained from hydrolyzates of methylated glycans containing 2-amino-2-deoxy-D-glucose residues is usually a part of the procedure involved in determining the mode of substitution on the aminodeoxyaldose in the original glycan. The methyl ethers of 2-amino-2-deoxy-D-glucose are not completely resolved by paper chromatography; however, a thin-layer chromatographic method in which the *N*-2,4-dinitrophenyl derivatives of the aminodeoxyaldose is used appears to offer a good procedure for the identification of the methyl ethers[1]. A gas–liquid chromatographic method, in which the fully acetylated methyl ethers of 2-amino-2-deoxy-D-glucitol are separated, has proved of value for the qualitative and quantitative analysis of mixtures of the methyl ethers of 2-amino-2-deoxy-D-glucopyranose[2]. This Note records the successful separation of all of the possible methyl ethers of 2-amino-2-deoxy-D-glucopyranose by an ion-exchange chromatographic method.

Fig. 1 is the chromatogram of a mixture of seven *O*-methylated derivatives of 2-amino-2-deoxy-D-glucose (approximately 0.2 μmole of each) and 2-amino-2-deoxy-D-galactose (0.2 μmole). 2-Amino-2-deoxy-D-galactose was eluted at 9 h 50 min,

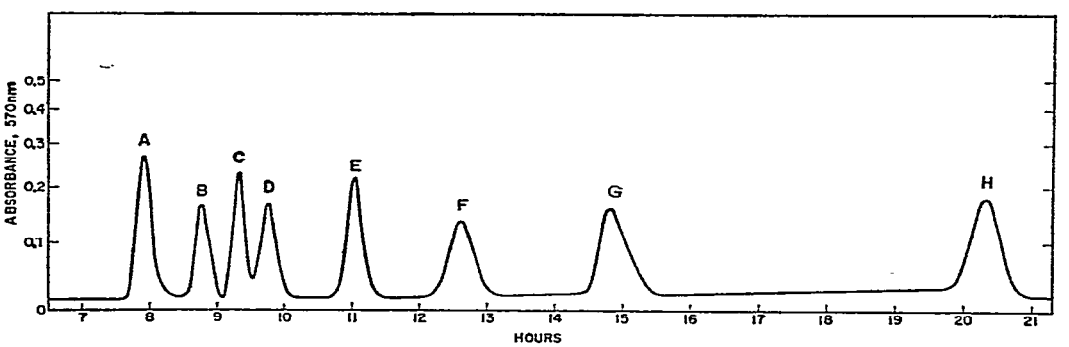

Fig. 1. Separation of the methyl ethers of 2-amino-2-deoxy-D-glucopyranose by ion-exchange chromatography: A, 2-amino-2-deoxy-3-*O*-methyl-D-glucose; B, 2-amino-2-deoxy-4-*O*-methyl-D-glucose; C, 2-amino-2-deoxy-6-*O*-methyl-D-glucose; D, 2-amino-2-deoxy-D-galactose; E, 2-amino-2-deoxy-4,6-di-*O*-methyl-D-glucose; F, 2-amino-2-deoxy-3,4-di-*O*-methyl-D-glucose; G, 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-D-glucose; and H, 2-amino-2-deoxy-3,6-di-*O*-methyl-D-glucose.

*Issued as NRCC No. 11070.

and can be used as an internal standard. 2-Amino-2-deoxy-D-glucose (not shown in Fig. 1) partially overlapped with 2-amino-2-deoxy-4-*O*-methyl-D-glucose and was not a satisfactory standard. All seven of the possible methyl ethers of 2-amino-2-deoxy-D-glucopyranose were completely resolved, and each peak was identified by chromatography of mixtures of the individual 2-amino-2-deoxy-D-glucose derivatives and the internal standard. Individual methyl ethers of 2-amino-2-deoxy-D-glucose each gave a single peak on the chromatograms.

All of the 2-amino-2-deoxy-mono-*O*-methyl-D-glucoses were eluted before 2-amino-2-deoxy-D-galactose, whereas 2-amino-2-deoxy-di-*O*-methyl-D-glucoses and 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-D-glucose were eluted after 2-amino-2-deoxy D-galactose. The peaks due to 2-amino-2-deoxy-3-*O*-methyl-D-glucose, 2-amino-2-deoxy-6-*O*-methyl-D-glucose, and 2-amino-2-deoxy-4,6-di-*O*-methyl-D-glucose were sharper than those due to 2-amino-2-deoxy-D-galactose. The peak due to 2-amino-2-deoxy-4-*O*-methyl-D-glucose had sharpness similar to that of 2-amino-2-deoxy-D-galactose. On the other hand, the peaks due to 2-amino-2-deoxy-3,4-di-*O*-methyl-D-glucose, 2-amino-2-deoxy-3,6-di-*O*-methyl-D-glucose, and 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-D-glucose were broader than that of 2-amino-2-deoxy-D-galactose.

EXPERIMENTAL

Materials. — The 3-, 4-, 6-*O*-methyl, 3,4-, 3,6-, 4,6-di-*O*-methyl, and 3,4,6-tri-*O*-methyl derivatives of 2-amino-2-deoxy-D-glucose hydrochloride were prepared in this laboratory by the methods of Jeanloz and coworkers[3] and they had physical and chemical properties in agreement with those recorded in the literature. They were pure by paper chromatography and, following reduction and acetylation, the resulting fully acetylated methyl ethers of 2-acetamido-2-deoxy-D-glucitol[2] gave from each aldose a single peak on gas–liquid chromatography.

Chromatography. — A Technicon NC-1 Amino Acid Analyzer was used. The column, 6 mm inside diameter by 132 cm long, was packed with Chromobeads Type A (Lot No. 137) and was jacketed at 60°. Between runs the column was washed for 30 min with 0.2M sodium hydroxide and then regenerated with pH 3.10 buffer. After the sample had been applied to the top of the column, it was eluted with pH 4.40 buffer for 21 h. The flow-rate throughout was 0.5 ml/min, with a back pressure of 220 $lb.in^{-2}$, and the effluent stream was analyzed (15 mm-flow cell, 570 nm) automatically after reaction with ninhydrin. The pH 3.10 buffer was made of 58.84 g of sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), 100 ml of 2M sodium hydroxide, and 40 ml of Brij-35 solution (Sigma Chemical Co., 100 g in 300 ml of water), and was adjusted to pH 3.10 with 6M hydrochloric acid, the final volume being 4000 ml. The pH 4.40 buffer was made to the same formula as the pH 3.10 buffer except that pH was adjusted with 6M hydrochloric acid to 4.40 instead of 3.10.

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Printed in Belgium

Note

Calculation of degree of substitution of polysaccharides : a FORTRAN computer program*

D. HORTON AND W. D. PARDOE
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210 (U. S. A.)
(Received July 5th, 1969)

Calculation of the degree of substitution (d.s.) of a polysaccharide derivative from analytical data obtained experimentally becomes very tedious when more than one substituent is involved. The computer program here presented was developed to facilitate such calculations for polysaccharide derivatives containing two substituents. The program generates analytical percentages for derivatives having degrees of substitution for each substituent from 0.01 (in steps of 0.02) to the maximum possible, and matches the percentages calculated to those obtained experimentally. Only those values that match the experimental data within the limits set are printed out. The limits are initially 0.30%; if no match is found, the limits are widened by 0.20% and the cycle is continued until a match is found, or until the limit reaches 2.00%, when the calculation is terminated.

The program (Fig. 1) is written in FORTRAN IV, and requires a processor that permits expressions of mixed mode. Five data-cards are necessary, as indicated in the program. It is recommended that the experimental data be corrected for the presence of ash before use with this program.

Three examples of the use of the program are presented. An amylose derivative prepared in this laboratory[1,2] had *O*-(*p*-tolylsulfonyl) and chlorodeoxy substituents, and it was supposed that it had a total d.s. of less than 1.0. The molecular formula was, thus,

$$C_6H_9O_4(OH)_{(1-l-m)}(C_7H_7O_3S)_lCl_m,$$

and the program was used to determine values of l and m that matched the experimental data (found: C, 47.73; H, 6.11; S, 7.38; Cl, 2.81). Accordingly, the following data† were read in to the program:

*Supported by the Agricultural Research Service, U. S. Department of Agriculture, Grant No. 12-14-9201(71) (O.S.U.R.F. Project 2573) administered by the Northern Utilization Research and Development Division, Peoria, Illinois. Computer facilities on the IBM System 360/75 were provided by The Computer Center, The Ohio State University.

†The molecular formula of the unsubstituted polysaccharide is expressed as follows: JJ is set equal to the number of hydroxyl groups for which it is desired that substitution be examined. This number of hydroxyl groups is then subtracted from the total molecular formula of the unsubstituted polysaccharide, and JC, JH, and JO are set to correspond with the remainder of the molecular formula. In this example, JJ = 1, and therefore JC = 6, JH = (10−1) = 9, and JO = (5−1) = 4.

JC = 6, JH = 9, JO = 4, JJ = 1
LC = 7, LH = 7, LO = 3, LS = 1, LN = LX = 0
MC = MH = MO = MN = MS = 0, MX = 1
LL = MM = 1
VC = 47.73, VH = 6.11, VO = 35.97, VN = 0.00, VS = 7.38, VX = 2.81
XX = 35.457

Three matches were obtained from the calculation, giving the following degrees of substitution (d.s.). The level of matching was 0.30%.

D.s. (*chlorodeoxy*)	*D.s.* O-(p-*tolylsulfonyl*)
0.19	0.61
0.21	0.63
0.23	0.63

An amylose derivative also prepared in this laboratory[1,3] had a double bond at C-2–C-3 and a trityloxy group at C-6. The data read in were as follows:

JC = 6, JH = 7, JO = 2, JJ = 3
LC = 19, LH = 15, LO = 1, LN = LS = LX = 0
MC = MH = MO = MN = MS = MX = 0
LL = 1, MM = 2
VC = 78.90, VH = 6.03, VO = 15.07, VN = VS = VX = 0.00
XX = 0.000

The calculation yielded 190 matches ranging from d.s. 0.65 and 1.17 (for the C-6 substituent and the C-2,3 double bond, respectively)* to 1.53 and 0.03. All of these values matched the experimental figures within 0.30%. From analysis of the precursor used in preparing the derivative, the degree of substitution on C-6 was known to be 0.79; thus, the 190 values could be reduced to the three that had DL = 0.79. These gave d.s. values for the C-2,3 double bond of 0.97, 0.99, and 1.01.

A 3,6-anhydroamylose derivative[4] having chlorodeoxy and *O*-(*p*-tolylsulfonyl) substituents at C-2 and C-3, and a d.s. of 1.0 in the anhydro group, was read in as follows:

JC = 6, JH = 7, JO = 3, JJ = 1
LC = LH = LO = LN = LS = 0, LX = 1
MC = MH = 7, MO = 3, MN = MX = 0, MS = 1
LL = MM = 1
VC = 51.53, VH = 5.26, VO = 30.46, VN = 0.00, VS = 9.16, VX = 3.59
XX = 35.457

*When a single, functional modification involves two carbon atoms (as with the C-2–C-3 double bond) and there is an additional site of substitution, the computer matches values up to a d.s. of 3.00/2 = 1.50, so that d.s. values greater than the theoretical maximum (1.00, in this case) may be printed out.

```
C     THIS PROGRAM DERIVES ANALYTICAL DATA FOR POLYSACCHARIDES CONTAINING
C     TWO SUBSTITUENT GROUPS, FOR EACH D.S. VALUE FROM 0.01 IN STEPS OF
C     0.02 TO TOTAL SUBSTITUTION, AND MATCHES THE VALUES CALCULATED TO
C     THOSE OBTAINED EXPERIMENTALLY.
C     THE SUBSTITUENT GROUPS MAY CONTAIN THE FOLLOWING ELEMENTS -
C     CARBON, HYDROGEN, OXYGEN, NITROGEN, SULFUR, AND ANY ONE OTHER.
C     THE FOLLOWING DATA ARE READ IN ON FIVE DATA-CARDS -
C     CARD 1.   COMPOUND CODE NAME (MAXIMUM 60 CHARACTERS)
C     CARD 2.   MOLECULAR FORMULA OF THE UNSUBSTITUTED POLYSACCHARIDE,
C     IN THE FORM  C-JC  H-JH  O-JO  (OH)-JJ, WHERE JJ IS THE TOTAL
C     NUMBER OF HYDROXYL GROUPS AVAILABLE FOR SUBSTITUTION.
C     CARD 3.   MOLECULAR FORMULAS OF THE TWO SUBSTITUENT GROUPS -
C     (C-LC H-LH O-LO N-LN S-LS X-LX) AND (C-MC H-MH O-MO N-MN S-MS X-MX)
C     LL AND MM SPECIFY HOW MANY CARBON ATOMS OF THE SUGAR EACH GROUP
C     IS BONDED TO.
C     FOR THE ANHYDRO GROUP, PUT LC=LH=LN=LS=LX= 0 AND LO= 1  LL= 2
C     FOR AN UNSATURATED LINKAGE, PUT LC=LH=LO=LN=LS=LX= 0  AND  LL= 2
C     CARD 4.   OBSERVED ANALYTICAL DATA.
C     CARD 5.   ATOMIC WEIGHT OF ELEMENT X.
0001      100 FORMAT(15A4)
0002      101 FORMAT(4I10)
0003      102 FORMAT(14I5)
0004      103 FORMAT(6F10.2)
0005      104 FORMAT(F10.3)
0006      105 FORMAT(//////2X,15A4)
0007      106 FORMAT(//5X,1HC,7X,1HH,7X,1HO,7X,1HN,7X,1HS,7X,1HX,6X,4HDS*L,
     1   14X,4HDS*M)
0008      107 FORMAT(/6F8.2,4X,4H----,4X,4H----/)
0009      108 FORMAT(8F8.2)
0010      109 FORMAT(/1X,29HDATA DO NOT FIT THIS COMPOUND)
0011      110 FORMAT(/1X,15HMATCH LEVEL =F6.2)
0012          LOGICAL ZA,ZB
0013          DIMENSION TAG(15)
0014       10 READ(5,100) (TAG(I), I=1,15)
0015          READ(5,101)JC,JH,JO,JJ
0016          READ(5,102)LC,LH,LO,LN,LS,LX,MC,MH,MO,MN,MS,MX,LL,MM
0017          READ(5,103)VC,VH,VO,VN,VS,VX
0018          READ(5,104)XX
0019          ZB= .FALSE.
0020          WRITE(6,105) (TAG(I), I=1,15)
0021          WRITE(6,106)
0022          WRITE(6,107)VC,VH,VO,VN,VS,VX
0023          GG= 0.30
0024          II= 100*JJ
0025       20 DO 60 IA=1,II,2
0026          ZA= .FALSE.
0027          DO 50 IB=1,II,2
0028          IF((IA*LL+IB*MM) .GT. II) GOTO60
0029          CC=12.01*(100*JC+IA*LC+IB*MC)
0030          CH=1.008*(100*(JH+JJ) + IA*(LH-LL) + IB*(MH-II))
0031          CO=16.*(100*(JO+JJ) + IA*(LO-LL) + IB*(MO-MM))
0032          CN=14.01*(IA*LN+IB*MN)
0033          CS=32.07*(IA*LS+IB*MS)
0034          CX=XX*(IA*LX+IB*MX)
0035          XT=(CC+CH+CO+CN+CS+CX)/100.
0036          CC=CC/XT
0037          CH=CH/XT
0038          CO=CO/XT
0039          CN=CN/XT
0040          CS=CS/XT
0041          CX=CX/XT
0042          IF(ABS(VN-CN) .GT. GG) GOTO40
0043          IF(ABS(VS-CS) .GT. GG) GOTO40
0044          IF(ABS(VX-CX) .GT. GG) GOTO40
0045          IF(ABS(VC-CC) .GT. GG) GOTO40
0046          DL=IA/100.
0047          DM=IB/100.
0048          WRITE(6,108)CC,CH,CO,CN,CS,CX,DL,DM
0049          ZA= .TRUE.
0050          ZB= .TRUE.
0051          GOTO50
0052       40 IF(ZA) GOTO60
0053       50 CONTINUE
0054       60 CONTINUE
0055          IF(ZB) GOTO80
0056          IF(GG .GT. 2.00) GOTO70
0057          GG= GG+0.20
0058          GOTO20
0059       70 WRITE(6,109)
0060          GOTO90
0061       80 WRITE(6,110)GG
0062       90 GOTO10
0063          END
```

Fig. 1. Computer program in FORTRAN IV for calculation of the degree of substitution of polysaccharide derivatives containing two substituents.

The calculation yielded five values within 0.50% of the experimental data, having the following degrees of substitution:

D.s. (*chlorodeoxy*)	*D.s.* O-(p-*tolylsulfonyl*)
0.23	0.69
0.23	0.71
0.23	0.73
0.25	0.73
0.25	0.75

The experimental analysis for hydrogen is not matched in the program given in Fig. 1; this is because the absolute change in the calculated percentages for this element over the range of d.s. values is usually small. If matching for hydrogen is desired, it can be effected by inserting an extra step, between steps numbered 45 and 46 in the program, as follows:

IF(ABS(VH—CH) .GT. GG) GOTO40

The program can readily be modified for use with derivatives containing three substituents, but substantially more computer time would be required for the calculation. With the present program, one minute of computer time is sufficient for computing compositions from 10–20 separate sets of analytical data.

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Note

Studies on alkali-soluble, rice-bran hemicelluloses

H. GREMLI AND B. O. JULIANO
Chemistry Department, International Rice Research Institute, Los Baños, Laguna (Philippines)
(Received April 28th, 1969; in revised form, July 10th, 1969)

Whereas the composition and structure of wheat-flour hemicelluloses (pentosans) have been extensively investigated[1,2], there has been little work on rice hemicelluloses[3-5]. Preliminary experiments at the International Rice Research Institute showed that rice bran-polish contains about 0.1% of water-soluble and 1% of alkali-soluble hemicelluloses. Since the alkali-soluble hemicelluloses apparently comprise the major portion of rice-bran hemicelluloses, a study was conducted to determine their composition.

The alkali-soluble, rice-bran hemicellulose preparation had an L-arabinose: D-xylose ratio of unity (Table I), which was in agreement with previous results[3-5]. D-Galactose was its only major hexose constituent. The pectin it contained must have been a contaminant since it was retained on the *O*-(diethylamino)ethylcellulose (DEAE-cellulose) column during fractionation. The protein content was eluted on DEAE-cellulose chromatography, mainly in the fraction eluted with 0.25M NaOH. Since the protein of this fraction was readily separated from the polysaccharide by gel filtration and disc electrophoresis, it must also have been a contaminant. The protein must be of low molecular weight since it was not precipitated during treatment

TABLE I

COMPOSITION AND INTRINSIC VISCOSITY OF FRACTIONS OBTAINED BY DEAE-CELLULOSE CHROMATOGRAPHY OF ALKALI-SOLUBLE, RICE-BRAN HEMICELLULOSE

Hemicellulose fraction	*Wt % of eluted carbohydrate*	*Composition (% dry basis)*						*[η] (ml/g)*
		L-*Ara*	D-*Xyl*	D-*Gal*	D-*Glc*	*Protein*	*Uronic acid*	
Whole		37	34	11	trace	8	9	
Fractions from DEAE-cellulose								
0.1M borate	4–9	40	40	20	0	0	0	37
0.3M borate	34–35	46	46	8	0	< 1	0	154
0.5M borate	18–26	38	38	21	0	2.8	0	184[a]
0.25M NaOH	28–38	32	35	9	trace	24	trace	148[a]

[a] The [η] of the protein fractions was considered negligible.

with trichloroacetic acid. Hemicelluloses of rye[6] and wheat flour[1,2,7] have L-arabinose: D-xylose ratios of less than unity.

Fractions from the DEAE-cellulose column also had essentially the same L-arabinose:D-xylose ratio of unity, but differed in content of D-galactose (Table I). Their constant L-arabinose: D-xylose ratio indicated that they are L-arabino-D-xylans containing D-galactose residues rather than mixtures of L-arabinans and D-xylans. A pure L-arabino-D-xylan fraction was not isolated. Differences in the L-arabinose: D-xylose ratio of fractions of alkali-soluble, wheat-flour hemicelluloses eluted from DEAE-cellulose have been reported[2,6].

The intrinsic viscosity of the three fractions eluted with borate increased with increasing concentration of borate. The four fractions had molecular weights of >200,000, based on their exclusion from Sephadex G-200 gel. Although these fractions may be degradation products of a single hemicellulose, it has been postulated that the conditions during alkaline extraction are too mild to cause degradation of hemicellulose[6,7]. On the other hand, solutions of these borate fractions in 0.1M NaOH were degraded (decrease in viscosity) on standing overnight at 25°, while the NaOH fraction was not affected. The fractions were fairly water-soluble, although they were water-insoluble in the rice bran. However, the water-soluble, rice-bran hemicellulose preparation had a higher L-arabinose:D-xylose ratio (2.0) than these alkali-soluble fractions.

The enzymic studies gave some insight into the structure of these hemicelluloses. The arabinose residues of the hemicellulose fractions were shown to have the α-L-furanoside configuration, which is also characteristic of wheat-flour pentosans[1]. From 35 to 50% of these residues were nonreducing end-groups of the polysaccharide and were readily hydrolyzed by the α-L-arabinofuranosidase. These L-arabinose branches must be short since their hydrolysis resulted only in a slight drop in the intrinsic viscosity of the hemicelluloses (Fig. 1). A D-xylose or D-galactose residue linked to one of the nonreducing OH groups in the remaining L-arabinose residues

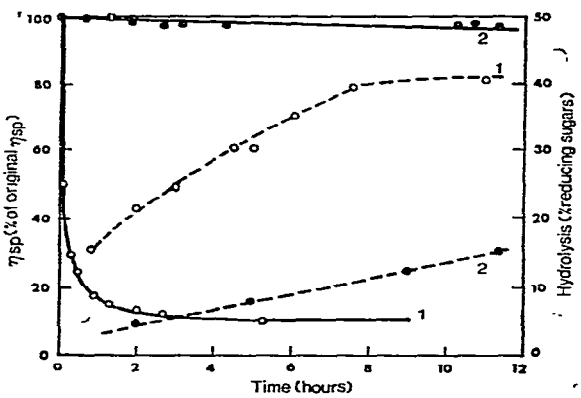

Fig. 1. Changes in specific viscosity (shown by the solid line) and percentage of hydrolysis during the enzymic degradation of the hemicellulose fraction eluted by 0.3M borate, with (1) Pectinol R-10 and (2) its purified α-L-arabinofuranosidase.

probably blocked the action of the α-L-arabinosidase in the original polymer. The viscosity of the hemicelluloses must be due primarily to the D-xylose residues, which were present in a greater proportion than D-galactose. Pectinol (containing an L-arabinosidase, a D-xylanase, a D-galactopyranosidase, and pectinase), but not its purified α-L-arabinosidase, appreciably decreased the viscosity of the hemicellulose (Fig. 1). The D-xylanase has been shown to degrade *O*-(carboxymethyl)-D-xylan[1]. The D-xylose and D-galactose residues probably have the β-D configuration since the hemicellulose had a high negative $[\alpha]_D$ value, as has been reported for wheat-flour hemicelluloses[2]. D-Mannose seemed also to be part of the polysaccharide moiety but it was detected only after enzymic removal of the bulk of the main sugar constituents. The presence of single D-mannose residues linked to the polysaccharide chain could explain the incomplete degradation of the hemicellulose to monosaccharides by Pectinol. This enzyme preparation has *endo*-D-mannanase activity but does not attack methyl D-mannosides[8].

EXPERIMENTAL

Rice bran-polish (270 g, variety IR-8) was repeatedly blended and washed with water, suspended in boiling water for 20 min, and cooled. The washed residue was digested[7] with α-amylase (Wallerstein Co., Staten Island, N. Y., negative for pentosanase). The starch-free residue (70 g) contained 14% of pentosans, as estimated with the orcinol reagent[9]. This crude preparation was extracted thrice with 0.5M NaOH under nitrogen[7]. The combined extract was neutralized with glacial acetic acid and centrifuged. The supernatant was treated with trichloroacetic acid to a final pH of 3.7, and centrifuged. The resulting supernatant was neutralized with NaOH and treated with ethanol to precipitate the hemicellulose. The precipitate was redissolved in water, dialyzed, and freeze-dried to a brown powder (2.3 g), $[\alpha]_D^{20}$ −200° (*c* 0.1, 0.5M pH 9.2 sodium borate) (Table I). This preparation was fractionated by chromatography on DEAE-cellulose in the borate form[10] (Cellex D, Bio-Rad Laboratories, Richmond, Calif., 0.76 meq/ml exchange capacity), with 0.1, 0.3, and 0.5M sodium borate (pH 9.2), and 0.25M NaOH in sequence for elution. The carbohydrate fractions eluted were pooled, dialyzed, and freeze-dried (Table I). The recovery of carbohydrate from the column was only 70%. The intrinsic viscosity of the four fractions was determined in 0.05M borate buffer at 25° in a Cannon–Ubbelohde semimicro dilution-viscometer No. 50. Gel filtration of these fractions on Sephadex G-200 with 0.05M borate buffer showed that the polysaccharides were eluted at the void volume. In the 0.25M NaOH fraction, a lower molecular-weight peak for protein was observed. Disc electrophoresis[11] of the NaOH fraction at pH 8.2 showed two fast-moving protein bands, and the polysaccharide remained at the point of application.

These fractions were also hydrolyzed at pH 4 with the methanol precipitate of Pectinol R-10 (Rohm and Haas, Philadelphia) and a purified α-L-arabinofuranosidase preparation from it (free of pectinases, D-xylanase, and D-galactopyranosidases, but containing cellulase and amylase impurities)[12]. Degradation was followed by

measurements of η_{sp} and reducing sugars (3,5-dinitrosalicylic acid–phenol method[1]). Hydrolysis of the hemicellulose fractions eluted by 0.3M borate with Pectinol R-10 resulted in a larger decrease in the viscosity and a greater extent of hydrolysis than with the purified α-L-arabinofuranosidase (Fig. 1). Essentially the same results were obtained with the other fractions. An arabinose was the only sugar detected by paper chromatography in the L-arabinosidase hydrolyzates but it constituted 35 to 50% of the total present in the polysaccharides. The Pectinol hydrolyzates contained L-arabinose, D-xylose, and D-galactose. Digestion of the residual polysaccharide with Pectinol after hydrolysis with L-arabinosidase also liberated the same three sugars. The oligosaccharides still present after exhaustive digestion of the hemicelluloses with Pectinol were found to contain D-mannose.

The total sugar or carbohydrate contents were determined by the phenol–sulfuric acid reagent[13], the pectin content by the carbazole method[14], and the protein content by the Folin–Ciocalteu reagent[15], with rice glutelin as standard. Hemicelluloses (5–20 mg) were hydrolyzed and subjected to descending chromatography on Whatman No. 1 paper with 8:2:1 ethyl acetate–pyridine–water as solvent[10], and the developed chromatograms were stained with aniline hydrogen phthalate[16]. For quantitative determinations, the sugars were eluted with water and assayed with the 3,5-dinitrosalicylic acid–phenol reagent[1]. The D-galactose content in the hydrolyzates was verified with D-galactose oxidase (Galactostat, Worthington Biochemicals Corp., Freehold, N. J.).

ACKNOWLEDGMENT

The authors are indebted to Prof. H. Neukom, Department of Agricultural Chemistry, Federal Institute of Technology, Zurich (Switzerland), for providing the purified L-arabinosidase.

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Note

Synthesis of substituted phenyl α-D-mannopyranosides

A. VERVOORT AND C. K. DE BRUYNE
Lab. Algemene en Biolog. Scheikunde, Ledeganckstraat 35, Ghent (Belgium)
(Received July 14th, 1969)

Nitrophenyl α-D-mannosides are useful chromogenic substrates for the quantitative determination of α-D-mannosidase activity, and 4-methylumbelliferyl α-D-mannoside and 6-bromo-2-naphthyl α-D-mannoside can be used for histochemical investigations, and for the localisation of α-D-mannosidase activity after analytical, disc electrophoresis on polyacrylamide and electrophoresis on agar gel. In the first case, the enzyme activity is detected by the u.v.-fluorescence of free 4-methylumbelliferone. In the second case; the polyacrylamide gel is incubated for 30 min in a solution of the bromonaphthyl mannoside and then treated with a solution (1 mg/ml) of diazotised 4,4′-bi-*o*-anisidine (4,4′-diamino-3,3′-dimethoxybiphenyl) (Fast Blue B, Fluka) in Tris buffer (pH 8.8). α-D-Mannosidase zones appear red–violet. The first method is the most specific and highly sensitive.

We have now prepared a series of substituted phenyl α-D-mannosides, by using the Helferich[1-3] method, for use in an investigation of the enzymic hydrolysis of these compounds. Fusion of α-D-mannose pentaacetate with the appropriate phenol in the presence of zinc chloride gave the compounds shown in Table I; the deacetylated derivatives are listed in Table II.

EXPERIMENTAL

The purity of the products was tested by t.l.c. on Silica Gel G (Merck) with ethyl acetate–benzene (3:7, v/v) for the mannoside acetates, and acetic acid–water–ethyl acetate (1:1:3, v/v) for the mannosides; detection was with 5% sulphuric acid in ethanol (10 min at 120°). Melting points were determined on a Mettler FP-2 instrument and are uncorrected. Optical rotations were measured with a Perkin–Elmer Model 141 polarimeter.

Preparation of glycosides. — α-D-Mannose pentaacetate[4] (0.05 mole) and the appropriate phenol (0.15–0.20 mole) were fused together and then treated with a solution of freshly fused zinc chloride (6 g) in a mixture (50 ml) of acetic acid–acetic anhydride (95:5, v/v). The melt was heated under diminished pressure for 1 h at 125–135°. The cooled mixture was dissolved in chloroform (500 ml) and washed successively with very dilute hydrochloric acid, ice-cold N sodium hydroxide, and water. The chloroform layer was dried (Na_2SO_4), treated with charcoal, and evapo-

TABLE I

ACETYLATED α-D-MANNOPYRANOSIDES

Aglycon group	*M.p.* (*degrees*)	*Yield* (%)	$[\alpha]_D^{22}$ (*degrees*) (c 2, *chloroform*)	*Found*(%) *C*	*H*	*Formula*	*Calc.*(%) *C*	*H*
p-Chlorophenyl	131–132	44	+77.5	52.3	5.4	$C_{20}H_{23}ClO_{10}$	52.3	5.1
p-Cresyl	80–81	53	+69.4	57.3	6.1	$C_{21}H_{26}O_{10}$	57.5	6.0
p-Methoxyphenyl	101–102	35	+95.9	55.6	5.7	$C_{21}H_{26}O_{11}$	55.5	5.4
p-Ethoxyphenyl	73–74	37	+66.4	56.4	5.9	$C_{22}H_{28}O_{11}$	56.4	6.0
p-Bromophenyl	132–133	33	+74.0	47.8	4.5	$C_{20}H_{23}BrO_{11}$	47.7	4.6
p-Ethylphenyl	103–104	48	+70.3	59.0	7.0	$C_{22}H_{28}O_{10}$	59.1	7.0
p-*tert*-Butylphenyl	111–113	28	+93.8	59.7	6.7	$C_{24}H_{30}O_{10}$	60.0	6.7
o-Nitrophenyl	131–133	26	+121.7	51.2	5.0	$C_{20}H_{23}NO_{12}$	51.2	4.9
m-Nitrophenyl	90–92	27	+81.2	51.3	4.9	$C_{20}H_{23}NO_{12}$	51.2	4.9
m-Cresyl	96–97	35	+72.7	57.5	6.0	$C_{21}H_{26}O_{10}$	57.5	6.0
m-Bromophenyl	95–96	35	+77.0	47.8	4.8	$C_{20}H_{23}BrO_{11}$	47.7	4.6
4-Methylumbelliferyl	160–161	37	+136.0	56.4	5.1	$C_{24}H_{27}O_{12}$	56.9	5.1
6-Bromo-2-naphthyl	169–170	45	—	52.0	4.6	$C_{24}H_{25}BrO_{10}$	52.1	4.7

TABLE II

α-D-MANNOPYRANOSIDES

Aglycon group	*M.p.* (*degrees*)	*Crystallisation solvent*	*Yield* (%)	$[\alpha]_D^{22}$ (*degrees*) (c 2, *methanol*)	*Found*(%) *C*	*H*	*Formula*	*Calc.*(%) *C*	*H*
p-Chlorophenyl	199–201	water	85	+127.5	49.6	5.4	$C_{12}H_{15}ClO_6$	49.6	5.2
p-Cresyl	166–167	water	88	+122.9	57.5	6.7	$C_{13}H_{18}O_6$	57.7	6.7
p-Methoxyphenyl	155–156	methanol	83	+122.6	54.3	6.3	$C_{13}H_{18}O_7$	54.5	6.3
p-Ethoxyphenyl	162–163	methanol	82	+107.0	55.7	6.6	$C_{14}H_{20}O_7$	56.0	6.7
p-Bromophenyl	207–209	water	84	+117.9[a]	43.7	4.6	$C_{12}H_{15}BrO_6$	43.6	4.5
p-Ethylphenyl	123–125	methanol	85	+106.3	59.0	7.0	$C_{14}H_{20}O_6$	59.1	7.0
p-*tert*-Butylphenyl	—	—	74	+110.7	60.4	7.7	$C_{16}H_{24}O_6$	60.4	7.7
o-Nitrophenyl	183–184	methanol	72	+99.8[b]	47.8	5.1	$C_{12}H_{15}NO_8$	47.8	5.0
m-Nitrophenyl	150–151	methanol	83	+130.8	47.7	5.1	$C_{12}H_{15}NO_8$	47.8	5.0
m-Cresyl	69–70	water	82	+115.9	57.5	6.7	$C_{13}H_{18}O_6$	57.8	6.7
m-Bromophenyl	133–134	water	88	+106.3	43.6	4.6	$C_{12}H_{15}BrO_6$	43.6	4.5
4-Methylumbelliferyl	222–225	water–*p*-dioxane	81	+178.2[a]	56.6	5.4	$C_{16}H_{19}O_8$	56.8	5.3
6-Bromo-2-naphthyl	246–248	dioxan	86	+144.0	49.7	4.3	$C_{16}H_{27}BrO_6$	49.8	4.4

[a] c 0.5; [b] c 1.0.

rated *in vacuo*. When dark-coloured, condensation products could not be removed with charcoal, solutions of the acetate in benzene were transferred to a column of alumina that was then eluted with more benzene; carbohydrate material was detected by the Molisch test. The products were crystallised from ethanol to constant m.p. and optical rotation (Table I).

The tetra-*O*-acetyl-α-D-mannopyranosides were catalytically deacetylated[5] by using barium methoxide for the nitrophenyl derivatives, and sodium methoxide for the other derivatives. The characteristics of the aryl α-D-mannopyranosides are shown in Table II. The *p-tert*-butylphenyl derivative, which has an amorphous structure and no defined m.p., dissolves immediately in all common solvents.

ACKNOWLEDGMENTS

We thank Professor Dr. L. Massart for his interest in this work, and Miss J. De Lat for performing the microanalyses.

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Note

Biological stability of the sulfate groups of chondroitin sulfate

J. PETER BENTLEY
University of Oregon Medical School, Portland, Oregon 97201 (U. S. A.)
(Received July 17th, 1969)

The sulfate groups on the 2-amino-2-deoxy-D-galactose residues of chondroitin sulfate are probably added following completion of a fairly large portion of the carbohydrate chain (for review see Ref. 1). This being so, it is reasonable to suppose that any preparation of chondroitin sulfate would contain a certain amount, albeit small, of undersulfated material, even though it may not be possible to demonstrate this undersulfation by analysis. It is thus not surprising that several authors[2-4] have been able to show that chondroitin sulfate, which is apparently fully sulfated, can accept additional sulfate groups under appropriate conditions, when measured by incorporation of radioactive sulfate. Addition of labeled sulfate groups to fully sulfated chondroitin sulfate could also occur if these groups were exchanged for non-radioactive sulfate on the carbohydrate chain. The experiment reported here was designed to determine whether such an exchange could in fact occur. This was done by incubating ^{35}S- or ^{14}C-labeled chondroitin sulfate with a supernatant fraction of chick embryo cartilage which contains a mucopolysaccharide sulfating system. The results indicate that no significant loss of previously incorporated sulfate groups occurred, but that the chondroitin sulfate was still capable of incorporating additional sulfate under these conditions.

EXPERIMENTAL

Ear cartilage from freshly killed 8-week old rabbits was cleaned and sliced on a commercial chopper, to a thickness of 0.2 mm. Portions of the cartilage slices (25 g) were incubated in Krebs-Ringer hydrogen carbonate buffer (85 ml), containing either 100 μc ^{14}C-uniformly labeled D-glucose or 100 μc sodium sulfate-^{35}S. We have previously described[5] the composition of the medium and the conditions of incubation. Following 4 h of incubation, the cartilage slices were rinsed with ice-cold physiological saline solution, homogenized in a Virtis-Homogenizer, and digested with papain. Chondroitin sulfate was isolated from the digest and analyzed as previously described[5]. The composition of this material is as follows: uronic acid 1.58 μmole/mg; hexosamine 1.31 μmole/mg; sulfate 1.35 μmole/mg. The hexosamine was shown by amino acid analysis to be 100% 2-amino-2-deoxygalactose. The polysaccharide was completely digested with testicular hyaluronidase. These data

are in agreement with the accepted structure of chondroitin sulfate. Analysis of this material with specific chondroitinases and sulfatases as described by Suzuki and his collaborators[6-8] showed that it consisted of 50% chondroitin 6-sulfate and 50% chondroitin 4-sulfate[9].

Reincubation with chick-embryo enzyme system. — The long-bone epiphyses from 24 15-day old chick-embryos were homogenized in a Potter-Elvehjem Homogenizer, and a 105,000-*g* supernatant fraction was prepared by differential centrifugation. Details of this preparation and characteristics of the mucopolysaccharide-sulfating system contained in this fraction have been described by Meezan and Davidson[4]. The volume of the supernatant was completed to a final volume of 50 ml with the buffer used for homogenization. Adenosine triphosphate (ATP, 5.7 μmole/ml) and nicotinamide adenine dinucleotide (NAD 0.14 μmole/ml) were added. Three series of 2-h incubations were performed using the prelabeled chondroitin sulfate described previously, and a nonradioactive sample of rabbit ear chondroitin sulfate prepared from fresh ear cartilage. Incubation conditions are given in Tables I and II.

TABLE I

INCORPORATION OF ^{35}S-LABELED SULFATE INTO RABBIT-EAR CHONDROITIN SULFATE BY A CHICK-EMBRYO ENZYME SYSTEM[a]

Experiment No.	*Incubation temperature (degrees)*	*D.p.m./μmole of uronic acid*
1		1,435
2	37	3,993
3		1,591
1		286
2	0	241
3		250

[a]Samples (8.42 mg) of unlabeled chondroitin sulfate were incubated for 2 h with a supernatant fraction of chick-embryo cartilage containing ATP (5.7 μmole/ml), NAD (0.14 μmole/ml), and 8 μc Na_2SO_4-^{35}S.

Following incubation, ethanol was added to a final concentration of 70% to all tubes, and the precipitate was recovered by centrifugation at 10,000 *g* for 1 h. The precipitate was washed with 70% ethanol and recentrifuged. The supernatant and washings were evaporated to dryness, and the residue was dissolved in water (0.2 ml) and counted in a liquid-scintillation spectrometer following suspension in fluor-containing Cab-o-sil thixotropic-gel powder (Packard Instruments Co.). The precipitated chondroitin sulfate was dissolved in water, and the content of uronic acid and the specific activity were determined.

The enzyme system is obviously capable of adding labeled sulfate to the apparently fully sulfated chondroitin sulfate (Table I), and this result confirms the work of Meezan and Davidson[4] who showed that this enzyme system mediated the incorporation of sulfate into several mammalian chondroitin sulfate preparations, and also

TABLE II

LOSS OF RADIOACTIVITY OF CHONDROITIN SULFATE INTO INCUBATION MEDIUM[a]

Substrate	*Expt.*	*Incubation temperatures (degrees)*	*Weight of substrate (mg)*	*Original specific activity (d.p.m./μmole of uronic acid)*	*Total activity (d.p.m.) incubated*	*D.p.m. in medium*	*Specific activity of recovered chondroitin sulfate (d.p.m./μmole of uronic acid)*	*Percentage of original specific activity*
Chondroitin sulfate-^{14}C	1					278	404	78.5
	2	37	8.31	514	7,017	252	412	80.1
	3					298	422	82.1
Chondroitin sulfate-^{14}C	1					241	401	78.0
	2	0	8.31	514	7,017	220	400	77.8
	3					274	432	84.0
Chondroitin sulfate-^{35}S	1					1,257	4,261	71.2
	2	37	8.32	5,978	74,018	1,126	4,168	69.7
	3					1,224	4,565	76.3
Chondroitin sulfate-^{35}S	1					814	4,316	72.2
	2	0	8.32	5,978	74,018	1,283	—	—
	3					1,028	4,402	73.6

[a]Labeled chondroitin sulfate was incubated for 2 h with a supernatant fraction of chick-embryo cartilage (2.5 ml, protein content 1.92 mg/ml, uronic acid content 1.24 μmole/ml, ATP content 5.7 μmoles/ml, and NAD content 0.14 μmole/ml).

into endogenous chick-embryo chondroitin sulfate. It is unlikely that the radioactivity present in the chondroitin sulfate was due to precipitation of an ethanol-insoluble salt, such as calcium sulfate-^{35}S, since the specific activity was much lower after incubation at 0°. Table II shows the results obtained after incubation of previously labeled chondroitin sulfate with the chick embryo system, and it can be seen that negligible amounts of label were found in the medium following a 2-h incubation. There was no difference between the radioactivities found in the medium after incubation at 37 or 0°, and since the percentage of the original activity found in the medium was roughly equivalent for both ^{35}S- and ^{14}C-labeled chondroitin sulfate consequently the small amount of activity observed in the medium can be explained by incomplete precipitation of chondroitin sulfate following incubation, and should not be attributed to a loss of sulfate groups previously incorporated into the polymer.

The specific activity of the chondroitin sulfate recovered after incubation was approximately 75% of the initial value, and again this figure is independent of incubation temperature or type of label.

The reduction in specific activity can be explained by dilution of the labeled chondroitin sulfate with a nonradioactive, uronic acid-containing material present in the cartilage preparation from the chick embryo. This material accounted for approximately 20% of the total uronic acid in the incubation mixture (Table II), and probably corresponds to the endogenous sulfate-acceptor characterized by Meezan and Davidson[10].

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Institutes of Health, U.S. Public Health Service (AM-9530, HE-10647, and HE-06336), and by a grant from the American Heart Association.

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Note

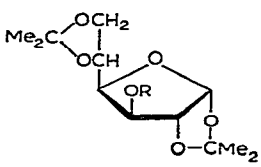
Sugars with reactive substituents: preparation of 3-*O*-(2,3-epoxypropyl and 2,3-epithiopropyl)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose

R. E. WING, W. M. DOANE, AND C. E. RIST

Northern Regional Research Laboratory, Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Peoria, Illinois 61604 (U. S. A.)

(Received March 26th, 1969; in revised form, July 18th, 1969)

This report describes methods whereby alkyl groups possessing oxirane and thiirane rings are etherified with 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**1**), a possible model for novel starch derivatives.



1 R = H

2 R = CH_2CHCH_2 (O)

3 R = Na

4 R = $CH_2CH{=}CH_2$

5a R = $CH_2CH(OH)CH_2Cl$

5b R = $CH_2CH(Cl)CH_2OH$

6 R = CH_2CHCH_2 (S)

3-*O*-(2,3-Epoxypropyl)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**2**) was prepared by five methods: (*a*) reaction of 1-chloro-2,3-epoxypropane with 1,2:5,6-di-*O*-isopropylidene-3-*O*-sodio-α-D-glucofuranose (**3**); (*b*) reaction of **1** with 1-chloro-2,3-epoxypropane and a Lewis acid catalyst[1–4]; (*c*) oxidation[5,6] of 3-*O*-allyl-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose[7] (**4**) with peroxybenzoic acid; (*d*) oxidation[8–10] of **4** with benzonitrile–50% hydrogen peroxide; and (*e*) addition[11] of hypochlorous acid to **4** to form chlorohydrins (**5a** and **5b**) followed by dehydrochlorination with sodium hydroxide[12].

Of these methods *a*, *c*, *d*, and *e* were easy to perform and gave **2** in almost quantitative yields. In method *b* the acidity of the Lewis acid catalysts was sufficient to open the oxirane ring of 1-chloro-2,3-epoxypropane[1–4] and to form the corresponding sugar chlorohydrin (**5a**); and also cause some deacetonation. Method *c* gave a quantitative yield of **2**, but the reaction was slow. The use of excess benzonitrile–50% hydrogen peroxide in method *d* did not improve the yield. In method *e*, care had to be taken to neutralize the hydrogen chloride as it was formed.

3-*O*-(2,3-Epithiopropyl)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**6**) was prepared[13,14] by three methods: (*a*) treatment of **2** with thiourea[15]; (*b*) treatment of **2** with potassium thiocyanate[16]; and (*c*) reaction of 1-chloro-2,3-epithiopropane[17] with **3**.

Compound **6** was easily made via the oxirane, and yields were quantitative. In method *a*, excess thiourea had to be added at the beginning of the reaction since degradation of **6** occurred if it were added later. Excess potassium thiocyanate can be added any time during the reaction without degradation of **6**. Quantitative yields of **6** were also obtained with 1-chloro-2,3-epithiopropane. However, the preparation of 1-chloro-2,3-epithiopropane[17] from 1-chloro-2,3-epoxypropane and thiourea was time-consuming because several fractional distillations of the product were necessary to remove unreacted starting materials.

Compounds **2** and **6** were obtained as syrups, which failed to crystallize. Each was homogeneous by t.l.c. and gave elemental analyses and spectral data consistent with their proposed structures. The oxirane and thiirane derivatives were soluble in all common solvents, even water. All solutions of **6** had to be evaporated at or below room temperature to prevent ring opening. On the other hand, the oxirane was stable in all hot solvents examined, including water.

EXPERIMENTAL

Optical rotations were determined with a Model 80 Rudolph* Polarimeter in 1-dm tubes. I.r. spectra were recorded on a Perkin–Elmer 137 Infracord spectrophotometer. For column chromatography, silica gel Adsorbosil (Applied Science Laboratories, Inc., P.O. Box 140, State College, Pennsylvania) 60–100 mesh neutralized to pH 7.0 with ammonium hydroxide was used with a graded hexane–chloroform eluant. T.l.c. was conducted on 20 × 20 cm plates of silica gel G (Brinkmann Instruments, Inc., Cantiague Road, Westbury, N.Y.) with 2:1 hexane–acetone as irrigant[18]. The plates were developed by spraying with a solution of 5% sulfuric acid in ethanol and heating for 20 min at 140°. Colored solutions were decolorized with Darco G-60 carbon.

3-O-(2,3-Epoxypropyl)-1,2:5,6-di-O-isopropylidene-α-D-glucofuranose (**2**). — *Method* a. Compound[19] **1** (10 g, R_F 0.41) was dissolved in anhydrous ether (100 ml) and was treated with excess sodium hydride (5 g) for 24 h at room temperature. The ether was removed under diminished pressure to yield **3**, which was treated with redistilled 1-chloro-2,3-epoxypropane (100 ml). After the reaction mixture had been stirred for 3 h at 90°, t.l.c. showed complete conversion into **2**. The reaction mixture was evaporated at 60° under diminished pressure and the product was extracted several times with ether. Evaporation of the ether extracts gave a syrup (12.2 g, 100%)

*The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

that contained only trace amounts of impurity. Column chromatography gave pure **2** as a syrup (12.1 g, 98%).

Method b[1–4]. Compound[19] **1** (1.0 g, R_F 0.41) was dissolved in 1-chloro-2,3-epoxypropane (20 ml) and boron trifluoride etherate (0.02 ml) was added. After 24 h at room temperature and 2 h at 50°, the reaction mixture was evaporated at 50° under diminished pressure. The resulting syrup (a mixture of **1** and **5a**) was dissolved in *p*-dioxane (50 ml) and powdered sodium hydroxide (2 g) was added. This mixture was stirred for 2 h at 50°. The solids were removed by filtration and the *p*-dioxane was removed at 60° under diminished pressure. The desired product (**2**) was present in the resulting syrup in about 30% yield as estimated by t.l.c.

Similar reaction and workup conditions but with an aluminum chloride catalyst in *p*-dioxane (20 ml), gave **2** in about 40% yield.

Method c[5,6]. Compound[7] **4** (4 g, R_F 0.77) was dissolved in benzene (5 ml) and a solution of peroxybenzoic acid[20] [0.55 g in chloroform (20 ml); 0.53 g required] was added. After the reaction mixture had been stirred for 4 days at room temperature, t.l.c. showed about a 90% conversion of **4** into **2**. An additional 0.05 g of peroxybenzoic acid was added. After a total of 7 days at room temperature, the reaction was complete. The solvent was removed at 30° and **2** was extracted into hexane. The remaining traces of benzoic acid were removed by column chromatography to yield **2** (95%).

Method d[8–10]. Compound[7] **4** (3.4 g, R_F 0.77) was dissolved in methanol (10 ml), and potassium hydrogen carbonate (0.34 g), benzonitrile (1.75 ml) and 50% hydrogen peroxide (1.16 g) were added. After the mixture had been stirred at room temperature overnight, t.l.c. showed about a 95% conversion into **2**. The filtrate from the mixture was evaporated at room temperature and then extracted with hexane. This hexane solution was extracted with several 150-ml portions of water to remove **2** from **4**. Upon evaporation of the water **2** remained as a pure syrup (3.4 g, 95%).

Method e[11,12]. Chlorine was bubbled through a solution of **4** (1.0 g, R_F 0.77) in *p*-dioxane (50 ml) and water (50 ml) for 30 min while a pH of 6 to 7 was maintained by the dropwise addition of 0.1N sodium hydroxide. The reaction mixture was extracted with chloroform (2 × 200 ml) and the combined extracts were dried with sodium sulfate. After filtration and evaporation of the dried extracts, the resulting syrup (1.12 g) contained about 80% of **5** (R_F 0.42 and 0.48) and 20% of degraded material (probably resulting from removal of isopropylidene groups) as estimated by t.l.c. I.r. analysis showed strong absorption at 3550 cm^{-1} (OH) and 725 cm^{-1} (C–Cl) but no absorption at 1650 cm^{-1} (C=C).

The syrup (1.12 g) was dissolved in *p*-dioxane (50 ml) and was treated with powdered sodium hydroxide (2 g) for 2 h at room temperature and for 1 h at 50°. T.l.c. showed complete conversion of **5** into **2**. Extraction of the evaporated filtrate from the reaction mixture with hexane left a syrup (1.01 g).

This procedure, repeated with calcium carbonate (5 g) in the reaction mixture to maintain neutrality, yielded only **5a** and **5b**.

Analysis of **2**. — The i.r. spectrum of the pure syrup showed no absorption at 3550 cm^{-1} (OH) but an absorption at 920 cm^{-1} (oxirane); $[\alpha]_D^{20}$ −20.5° (*c* 2, $CHCl_3$); R_F 0.50.

Anal. Calc. for $C_{15}H_{24}O_7$: C, 56.96; H, 7.59. Found: C, 56.76; H, 7.68.

3-O-(2,3-epithiopropyl)-1,2:5,6-di-O-isopropylidene-α-D-glucofuranose (**6**). — *Method* a[13–15]. First **2** (1.49 g) was dissolved in methanol (50 ml) and then thiourea (1.07 g) was added. After the reaction mixture had been stirred overnight at room temperature, t.l.c. showed that conversion into **6** was nearly complete. The reaction mixture was evaporated at room temperature and the residue was extracted with hexane (2 × 150 ml). Evaporation of the hexane afforded a syrup (1.56 g, 100%). The trace of **2** remaining was removed by column chromatography.

Method b[13,14,16]. Compound[7] **2** (1.77 g) was dissolved in acetone (50 ml) and potassium thiocyanate (1.65 g) was added. After the reaction mixture had been heated overnight under reflux, t.l.c. showed about 80% conversion into **6**. The reaction mixture was cooled and additional potassium thiocyanate (1.0 g) was added. After an additional 24 h of refluxing, t.l.c. showed complete conversion into **6**. The reaction mixture was cooled and hexane was added to precipitate almost all of the salt. After filtration and evaporation, the residue was extracted into hexane which was evaporated to give **6** (1.86 g, 100%).

Method c. Compound[19] **1** (1.0 g) was dissolved in anhydrous ether (50 ml) and treated with excess sodium hydride (2 g) for 24 h. The ether was evaporated at room temperature to give **3**. 1-Chloro-2,3-epithiopropane[17] (25 ml) was added and the mixture was stirred at 60°. After 4 h t.l.c. showed that reaction was complete. Evaporation gave a syrup, which was extracted into hexane and the hexane was evaporated to give **6** (1.27 g, 100%).

Analysis of **6**. — The i.r. spectrum of the pure syrup showed no absorption at 3550 cm^{-1} (OH) or 920 cm^{-1} (oxirane) and a weak absorption at 640–650 cm^{-1} (C–S); $[\alpha]_D^{20}$ −16° (*c* 2, $CHCl_3$); R_F 0.64.

Anal. Calc. for $C_{15}H_{24}O_6S$: C, 54.22; H, 7.23; S, 9.64. Found: C, 54.38; H, 7.34; S, 9.49.

ACKNOWLEDGMENTS

We are grateful to Mrs. Bonita Heaton and Mrs. Karen Jones for the microanalyses..

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Note

Quantitative micro-determination of glycolic acid in complex mixtures obtained from periodate oxidation of carbohydrates

S. Robert Sarfati and Patricia Szabó
Institut de Biochimie, Faculté des Sciences, 91—Orsay (France)
(Received July 28th, 1969)

Glycolic acid is known to be one of the products formed when ketoses are oxidised with periodate[1]. Methods previously described for the determination of this acid all require its prior isolation from the oxidation mixtures, since other products of the reaction, as well as the oxidant itself, interfere with the estimations[2,3]. In connection with work on the periodate oxidation of ketoses in the conditions[4] of the "cold acid" method, a simple and direct procedure for the determination of very small quantities of glycolic acid was required. A colorimetric method described by Calkins[5] has therefore been suitably modified. Interference by glyoxylic acid, formaldehyde, periodate, and iodate has been eliminated so that glycolic acid can be estimated directly in the oxidation mixtures.

EXPERIMENTAL

Analytical procedure. — (*a*) *Glycolic acid calibration curve.* Six standard solutions of potassium D-gluconate (0.6mM) and of sodium metaperiodate (6.6mM) in 0.1N sulphuric acid containing, respectively, 0 (blank), 10, 20, 30, 40, and 50 ml of a solution of glycolic acid (1.2mM) in 0.1N sulphuric acid, in a total volume of 100 ml, were prepared at 0° and 48 h before utilisation (to allow oxidation of the gluconate to go to completion), and were stored at 0°. These solutions are stable for at least 2 months. Calibration curves were done with each set of glycolic acid determinations.

(*b*) *Reagents:* (1) an aqueous solution of sodium acetate trihydrate (40% w/v); (2) a freshly prepared, aqueous solution of sodium borohydride (5% w/v); (3) 8N sulphuric acid; (4) a solution (0.01% w/v) of 2,7-dihydroxynaphthalene (recrystallised from water) in conc. sulphuric acid.

(*c*) *Method.* Portions (4 ml) of the six standard solutions and of the sample solutions (0.6mM with respect to the ketose and 6.6mM with respect to periodate, in 0.1N sulphuric acid at 0°) were pipetted into stoppered test-tubes, sodium acetate solution (0.55 ml) was added, and the tubes were kept for 24 h in the dark at room temperature (to allow destruction of glyoxylic acid). Sodium borohydride solution (1 ml) was added and, after 2 h, 8N sulphuric acid (4.45 ml), and the tubes were shaken thoroughly. Aliquots (1 ml) from each tube were treated with 2,7-dihydroxy-

naphthalene solution (10 ml), and the tubes were shaken, well immersed in a boiling-water bath and heated for 20 min, cooled under running water, and kept for 15 min at room temperature. The absorbances were then read at 530 nm against the standard blank solution. The amounts of glycolic acid present in the oxidation mixtures were determined from the calibration curve.

DISCUSSION

The method of Calkins[5] for the quantitative determination of glycolic acid is based on a sensitive colour reaction first described by Eegriwe[6]. When glycolic acid is heated with conc. sulphuric acid, formaldehyde is released; this condenses with 2,7-dihydroxynaphthalene to form colourless tetrahydroxydinaphthylmethane which is then oxidised to a violet–red dye. It is obvious, therefore, that formaldehyde, as well as substances which release formaldehyde when heated with conc. sulphuric acid, will interfere with the test, as will also such oxidising compounds as periodate and iodate.

Since Calkins[5] showed that glyoxylic and oxalic acids can be estimated as glycolic acid after reduction with magnesium powder, attempts were made to reduce the interfering compounds with this reagent in solutions of periodate (6.6mM)-oxidised mannitol that were 0.6mM in 0.1N sulphuric acid. It was found that the solutions thus treated still produced considerable and variable amounts of coloration in the glycolic acid test. When sodium borohydride was used in place of magnesium, the solutions obtained were only very slightly tinted. This faint colour did not interfere with the estimation of glycolic acid, as was shown by treating periodate-oxidised mannitol solutions, to which various amounts of glycolic acid had been added, with sodium borohydride and then with the 2,7-dihydroxynaphthalene reagent; the colour yields obtained were directly proportional to the amount of glycolic acid present, in the range of 3 to 20 μg (final dilution of the solution treated with 2,7-dihydroxy-naphthalene), *i.e.*, in the range of concentration expected to be present in oxidation mixtures of 0.6mM ketose solutions.

In addition to formaldehyde, periodate, and iodate, the periodate-oxidised solutions of ketoses also contain formic and glyoxylic acids (the latter is not oxidised by periodate in the conditions of the "cold acid" method[7]). The former does not produce colour in the glycolic acid test[5], and although it is partially reduced by magnesium[8], it is not attacked by borohydride[9]. Glyoxylic acid is reduced to glycolic acid by borohydride, but this transformation is apparently not quantitative in the conditions used; known amounts of glyoxylic acid, generated from D-gluconic acid by periodate oxidation, after treatment with borohydride, did not yield the expected amounts of dye in the glycolic acid test. Consequently, for accurate determination of glycolic acid, it is necessary to destroy any glyoxylic acid present before the reduction step with borohydride. This can be easily achieved[9] by adjusting the pH of the mixture to 5, since, at this pH and at room temperature, glyoxylic acid is oxidised quantitatively in less than 20 h; glycolic acid remains unaffected by periodate in

these conditions[10]. By using standard solutions containing formaldehyde, periodate, iodate, and glyoxylic acid (obtained by treating D-gluconic acid with periodate), in concentrations similar to those present in the oxidation mixtures of ketoses, and glycolic acid in various amounts, it was found that, after destruction of the glyoxylic acid followed by borohydride reduction, the colour produced in the glycolic acid test was directly proportional to the amounts of glycolic acid present. However, it was noted that the colour yields from glycolic acid in these solutions was lower than those obtained from equimolar solutions of glycolic acid in 0.1N sulphuric acid treated with sodium acetate and borohydride. For this reason, the amounts of glycolic acid produced upon periodate oxidation of ketoses were determined from calibration curves prepared by using the above-mentioned standard solutions. Calibration curves were linear over the range mentioned above; at concentrations lower than 10 μg/ml, the standard error, which is about $\pm 4\%$ from 10 to 20 μg/ml, increases to a maximum of $\pm 10\%$. In very dilute oxidation-mixtures, 10 μg represents about 0.2 molar equivalent of glycolic acid per mole of sugar; the error, on an absolute basis, is therefore quite small.

It was also shown that oxalic acid, which is not oxidised by periodate, gave no reaction in this test, even when present in ten-fold excess.

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Note

Preparation of inositol hexasulfate*

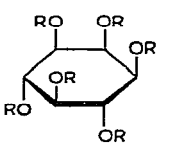
ALEXANDER J. FATIADI

Division of Analytical Chemistry, Institute for Materials Research, National Bureau of Standards, Washington, D. C. 20234 (U. S. A.)

(Received September 5th, 1969)

The only literature that reports on the preparation of *myo*-inositol hexasulfate (**1**) are those of Takahashi and Egami[1], and of Kimura and Noda[2]. The multistep procedure[1] involved the treatment of *myo*-inositol (1,2,3,5/4,6-inositol) suspended in ice-cold pyridine with chlorosulfonic acid as the sulfating agent; product **1** was isolated as the calcium salt, and this was converted into the hexapotassium salt, **2**, but the yield was not stated.

It has now been found that inositol hexasulfate (**1**) can conveniently be prepared by direct interaction of *myo*-inositol with either fuming sulfuric acid or chlorosulfonic acid (when a spray of sulfuric acid is applied to inositol spots on a t.l.c. plate, it does not cause charring, even on slight heating, in contrast to spots of sugars or alditols). The former process involves the gradual addition of inositol to warm, stirred, fuming sulfuric acid until a solution is obtained (see experimental procedures A and B). The reaction may also proceed with chlorosulfonic acid, which is less oxidizing than sulfuric acid, and is therefore recommended for esterification of the more sensitive inositols (Procedure C). As yet another alternative, sulfur trioxide, dissolved in *N*,*N*-dimethylformamide, may be used as the sulfating agent.



1 R = SO_3H
2 R = SO_3K
3 R = Ac
4 R = Bz

RESULTS AND DISCUSSION

The temperature at which the esterification is conducted should be kept at 60–66° when fuming sulfuric acid is used, and somewhat higher (70–85°) when chlorosulfonic acid is used. These esterifications are exothermic, and, during most of the time required for reaction, are self-sustaining. Control of the temperature is necessary, because higher temperatures cause darkening of reaction mixtures, and result in decreased yields.

*Part VI. Methods in Inositol Chemistry. For Part V, see *Carbohyd. Res.*, 12 (1970) 130. This paper was released (for publication) by the Patent Advisory Board of the National Bureau of Standards on July 2, 1969.

Hexasulfate **1** may be isolated from the reaction mixture as its hexapotassium salt (**2**) (generally, in 55–62 percent yield) by an exchange reaction (Procedure A) with potassium chromate, the salt of an acid of approximately the same strength as sulfuric acid (and of hexasulfate **1**); the moderately soluble salt **2** crystallizes out, leaving free chromic acid and sulfuric acid in the solution.

In an alternative procedure (Procedure B), at completion of sulfation, the inositol hexasulfate is neutralized by adding potassium hydroxide (3.6 equivalents) to the sulfation mixture; organic and inorganic salts of potassium are formed (in equilibrium), but salt **2** crystallizes out first.

Use of inexpensive, common reagents, namely, *myo*-inositol (a side-product in the corn industry) and fuming sulfuric acid or chlorosulfonic acid, and simplicity of operation make this process both very economical and applicable to preparation on a larger scale than that reported.

The hexapotassium salt of *myo*-inositol hexasulfate (**2**) is a relatively stable compound. For example, when the crude salt is stored in a dry place for 2 years at room temperature, it develops only a light-pink coloration that can readily be removed by recrystallization from hot water, after treatment with carbon; however, after 6 months of storage at room temperature in the presence of moist air, darkening and partial decomposition occur. Free inositol hexasulfate (**1**) can be obtained from a very dilute solution of **2** by treatment with Amberlite IR-120 (H^+) cation-exchange resin; free acid **1** is a syrup, and is a very strong acid having $pK \sim 0.88$ (by titration). Hexapotassium salt **2** was readily converted into hexaacetate **3** (m.p. 214–216°) and hexabenzoate **4** (m.p. 256–258°) in 80% and 75% yield, respectively.

In contrast to the well-explored chemistry of inositol hexaphosphate (phytic acid)[3–5] and sulfuric acid esters of sugars[6], the chemistry of inositol hexasulfates (or partially esterified derivatives) has not yet been explored, except for a few biochemical applications of **2**. In these, it was used for selective separation of the basic protein clupeine by forming a salt-like complex between the sulfate groups and the arginyl residues of the protein[7,8]. As a consequence of its ability to interact with proteins, the possibility of existence of **2** or partially sulfated inositols in Nature is not excluded.

The above procedures can be used for the preparation of hexasulfates of other inositols, and these will be described in a forthcoming publication.

EXPERIMENTAL

General. — Spectra were recorded with a Perkin–Elmer Model 257 grating i.r. spectrophotometer*. Analyses were made by William Schmidt of the Microchemical Analysis Section of this Division.

*Mention of a commercial instrument in this paper does not imply recommendation or endorsement by the National Bureau of Standards.

myo-*Inositol (hexapotassium hexasulfate)* (**2**). — *Procedure A.* Fuming sulfuric acid (300 g, containing 30% of sulfur trioxide, 4.87 moles) was placed in an Erlenmeyer flask provided with a magnetic stirring-bar, a thermometer extending to the bottom of the flask, and a loosely stoppered drying-tube containing calcium chloride. The acid was heated to 60° (oil bath), and finely powdered *myo*-inositol (30 g, 166 mmoles) was introduced in small increments during 2.75–3 h. The reaction is exothermic, and the temperature of the reaction mixture should be kept at 60–66° by cooling. Sometimes, near the end of the reaction, the temperature must be raised to 70–75° for 10 to 15 min, in order to dissolve any unreacted material. If the temperature exceeds 80°, some decomposition usually ensues, as shown by darkening of the solution. The light-yellow to brownish-yellow solution containing *myo*-inositol hexasulfate was then cooled to about 35°, and was carefully* poured into a stirred slurry of a solution of potassium chromate (120 g, a 10% excess) in water (500 ml) with crushed ice (about 700 g). The greenish-yellow suspension was then diluted with cold methanol (300 ml), stirred for 30 min at 0–3°, and filtered. The precipitate was washed successively with ice-cold water (100 ml) and ice-cold 1:1 water–methanol (200 ml). The wet, somewhat colored product was then recrystallized from hot water (about 700 ml); cooling and washing produced snow-white needles of **2** (as the dihydrate when dried *in vacuo* at room temperature); yield 82–85 g (53–55%); the test for SO_4^{2-} by addition of Ba^{2+} was negative.

Anal. Calc. for $C_6H_6K_6O_{24}S_6 \cdot 2H_2O$: C, 7.78; H, 1.08; S, 20.78. Found: C, 7.82; H, 0.96; S, 20.44.

The twice-recrystallized sample of salt was dried for 4 h at 196°/0.05 mmHg to give an anhydrous product, m.p. 362–364° (dec.; darkening at 352°); a trace of moisture was somewhat difficult to remove.

Anal. Calc. for $C_6H_6K_6O_{24}S_6$: C, 8.11; H, 0.68; S, 21.63. Found: C, 7.99; H, 0.77; S, 21.32.

An air-dried sample of hexapotassium salt **2** (0.2 g) was dried for 4 h at 140°/0.1 mmHg; the loss in weight corresponded to two molecules of water per molecule.

Compound **2** is best characterized by its i.r. spectrum, particularly a quintet in the region of 1070 to 1000 cm^{-1}. Because the spectrum of a potassium bromide pellet containing **2** shows a little deformation (apparently due to interaction with the bromide), the i.r. spectrum of **2** in Nujol mull is given: ν_{max}^{Nujol} 3500 w (OH), 2740 m (C–H), 1640 w (OH), 1235 s (S=O)[6,9], 1175 sh, 1150 w, 1130 m, 1070 m, 1052 m, 1040 m, 1020 s, 1000 m, 960 sh, 950 s, 872 s, 838 s (C–O–S)[6,9], 790 s, 700 s, and 650 s cm^{-1}.

Procedure B. — *myo*-Inositol (30 g, 166 mmoles) was treated with fuming sulfuric acid (300 g) as described in Procedure A. The light-yellow solution was cooled, mixed with 95% ethyl alcohol (500 ml), and cooled to −15 to −20° by

*Caution: this is an exothermic reaction; a hood, an ice–salt cooling bath, and an efficient, mechanical stirrer should be used.

addition of solid carbon dioxide. The clear, cold solution was then neutralized with a solution of potassium hydroxide (200 g) in water (550 ml), with stirring and cooling. The white, crystalline precipitate was separated, washed with cold water (75 ml), and recrystallized from hot water (750 ml); yield of crystalline dihydrate **2**, 65 g (42%); the test for SO_4^{2-} was negative.

Procedure C. — Inositol (25 g, 138 mmoles) was treated with chlorosulfonic acid (250 g) for about 2 h at 70–85°. (Because of vigorous evolution of hydrogen chloride, the use of a hood is imperative). The increments of inositol added were lessened toward the end of the reaction, because of the considerable heat of reaction (external cooling is necessary) and formation of foam. The almost colorless solution was then decomposed (extreme caution is necessary because of the very vigorous, exothermic reaction) with an ice-cold slurry of a solution of potassium chromate (110 g) in water (500 ml) with crushed ice (1 kg). The temperature of the reaction mixture was kept at 0–5° throughout this procedure; yield of the dihydrate **2**, 70–72 g (54–56%).

Identification of myo-*inositol in milligram quantities.* — *myo*-Inositol (2 mg) was stirred with fuming sulfuric acid (0.2 ml) for 10 min at 70° (water bath); then, ice-cold, 0.05M potassium chromate solution (0.2 ml), crushed ice (1 g), and cold methanol (1 ml) were added; this gave salt **2**, 7–8 mg (70–80%), readily identified by its i.r. spectrum.

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Note

Aryl glycosides of oligosaccharides
Part II. Bromo-, chloro-, and iodo-phenyl β-D-glycosides of some disaccharides

I. C. M. DEA
Department of Chemistry, University of Stirling, Stirling (Great Britain)
(Received July 28th, 1969)

An understanding of the preferred conformations of polysaccharide molecules awaits detailed knowledge of the molecular geometry of the glycosidic linkage between sugar residues. X-ray diffraction analysis of oligosaccharides is an obvious candidate for the investigation of this problem. The *p*-bromophenyl β-glycosides of cellobiose, lactose, and maltose[1], and the halophenyl β-D-glycosides listed in Tables I and II have been prepared for this purpose. X-ray studies of the suitably crystalline derivatives will be described elsewhere.

The hepta-*O*-acetyl derivatives (Table I) of the aryl β-glycosides of cellobiose, lactose, and maltose were synthesised by an adaptation[1] of Griebel and Helferich's method[2]. For the bromophenyl and chlorophenyl β-D-glycosides, the yields decrease in the series $p>m>o$; the *p*-iodophenyl β-D-glycosides were obtained in much higher yield than the *o*-iodophenyl analogues. Such decreases in yield might be expected to be due to steric hindrance. However, the relatively small differences in yield between the peracetylated derivatives of the *m*- and *o*-substituted phenyl β-D-glycosides (much smaller than the difference between the yields of the *p*- and *m*-substituted derivatives) suggests that steric hindrance might not be the only factor.

An interesting finding in these studies is that, for the eight types of aryl β-D-glycosides, there is always a decrease in yield for the series cellobiose > lactose > maltose. Earlier syntheses of aryl β-D-glycosides of disaccharides support these findings. Thus, the percentage yields of the hepta-*O*-acetyl derivatives of phenyl β-cellobioside, phenyl β-lactoside, and phenyl β-maltoside formed under similar reaction conditions to those employed in the present study were, respectively, 75 (ref. 3), 52 (ref. 3), and 45% (ref. 4). The reason for this trend is not yet clear.

The bromo-, chloro-, and iodo-phenyl β-cellobiosides were obtained by deacetylation of the corresponding hepta-*O*-acetyl derivatives by using methanolic sodium methoxide. The physical properties of these compounds are listed in Table II. Recrystallisation from water gave single crystals of *o*-bromophenyl β-cellobioside and *o*-iodophenyl β-cellobioside large enough for study by X-ray diffraction. The other aryl β-cellobiosides were obtained either as very small, single crystals or as spherulite crystals.

TABLE I

PHYSICAL DATA FOR ARYL HEPTA-*O*-ACETYL-β-D-GLYCOSIDES

Hepta-O-acetyl derivative	*M.p.* (*degrees*)	$[\alpha]_D$ (*degrees*)	*Yield* (%)	*Found* (%)		*Formula*	*Calc.* (%)	
				C	*H*		*C*	*H*
m-Bromophenyl β-cellobioside	183–185	−27.8	44	48.57	5.00			
m-Bromophenyl β-lactoside	153–156	−21.4	31	48.52	4.96			
m-Bromophenyl β-maltoside	133–134	+33.2	19	48.33	5.06			
o-Bromophenyl β-cellobioside	189–191	−39.7	39	48.51	5.12	$C_{32}H_{39}BrO_{18}$	48.53	4.96
o-Bromophenyl β-lactoside	185–186	−37.2	24	48.41	5.01			
o-Bromophenyl β-maltoside	141–144	+9.1	13.5	48.51	4.92			
p-Chlorophenyl β-cellobioside	223–224	−29.3	54	51.51	5.33			
p-Chlorophenyl β-lactoside	149–151	−21.7	39	51.48	5.26			
p-Chlorophenyl β-maltoside	174–176	+44.7	35	51.35	5.25			
m-Chlorophenyl β-cellobioside	186–187	−30.6	48	51.69	5.34			
m-Chlorophenyl β-lactoside	138–140	−26.8	25	51.56	5.34	$C_{32}H_{39}ClO_{18}$	51.52	5.27
m-Chlorophenyl β-maltoside	155–157	+57.1	22	51.41	5.30			
o-Chlorophenyl β-cellobioside	181–183	−36.0	40	51.34	5.26			
o-Chlorophenyl β-lactoside	188–190	−38.3	20	51.61	5.28			
o-Chlorophenyl β-maltoside	134–137	+46.8	18	51.43	5.28			
p-Iodophenyl β-cellobioside	239–241	−16.0	55	45.74	4.60			
p-Iodophenyl β-lactoside	165–167	−17.5	36	45.69	4.66			
p-Iodophenyl β-maltoside	131–133	+39.6	31	45.59	4.63			
o-Iodophenyl β-cellobioside	184–186	−31.2	41	45.72	4.70	$C_{32}H_{39}IO_{18}$	45.77	4.69
o-Iodophenyl β-lactoside	143–145	−41.5	19	45.74	4.70			
o-Iodophenyl β-maltoside	149–150	+59.4	19	45.76	4.72			

TABLE II

PHYSICAL DATA FOR ARYL β-CELLOBIOSIDES

Glycoside	*M.p.* (*degrees*)	$[\alpha]_D$ (*degrees*)	*Found* (%)		*Formula*	*Calc.* (%)	
			C	*H*		*C*	*H*
m-Bromophenyl	231–233	−47.5	43.27	5.21	$C_{18}H_{25}BrO_{11}$	43.45	5.07
o-Bromophenyl	223–226	−46.4	43.29	5.12			
p-Chlorophenyl	214–215	−55.3	47.78	5.62	$C_{18}H_{25}ClO_{11}$	47.74	5.57
m-Chlorophenyl	228–230	−56.6	47.60	5.60			
o-Chlorophenyl	217–219	−49.1	47.59	5.57			
p-Iodophenyl	229–231	−45.6	39.56	4.80	$C_{18}H_{25}IO_{11}$	39.71	4.63
o-Iodophenyl	223–225	−41.7	39.52	4.75			

EXPERIMENTAL

Melting points are uncorrected. Rotary evaporations were carried out at or below 40°. Specific rotations were determined at 589 nm by using a Perkin–Elmer 141 polarimeter at 23°. N.m.r. spectra were measured on a Perkin–Elmer R-10 spectrometer for solutions in carbon tetrachloride, with tetramethylsilane as internal standard. The acetobromo derivatives of the disaccharides were synthesised from the β-D octa-*O*-acetyl derivatives by the standard literature methods[5,6].

Halophenyl hepta-O-acetyl-β-D-glycosides of disaccharides[1]. — Solutions of the potassium salt of the phenol in water were mixed with a solution of the acetobromo derivative of the disaccharide in acetone and kept for 24 h. After extraction of the product with benzene, the peracetylated β-D-glycosides were crystallised from ethanol. N.m.r. measurements confirmed their identity and purity.

Halophenyl β-cellobiosides. — Halophenyl hepta-*O*-acetyl-β-cellobiosides were deacetylated by refluxing with 0.01N methanolic sodium methoxide[1], and the products were recrystallised from ethanol.

ACKNOWLEDGMENTS

The author thanks Professor R. P. Bell and Dr. P. Murray-Rust for the interest and encouragement they have afforded this work, and Mr. Cameron of the Analytical Section, Department of Chemistry, University of Glasgow, for performing the microanalyses.

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CORRIGENDUM

Carbohyd. Res., 11 (1969) page 544, legend for Fig. 2 should read:

Fig. 2. Plot of $\log (k'_1 + k'_2)$ *vs.* $\log [G_t]$ for the mutarotation of D-glucose in dry pyridine.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

NUCLEOSIDE VON FLUOR-ZUCKERN

TEIL III*. SYNTHESE 5'-FLUORSUBSTITUIERTER DESOXYRIBONUCLEOSIDE

G. KOWOLLIK, K. GAERTNER, G. ETZOLD, UND P. LANGEN
Institut für Biochemie der Deutschen Akademie der Wissenschaften zu Berlin, Berlin-Buch (D. D. R.)
(Eingegangen den 24. Juli 1969)

ABSTRACT

2',5'-Dideoxy-5'-fluororibonucleosides are conveniently obtained by treatment of the corresponding 5'-*O*-tosyl† derivatives with an excess of tetrabutylammonium fluoride in *N*,*N*-dimethylformamide at 50°. The p.m.r. spectra of the fluoro derivatives establish unequivocally their structure. Surprisingly, treatment of 3',5'-di-*O*-mesylthymidine‡ with potassium fluoride or with tetrabutylammonium fluoride gave mesylanhydrothymidines rather than fluorine-containing products. Under similar conditions, 5'-deoxy-5'-iodothymidine was converted mainly into 4',5'-unsaturated 5'-deoxythymidine.

ZUSAMMENFASSUNG

Es wird gezeigt, daß 2',5'-Didesoxy-5'-fluorribonucleoside in einfacher Weise aus den entsprechenden 5'-*O*-tosylierten Derivaten durch Umsatz mit Tetrabutylammoniumfluorid in *N*,*N*-Dimethylformamid bei 50° erhalten werden. Die p.m.r.-Spektren der Fluor-Derivate erweisen einwandfrei deren Struktur. Überraschenderweise bildet 3',5'-Di-*O*-mesylthymidin mit Kaliumfluorid oder mit Tetrabutylammoniumfluorid kein fluorhaltiges Produkt, sondern mesylierte Anhydrothymidine. Aus 5'-Desoxy-5'-jodthymidin entsteht unter ähnlichen Bedingungen vorwiegend 5'-Desoxy-4',5'-didehydrothymidin.

EINLEITUNG

Wie wir kürzlich zeigen konnten, ist 5'-Desoxy-5'-fluorthymidin (**2**) ein Inhibitor-Analoges von Thymidin-5'-monophosphat bei der Thymidin-5'-monophosphat–Kinase-Reaktion und hemmt daher selektiv die Desoxyribonucleinsäure-Synthese[2]. Dieser Befund veranlaßte uns, nach allgemeinen anwendbaren Verfahren für die Darstellung von weiteren im Zuckerteil fluor-substituierten 2'-Desoxyribonucleosiden zu suchen und die erhaltenen Verbindungen in der angegebenen Richtung biochemisch zu testen.

*Teil II, Ref. 1.
†Tosyl = *p*-toluenesulfonyl.
‡Mesyl = methanesulfonyl.

Im Zuckerteil fluor-substituierte Pyrimidin- und Purin-Nucleoside der Ribose und der Arabinose sind durch Spaltung geeigneter Nucleosid-Derivate mit wasserfreiem Fluorwasserstoff[3] oder durch Kondensation von Pyrimidin- und Purin-Basen mit dem entsprechenden Fluor-Zucker[4] erhalten worden. Analoge Fluor-Nucleoside der 2-Desoxy-D-*erythro*-pentose waren bisher nicht bekannt. Zur Darstellung primärer Fluor-Derivate von Desoxyribonucleosiden haben wir die nucleophile Substitution von *p*-Tosyloxy-Gruppen durch Fluor in einer Reihe von 5'-*O*-Tosyl-desoxyribonucleosiden eingehend untersucht. Über Versuche zur Einführung von Fluor am C-3' von Desoxyribonucleosiden zwecks Darstellung sekundärer Fluor-Derivate wird in Kürze berichtet[1,5].

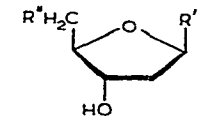
ERGEBNISSE UND DISKUSSION

Ausgangsprodukte waren die entsprechenden 5'-*O* tosylierten Desoxyribonucleoside: 5'-*O*-Tosylthymidin (**1**)[6], 2'-Desoxy-5'-*O*-tosyl-5-fluoruridin (**5**)[7], 2'-Desoxy-5'-*O*-tosyladenosin (**9**)[8] und das bisher unbekannte 2'-Desoxy-5'-*O*-tosyluridin (**3**). Sie wurden durch Umsetzung des entsprechenden Nucleosids mit Tosylchlorid in Pyridin gewonnen. 2'-Desoxy-5'-*O*-tosylcytidin (**7**)[9-11] ließ sich auf diesem direkten Weg jedoch nur in *ca.* 12-proz. Ausbeute aus 2'-Desoxycytidin gewinnen. Dagegen konnte *N*-Acetyl-2'-desoxycytidin[12] mit Tosylchlorid in Pyridin bei Raumtemperatur leicht in *N*-Acetyl-2'-desoxy-5'-*O*-tosylcytidin (**11**) übergeführt werden. Wir bevorzugten daher diese Verbindung zur Darstellung von 2',5'-Didesoxy-5'-fluorcytidin (**8**). Sowohl 2'-Desoxy-5'-*O*-tosylcytidin (**7**) als auch *N*-Acetyl-2'-desoxy-5'-*O*-tosylcytidin gehen mit überschüssigem Acetanhydrid in kristallines *N*,3'-*O*-Diacetyl-2'-desoxy-5'-*O*-tosylcytidin über, eine Substanz, die bisher nur als "solid foam" beschrieben[10] ist.

Zur Überführung der Tosyl- in die entsprechenden Fluor-Nucleoside verwendeten wir zunächst Kaliumfluorid. So konnte 5'-Desoxy-5'-fluorthymidin (**2**) erstmals durch kurzzeitiges Erhitzen von 5'-*O*-Tosylthymidin (**1**) mit Kaliumfluoriddihydrat in Methanol auf 150° gewonnen werden[2]. Es war nicht überraschend, daß unter diesen überwiegend S_N1-Bedingungen durch Einbeziehung des Lösungsmittels in die Reaktion Nebenprodukte entstanden, wodurch eine verhältnismäßig geringe Ausbeute (16%) an 5'-Desoxy-5'-fluorthymidin (**2**) resultierte. Auch in aprotonischen Lösungsmitteln (z.B. mit wasserfreiem Kaliumfluorid in Acetamid), erhielten wir unbefriedigende Ergebnisse. Das veranlaßte uns, das von Foster, Hems, *et al.*[13] zur Darstellung primärer und insbesondere sekundärer Fluor-Kohlenhydrate eingeführte Tetrabutylammoniumfluorid auf seine Verwendbarkeit zur Einführung von Fluor in Nucleoside zu prüfen. Tetrabutylammoniumfluorid ist in aprotonischen Lösungsmitteln gut löslich und gestattet damit Fluor unter überwiegend S_N2-Bedingungen einzuführen.

Mittels Tetrabutylammoniumfluorid konnten wir alle vorher genannten 5'-*O*-Tosyl-desoxyribonucleoside (**1, 3, 5, 7, 9**) mit teilweise guten Ausbeuten in die entsprechenden 5'-Desoxy-5'-fluor-Derivate (**2, 4, 6, 8, 10**) überführen.

1 R′ = 1-(Thyminyl); R″ = TsO
2 R′ = 1-(Thyminyl); R″ = F
3 R′ = 1-(Uracilyl); R″ = TsO
4 R′ = 1-(Uracilyl); R″ = F
5 R′ = 1-(5-Fluoruracilyl); R″ = TsO
6 R′ = 1-(5-Fluoruracilyl); R″ = F
7 R′ = 1-(Cytidinyl); R″ = TsO
8 R′ = 1-(Cytidinyl); R″ = F
9 R′ = 9-(Adeninyl); R″ = TsO
10 R′ = 9-(Adeninyl); R″ = F
11 R′ = 1-(*N*-Acetylcytidinyl); R″ = TsO
17 R′ = 1-(Thyminyl); R″ = I
18 R′ = 1-(Thyminyl)



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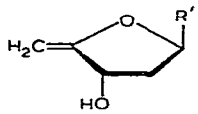

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Allgemein erwiesen sich die folgenden Bedingungen als besonders geeignet : *N*,*N*-Dimethylformamid als Lösungsmittel, Verwendung eines etwa 6M Überschusses an Tetrabutylammoniumfluorid (mit Ausnahme von *N*-Acetyl-2′-desoxy-5′-*O*-tosylcytidin (**11**), für das ein 50–80M Fluorid-Überschuß zur Erzielung einer guten Ausbeute erforderlich ist), Reaktionstemperatur 50°. Nach Abdestillieren des *N*,*N*-Dimethylformamids wurde in Wasser gelöst und das überschüssige Tetrabutylammoniumfluorid durch kombinierte H^+-Ionenaustauscher–Calciumcarbonat-Behandlung entfernt. Die Fluor-Nucleoside wurden durch Säulenchromatographie isoliert. Durch Jodieren von 2′,5′-Didesoxy-5′-fluoruridin (**4**) nach der Methode von Prusoff[14] erhielten wir außerdem 2′,5′-Didesoxy-5′-fluor-5-joduridin.

Außer durch die üblichen physikalischen Daten und durch Elementaranalyse wurden die 2′,5′-Didesoxy-5′-fluorribonucleoside durch p.m.r.-Spektren charakterisiert. Wesentliche Werte der chemischen Verschiebungen sind in Tabelle I zusammengestellt. Die Kopplungseffekte des Fluors mit den H-5′-Protonen und dem H-4′-Proton erweisen zweifelsfrei die Struktur der Verbindungen. In allen Spektren überlagert sich die eine Hälfte der aufgespaltenen Signale für die H-5′-Protonen und für das H-4′-Proton zum Teil mit den Signalen von H-3′, so daß ein breites Multiplett bei etwa τ 5,70 resultiert. Weitere Signale erscheinen für ein H-5′, als Duplett bei etwa τ 4,95 und für 0,5 H-4′ als Quadruplett bei τ 6,0–6,2. Die Lage der übrigen Signale ist gegenüber den unsubstituierten Nucleosiden nahezu unverändert.

In der Absicht, selektiv die primäre Mesyloxy-Gruppe in 3′,5′-Di-*O*-mesylthymidin (**12**)[15] durch Fluor zu substituieren, bezogen wir auch diese Verbindung in unsere Untersuchungen ein. Überraschenderweise konnten wir jedoch weder beim Umsatz mit Tetrabutylammoniumfluorid noch mit Kaliumfluorid die Bildung eines fluorhaltigen Produktes nachweisen. Dagegen entstanden z. B. mit Kaliumfluorid in Dimethylsulfoxid bei 90° 2,3′-Anhydro-1-(2′-desoxy-5′-*O*-mesyl-β-D-*threo*-pentofuranosyl)thymin[15–17] (**13**) und 2,5′-Anhydro-1-(2′-desoxy-3′-*O*-mesyl-β-D-*erythro*-pentofuranosyl)thymin[18] (**14**) im Verhältnis 2:1. Diese Verbindungen reagieren mit Natronlauge zu charakteristischen Folgeprodukten: **13** geht quantitativ in 1-(3′,5′-Anhydro-2′-desoxy-β-D-*threo*-pentofuranosyl)thymin (**15**), **14** zu 90% in 1-(2′-

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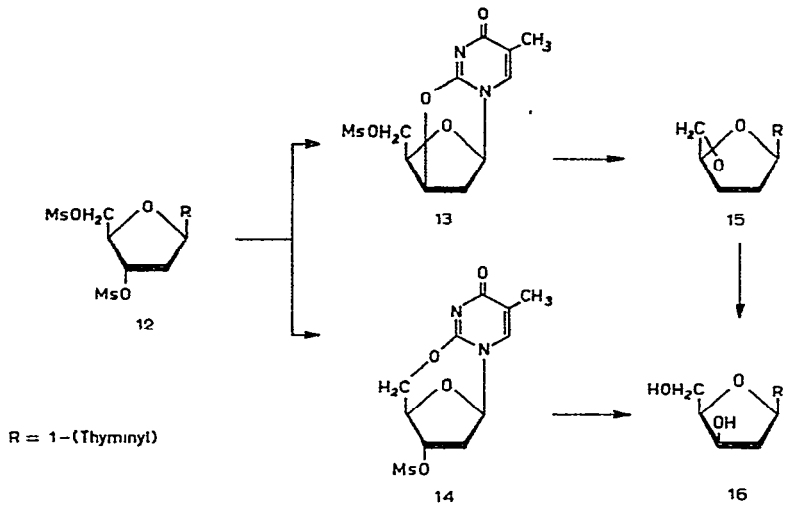
TABELLE I

CHEMISCHE VERSCHIEBUNGEN IN τ (P.P.M.) UND H,F-KOPPLUNGSKONSTANTEN (C.P.S.) VON 2',5'-DIDESOXY-5'FLUORRIBONUCLEOSIDEN[a]

Verbindung	*H-1'*	*2 (H-2')*	*1 (H-5')+H-3' +0,5 (H-4')*	*0,5 (H-4')*	*1 (H-5')*	*NH*	*NH_2*	$J_{H-5',F}$	$J_{H-4',F}$
5'-Desoxy-5'-fluor-thymidin (2)[b]	3,76 (t)	7,70–8,02 (m)	5,55–6,05 (m)	6,23 (q)	4,98 (d)	−1,28	—	48,5	25
2',5'-Didesoxy-5'-fluor-uridin (4)[b]	3,77 (t)	7,65–8,02 (m)	5,60–6,00 (m)	6,25 (q)	4,99 (d)	−0,33 bis −1,67	—	47,5	25
2',5'-Didesoxy-5,5'-difluor-uridin (6)[b]	3,81[c] (t)	7,67–7,92 (m)	5,60–5,93 (m)	6,22 (q)	4,98 (d)	−0.33 bis −1,25	—	48	25
2',5'-Didesoxy-5'-fluor-cytidin (8)[d]	3,55[c] (t)	7,47–7,81 (m)	5,40–5,73 (m)	5,97 (q)	4,96	—	2,57 (s)	47	25
2',5'-Didesoxy-5'-fluor-adenosin (10)[b]	3,54 (t)	7,00 (m)	5,23–5,87 (m)	6,05 (q)	4,95 (d)	—	2,70 (s)	47,5	23

[a]s = Singulett; d = Duplett; t = Triplett; q = Quadruplett; m = Multiplett. [b]Gemessen mit einem Gerät Varian A-60 in Dimethylsulfoxid-d_6 und Tetramethylsilan als internem Standard. [c]Mit Unteraufspaltungen. [d]Gemessen mit einem Gerät Varian HA-100 in Dimethylsulfoxid-d_6 mit Hexamethyldisiloxan als externem Standard.

Desoxy-β-D-xylofuranosyl)thymin (**16**) über. Dementsprechend entstehen — bedingt durch das alkalische Reaktionsmilieu — ausschließlich diese Folgeprodukte bei der Umsetzung von **12** mit Kaliumfluoriddihydrat in Methanol aus primär gebildetem **13** und **14**.



Bei der Umsetzung von 5′-Desoxy-5′-jodthymidin (**17**) mit Kaliumfluorid oder mit Tetrabutylammoniumfluorid wurde unter allen geprüften Bedingungen überwiegend 4′,5′-Didehydro-5′-desoxythymidin (**18**) erhalten. Die optimalen Bedingungen für diese Reaktion, die dem bekannten Silberfluorid–Pyridin-Verfahren[19] zur Darstellung ungesättigter Kohlenhydrat-Derivate mit exocyclischer Doppelbindung analog ist, haben wir jedoch nicht untersucht.

Über die biochemischen Wirkqualitäten einiger der dargestellten Verbindungen wird an anderer Stelle berichtet.

EXPERIMENTELLER TEIL

Allgemeines. — Schmelzpunkte wurden auf dem Mikroheiztisch nach Boëtius (korr. Werte) bestimmt, u.v. Spektren mit dem selbstregistrierenden Spektralphotometer Beckman DK-2 aufgenommen. Die folgenden Fließmittel wurden für die Säulen- und Dünnschichtchromatographie benutzt : (*A*) 12:1:6 Essigsäureäthylester–2-Propanol–Wasser; (*B*) 8:2 Chloroform–Äthanol; (*C*) 9:1 Chloroform–Äthanol; und (*D*) wassergesättigter Butylalkohol. Die Dünnschichtchromatographie verlief aufsteigend auf Kieselgel HF_{254} (E. Merck, Darmstadt). Die Substanzen wurden im u.v. Licht durch Fluoreszenzlöschung und nach Besprühen mit Schwefelsäure lokalisiert. Zur Säulenchromatographie wurden verwendet : Kurzsäulen mit „Kieselgel G (oder H) zur Dünnschichtchromatographie" (E. Merck, Darmstadt). Methodik : Füllung einer Glassäule der Abmessung 45 × 250 mm mit 100 g Adsorbens durch Einschlämmen und Absetzenlassen mit dem jeweiligen Fließmittel, Betrieb

der Säule unter geringem Überdruck. Normale Säulen mit Celite (Hyflo-Super-Cel, Johns-Manville Co.) (zur Methodik siehe Lit.[20]) oder mit "Kieselgel zur Säulenchromatographie" (E. Merck, Darmstadt); der Verlauf der säulenchromatographischen Trennungen wurde mit Hilfe des u.v. Durchflußphotometers LKB Uvicord Typ 8301 A bei 254 nm registriert und/oder dünnschichtchromatographisch verfolgt. Die Elementaranalysen wurden nach mehrstündigem Trocknen der Substanzen *in vacuo* über Phosphorpentoxid mit dem Perkin-Elmer 240 Elemental Analyzer angefertigt.

5'-Desoxy-5'-fluorthymidin (**2**). — Eine Lösung von 5'-*O*-Tosylthymidin (**1**) (4 g, 10 mmol) und Tetrabutylammoniumfluorid (15 g, 57,5 mmol) in *N,N*-Dimethylformamid (100 ml) wird während 24 Stdn. auf 50° erhitzt. Danach wird das *N,N*-Dimethylformamid abdestilliert und der ölige Rückstand (18 g) in Wasser (500 ml) gelöst. Die wässrige Lösung gibt man auf eine mit Dowex-50W X-8, (50–100 mesh, H^+, 40 ml) gefüllte Säule und eluiert mit Wasser, bis im Eluat keine u.v. Absorption mehr nachweisbar ist. Das fluorwasserstoffsaure Eluat läßt man in eine intensiv gerührte Calciumcarbonat-Suspension (10 g in *ca.* 50 ml Wasser) eintropfen. Nach Absaugen und Einengen *in vacuo* wird der Rückstand (5,3 g) in der unteren (wässrigen) Phase des Fließmittels (*A*) gelöst (9 ml), mit Celite (25 g) gleichmäßig verrührt und an einer Celite-Säule (400 g) mit der oberen (organischen) Phase des Fließmittels (*A*) chromatographiert. Man erhält ein angereichertes Produkt (1,4 g), das nach der Chromatographie an einer Kieselgel-Säule (250 g) mit Fließmittel (*B*) analysenreines **2** (0,60 g, 24,6%) vom Schmp. 201–203° ergibt (Lit.[2]: Schmp. 203–205°).

2'-Desoxy-5'-O-tosyluridin (**3**). — Zu einer Lösung von 2'-Desoxyuridin (5 g, 22 mmol) in trockenem Pyridin (4,5 ml) wird bei 0° unter Rühren eine Lösung von Tosylchlorid (5 g, 26,2 mmol) in Pyridin (4,5 ml) getropft. Man beläßt 16 Stdn. bei 0° und gießt danach in Eiswasser (250 ml). Die wässrige Lösung extrahiert man mit Chloroform (3 × 100 ml) und wäscht die vereinigten Chloroform-Extrakte nacheinander mit gesätt. Natriumhydrogencarbonat-Lösung und mit Wasser. Nach dem Trocknen mit Natriumsulfat wird *in vacuo* zur Trockne eingeengt und zum Rückstand mehrfach absol. Äthanol zugesetzt und *in vacuo* wieder abdestilliert. Der verbleibende Rückstand wird aus 90%. Äthanol umkristallisiert und ergibt **3** (4,76 g, 57%). Ein analysenreines Produkt wird durch weiteres Umkristallisieren aus 96% Äthanol unter Zusatz von wenig Aktivkohle erhalten. Schmp. 160–162° (Zers.); u.v. Spektrum (Methanol): λ_{max} 262 (ε 11,700) und 223 nm (ε 18,000); λ_{min} 241 nm (ε 5850).

Anal. Ber. für $C_{16}H_{18}N_2O_7S$ (382,39) : C, 50,25; H, 4,74; N, 7,33. Gef. : C, 50,56; H, 4,60; N, 7,60.

2',5'-Didesoxy-5'-fluoruridin (**4**). — Ein Gemisch aus **3** (3,82 g, 10 mmol), Tetrabutylammoniumfluorid (15 g, 57,5 mmol) und *N,N*-Dimethylformamid (100 ml) wird während 24 Stdn. auf 50° erhitzt. Die Aufarbeitung erfolgt analog der von **2**. Man erhält **4** (0,55 g, 24%) nach chromatographischer Reinigung an einer Celite-Säule (800 g) mit Fließmittel (*A*) durch Kristallisation aus Aceton–Petroläther; Schmp. 158,5–159,5°; $[\alpha]_D^{20}$ +25,3° (*c* 1,46, Wasser); u.v. Spektrum (Methanol) : λ_{max} 262 nm (ε 10,350); λ_{min} 230 nm (ε 2320).

Anal. Ber. für $C_9H_{11}FN_2O_4$ (230,19) : C, 46,96; H, 4,82; F, 8,25; N, 12,17. Gef. : C, 47,31; H, 4,98; F, 8,70; N, 12,20.

Das *3'-O-Acetyl-Derivat* wurde durch Kristallisation aus Aceton erhalten, Schmp. 190–193°.

Anal. Ber. für $C_{11}H_{13}FN_2O_5$ (272,23) : C, 48,53; H, 4,81; N, 10,29. Gef. : C, 48,50; H, 4,53; N, 10,02.

2',5'Didesoxy-5'-fluor-5-joduridin. — **4** (0,1 g) wird mit Jod (0,1 g) in Chloroform (1 ml) und M Salpetersäure (2 ml) während 2 Stdn. unter Rückfluß erhitzt. Es scheiden sich Kristalle ab, die abgesaugt und mit Wasser und Äther gewaschen werden (55 mg, 36%). Nach Umkristallisieren aus Wasser werden farblose Nadeln erhalten, die sich oberhalb 190° allmählich zersetzen; u.v. Spektrum (Wasser) : λ_{max} 285 nm (ε 8700), λ_{min} 247 nm (ε 3000).

Anal. Ber. für $C_9H_{10}FJN_2O_4$ (356,10) : C, 30,36; H, 2,83; N, 7,87. Gef. : C, 30,65; H, 2,72; N, 7,85.

2',5'-Didesoxy-5,5'-difluoruridin (**6**). — Ein Gemisch aus **5** (2 g, 5 mmol), Tetrabutylammoniumfluorid (8 g, 30,6 mmol) und *N*,*N*-Dimethylformamid (50 ml) wird während 90 Min. auf 50° erhitzt. Die Aufarbeitung erfolgt analog der von **2**. Nach chromatographischer Reinigung an einer Kurzsäule (Kieselgel G) mit Fließmittel (*C*) erhält man durch Kristallisation aus Äthanol **6** (1,15 g, 92,5%), Schmp. 184°; $[\alpha]_D^{23}$ +14.3° (*c* 1,48, Wasser); u.v. Spektrum (Methanol) : λ_{max} 268 nm (ε 10,250), λ_{min} 236 nm (ε 3640).

Anal. Ber. für $C_9H_{10}F_2N_2O_4$ (248,19) : C, 43,56; H, 4,06; F, 15,25; N, 11,29. Gef. : C, 43,58; H, 4,05; F, 15,31; N, 11,28.

Das *3'-O-Acetyl-Derivat* wurde durch Kristallisation aus Aceton–Petroläther erhalten, Schmp. 148–149°.

N-*Acetyl-2'-desoxy-5'-O-tosylcytidin* (**11**). — Zu einer Suspension von *N*-Acetyl-2'-desoxycytidin (3 g, 10 mmol) in Pyridin (20 ml) wird unter Rühren bei 0° eine Lösung von Tosylchlorid (5,2 g, 27,4 mmol) in Pyridin (20 ml) zugetropft. Die Eiskühlung wird entfernt und das Gemisch während 4 Stdn. bei Raumtemperatur gerührt. Danach tropft man unter Rühren in Eiswasser (300 ml) und extrahiert mit Chloroform (3 × 50 ml). Die vereinigten Chloroform-Extrakte werden nacheinander mit gesätt. Natriumhydrogencarbonat-Lösung und mit Wasser gewaschen, mit Natriumsulfat getrocknet und *in vacuo* zur Trockne eingeengt. Das Rohprodukt (4,32 g) wird an zwei Kurzsäulen (Kieselgel H) mit Fließmittel (*A*) gereinigt. Man erhält **11** (2,3 g, 54%) als farblosen Schaum, der beim Stehen in Gegenwart von Äthanol in der Kälte in farblose Prismen vom Schmp. 132–134° (Zers.) übergeht; $[\alpha]_D^{20}$ +45,4° (*c* 1,41, Methanol); u.v. Spektrum (Methanol) : λ_{max} 213, 248, und 297 nm (ε 17,400, 17,450 und 9580), Schulter bei 227 nm, λ_{min} 239 und 273 nm (ε 15,300 und 6350); n.m.r. Spektrum : Singuletts bei τ 7,87 (*N*-Acetyl) und bei τ 7,52 (aromatische Me-Gruppe).

Anal. Ber. für $C_{18}H_{21}N_3O_7S$ (423,44) : C, 51,05; H, 5,00; N, 9,92. Gef. : C, 51,45; H, 5,13; N, 9,43.

Das *Pikrat* wurde durch Kristallisation aus 96% Äthanol erhalten, Schmp. 169–171° (Zers.).

Anal. Ber. für $C_{24}H_{24}N_6O_{14}S$ (652,55) : C, 44,17; H, 3,71; N, 12,88. Gef. : C, 43,94; H, 3,90; N, 13,35.

2'-Desoxy-5'-O-tosylcytidin (7). — Zu einer Suspension von 2'-Desoxycytidin (4,5 g, 19,8 mmol) in Pyridin (30 ml) wird bei 0° unter Rühren eine Lösung von Tosylchlorid (6 g, 31,5 mmol) in Pyridin (40 ml) zugetropft. Man beläßt während 24 Stdn. bei 0°. Danach wird die Lösung unter Rühren in Eiswasser (250 ml) getropft und die wässrige Lösung mit Chloroform (3 × 100 ml) extrahiert. Die vereinigten Chloroform-Extrakte werden nacheinander mit gesätt. Natriumhydrogencarbonat-Lösung und mit Wasser gewaschen, mit Natriumsulfat getrocknet und *in vacuo* zur Trockne eingeengt. Der Rückstand wird in 5:4:1 Methanol–Chloroform–Wasser (100 ml) gelöst und die Lösung auf eine Säule mit Dowex 50-W X-8 (50–100 mesh, H^+, 20 ml) gegeben. Mit Methanol wird *N*,3'-*O*-Di-tosyl-2'-desoxycytidin, danach mit 5M Ammoniak (*ca.* 1 Liter) das mit 2'-Desoxycytidin verunreinigte Hauptprodukt eluiert. Die ammoniakalische Lösung wird *in vacuo* zur Trockne eingeengt. Der Rückstand wird mit Eiswasser (2 × 20 ml) verrieben, wobei 2'-Desoxycytidin gelöst und das Hauptprodukt verfestigt wird. Nach Umkristallisieren aus Methanol wird **7** (0,89 g, 11,8%) als farbloses, amorphes Produkt vom Schmp. 145–147° erhalten. Die analysenreine Substanz schmilzt bei 149–152° (Lit.[9] : 120–123°); $[\alpha]_D^{22}$ +45,9° (*c* 1,50, Methanol); u.v. Spektrum (Wasser) : λ_{max} 227 und 270 nm (ε 20,200 und 9240), λ_{min} 212 und 250 nm (ε 16,200 und 6870). N.m.r. Spektrum : Singulett bei τ 7,56 (aromatische Me-Gruppe), breites Singulett bei τ 2,75 (NH_2).

Anal. Ber. für $C_{16}H_{19}N_3O_6S$ (381,40) : C, 50,38; H, 5,02; N, 11,02; S, 8,41. Gef. : C, 50,52; H, 5,05; N, 10,94; S, 8,81.

N,3'-O-Diacetyl-2'-desoxy-5'-O-tosylcytidin. — Diese Verbindung entsteht quantitativ aus **7** oder aus **11** nach Behandlung mit überschüssigem Acetanhydrid in Pyridin bei Raumtemperatur. Das Rohprodukt wurde an einer Kurzsäule (Kieselgel H) mit Fließmittel (*A*) gereinigt. Nach Stehen über Äther wird aus 96% Äthanol umkristallisiert. Farblose Prismen vom Schmp. 138–141° (Zers.); $[\alpha]_D^{22}$ +38° (*c* 1,44, Methanol); u.v. Spektrum (Methanol) : λ_{max} 214, 248,5 und 297 nm (ε 29,900, 18,400 und 9300), Schulter bei 227 nm, λ_{min} 238,5 und 275 nm (ε 15,500 und 6370). N.m.r. Spektrum : Singuletts bei τ 7,95 (*O*-Ac), τ 7,86 (*N*-Ac) und τ 7,58 (aromatische Me-Gruppe), breites (schwach aufgespaltenes) Singulett bei τ −0,9 (NH).

Anal. Ber. für $C_{20}H_{23}N_3O_8S$ (465,47) : C, 51,60; H, 4,98; N, 9,03. Gef. : C, 51,45; H, 4,96; N, 8,91.

2',5'-Didesoxy-5'-fluorcytidin (8). — (*a*) Ein Gemisch aus **7** (0,33 g, 0,87 mmol), Tetrabutylammoniumfluorid (1,3 g, 5 mmol), und *N*,*N*-Dimethylformamid (9 ml) wird während 24 Stdn. auf 50° erhitzt. Danach wird das *N*,*N*-Dimethylformamid *in vacuo* abdestilliert und der Rückstand (1,7 g) in Wasser (10 ml) gelöst. Die wässrige Lösung gibt man auf eine Säule mit Dowex-50W X-8 (50–100 mesh, H^+, 10 ml) und eluiert mit Wasser, bis das Eluat neutral abläuft. Das Eluat wird verworfen. Danach werden mit 15% Ammoniak die nucleosidischen Bestandteile eluiert. Die ammonia-

kalische Lösung wird zur Trockne eingeengt und das Substanzgemisch an einer Kurzsäule (Kieselgel H) mit Fließmittel (*D*) getrennt. Man erhält **8** (0,13 g, 64%), das aus absol. Äthanol umkristallisiert wird und farblose Prismen vom Schmp. 215–220° bildet.

(*b*) Ein Gemisch aus **11** (0,28 g, 0,66 mmol), Tetrabutylammoniumfluorid (15 g, 57,5 mmol), und *N,N*-Dimethylformamid (15 ml) wird während 20 Stdn. auf 50° erhitzt. Danach wird das *N,N*-Dimethylformamid *in vacuo* abdestilliert. Den Rückstand löst man in Methanol (10 ml) und gibt die methanolische Lösung zu einer wässrigen Calciumcarbonat-Suspension (8 g in 100 ml Wasser). Unter Rühren wird Dowex-50W X-8 (50–100 mesh, H^+, 40 ml) zugesetzt*. Nach 30 Min. Rühren wird über eine G4-Fritte abgesaugt und die wässrige Lösung *in vacuo* zur Trockne eingeengt. Zum Rückstand wird mehrfach absol. Äthanol zugesetzt und *in vacuo* wieder abdestilliert. Man erhält ein rötliches Öl (1,3 g), aus dem durch Chromatographie an einer Kurzsäule (Kieselgel H) mit Fließmittel (*B*) *N*-Acetyl-2',5'-didesoxy-5'-fluorcytidin (0,15 g, 84%) isoliert wird. Dieses wird durch 20stdg. Stehen in methanolischem Ammoniak (bei 0° halbgesättigt) quantitativ in **8** übergeführt. Nach Abdestillieren des Methanols wird das Rohprodukt aus absol. Äthanol umkristallisiert, Schmp. 210–216° (Zers.); u.v. Spektrum (Wasser) : λ_{max} 271 nm (ε 9780). λ_{min} 248 nm (ε 6170); (0,1M Salzsäure) : λ_{max} 278,5 nm, λ_{min} 241 nm.

Anal. Ber. für $C_9H_{12}FN_3O_3$ (229,21) : C, 47,16; H, 5,27; N, 18,33. Gef. : C, 46,81; H, 5,33; N, 17,01.

Das *Pikrat.* — Zers. P. 204-206°, Äthanol.

Anal. Ber. für $C_9H_{12}FN_3O_3 \cdot C_6H_3N_3O_7$ (458,32) : F, 4,15. Gef. : F, 4,45.

Das N,*3'*-O-*Diacetyl-Derivat* wurde durch Kristallisation aus absol. Äthanol erhalten. Prismen, Schmp. 186–188,5°; u.v. Spektrum (Wasser) : λ_{max} 246 und 297 nm (ε 16,250 und 9400), λ_{min} 270,5 nm (ε 4480).

Anal. Ber. für $C_{13}H_{16}FN_3O_5$ (313,28) : C, 49,81; H, 5,15; F, 6,06; N, 13,41. Gef. : C, 50,01; H, 5,45; F, 6,00; N, 13,20.

2',5'-Didesoxy-5'-fluoradenosin (**10**). — Ein Gemisch aus **9** (4,4 g, 10,8 mmol), Tetrabutylammoniumfluorid (17,7 g, 66,9 mmol) und *N,N*-Dimethylformamid (110 ml) wird während 48 Stdn. auf 50° erhitzt. Tetrabutylammoniumfluorid wird nach der bei **8**, Verfahren (*b*), beschriebenen Methode abgetrennt. Der nach Einengen der wässrigen Lösung verbleibende Rückstand (9,2 g) wird an zwei Kurzsäulen (Kieselgel G) mit Fließmittel (*B*) chromatographiert, wobei **10** (1,98 g, 72,2%) erhalten wird. Das analysenreine Produkt schmilzt bei 215–217° (Zers.) nach Kristallisation aus Methanol; $[\alpha]_D^{23}$ −21,3° (*c* 1,35, Methanol); u.v. Spektrum (Methanol) : λ_{max} 258,5 nm (ε 15,600), λ_{min} 227 (ε 3920).

*Bei Anwendung einer Dowex-Säule werden die 2'-Desoxycytidin-Derivate auf der Säule festgehalten. Man wäscht in diesem Falle mit Wasser, bis das Eluat neutral abläuft und eluiert darauf die nucleosidischen Bestandteile mit 5–10% Ammoniak in bereits entacetylierter Form. Nach Einengen der ammoniakalischen Lösung wird nach Chromatographie an einer Kurzsäule mit Fließmittel (*D*) **8** erhalten.

Anal. Ber. für $C_{10}H_{12}FN_5O_2$ (253,24) : C, 47,43; H, 4,78; F, 7,50; N, 27,66. Gef. : C, 47,26; H, 4,75; F, 7,45; N, 27,63.

Umsetzung von 3',5'-Di-O-mesylthymidin (**12**) *mit Kaliumfluorid.* — Ein Gemisch aus **12** (4 g, 10 mmol), trockenem Kaliumfluorid (2,5 g, 43 mmol) und Dimethylsulfoxid (100 ml) wird 3,5 Stdn. auf 90° erhitzt. Anschließend wird das Dimethylsulfoxid *in vacuo* weitgehend abdestilliert. Der Rückstand wird an einer Celite-Säule (1,1 kg) mit Fließmittel (*A*) chromatographiert, wobei neben nicht umgesetzten Ausgangsmaterial und geringen Mengen unbekannter Produkte 2,3'-Anhydro-1-(2'-desoxy-5'-*O*-mesyl-β-D-*threo*-pentofuranosyl)thymin (**13**) (0,55 g, 17,7%, nach Umkristallisieren aus Wasser) und 2,5'-Anhydro-1-(2'desoxy-3'-*O*-mesyl-β-D-*erythro*-pentofuranosyl)thymin (**14**) (0,33 g, 10,5%) erhalten werden.

Verbindung **13** ergab nach Kristallisation aus Wasser : Schmp. 175–178° (Zers.); $[\alpha]_D^{22}$ −59,5° (*c* 1,69, *N,N*-Dimethylformamid); [Lit[16] : Schmp. 177–178° (Zers.); $[\alpha]_D^{23}$ −61° (*c* 1,23, *N,N*-Dimethylformamid)]; u.v. Spektrum (Methanol) : λ_{max} 204 und 248 nm, λ_{min} 219 nm.

Das Anhydro-Nucleosid geht bei 24 stdg. Behandeln mit M Natriumhydroxyd bei Raumtemperatur quantitativ in 1-(2'-Desoxy-3',5'-anhydro-β-D-*threo*-pentofuranosyl)thymin (**15**) vom Schmp. 193–194° nach Kristallisation aus Äthanol über; $[\alpha]_D^{20}$ −123,8° (*c* 1,11, Wasser); [Lit.[17] : 193,5–194°; $[\alpha]_D^{24}$ −127° (*c* 1,0, Wasser)].

Verbindung **14** ergab Schmp. 178–180° (Zers.) nach Kristallisation aus 95% Methanol; $[\alpha]_D^{22}$ +50° (*c* 1,4, *N,N*-Dimethylformamid); [Lit.[18] : Schmp. 182–183° (Zers.); $[\alpha]_D^{26}$ +52° (*c* 0,4, *N,N*-Dimethylformamid)]; u.v. Spektrum (Methanol) : λ_{max} 249 nm, λ_{min} 218 nm. Dieses Anhydro-Nucleosid bildet bei 2 stdg. Behandeln mit M Natriumhydroxyd bei Raumtemperatur neben wenig 3'-*O*-Mesylthymidin 90% 2'-Desoxy-β-D-*threo*-pentofuranosylthymin (**16**).

4',5'-Didehydro-5'-desoxythymidin (**18**). — Ein Gemisch aus 5'-Desoxy-5'-jodthymidin (**17**, 3,5 g, 10 mmol), trockenem Kaliumfluorid (2,4 g, 41,4 mmol) und Dimethylsulfoxid (100 ml) wird unter Rühren während 7 Stdn. auf 90° erhitzt. Danach wird vom Ungelösten abgetrennt und das Dimethylsulfoxid *in vacuo* (80° Badtemperatur) weitgehend abdestilliert. Das restliche Lösungsmittel wird durch mehrfache Äther-Extraktion beseitigt. Der Rückstand wird an einer Celite-Säule (550 g) mit Fließmittel (*A*) chromatographiert und ergibt **18** (0,35 g, 15,6%), das nach zweimaligem Umkristallisieren aus 96% Äthanol bei 196–197,5° (Zers.) schmilzt. Aus den Mutterlaugen und den Mischfraktionen der säulenchromatographischen Trennung kann weiteres Produkt gewonnen werden. Die Substanz entfärbt wässrige Jod-Lösung sofort; $[\alpha]_D^{22}$ +2,1° (*c* 0,57, Methanol); u.v. Spektrum (Wasser) : λ_{max} 266 nm (ε 9560), λ_{min} 235 nm (ε 3430). N.m.r. Spektrum : die 5'-Protonen erscheinen als AB-System bei τ 4,07 und τ 4,20. Die Signale sind durch „long-range"-Kopplung mit H-3' schwach aufgespalten. Ein für H-4' charakteristisches Signal tritt nicht auf.

Anal. Ber. für $C_{10}H_{12}N_2O_4$ (224,21) : C, 53,55; H, 5,40; N, 12,51. Gef. : C, 53,64; H, 5,66; N, 12,27.

DANK

Wir danken Herrn Prof. M. Pletscher, Hoffmann-La Roche and Co., AG, Basel, für die Überlassung von 2'-Desoxy-5-fluoruridin und Herrn Dr. A. Holý, Prag, für die Überlassung von 2'-Desoxyuridin. Herrn Dr. E. Gründemann, Institut für Organische Chemie der Deutschen Akademie der Wissenschaften zu Berlin, Berlin-Adlershof, sind wir für die Aufnahme und Hilfe bei der Diskussion der p.m.r. Spektren, Herrn Dr. J. Malur aus unserem Institut für die Aufnahme der u.v. Spekten zu Dank verpflichtet.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

THE SYNTHESIS, EQUILIBRATION, AND CONFORMATIONS OF DIASTEREOISOMERIC 1,2-*O*-ISOPROPYLIDENE-3,5-*O*-(METHOXYMETHYLIDENE)-6-*O*-*p*-TOLYLSULFONYL-α-D-GLUCOFURANOSES. CONFORMATIONAL EVIDENCE FROM A NUCLEAR OVERHAUSER EFFECT

BRUCE COXON
Institute for Materials Research, National Bureau of Standards, Washington, D. C. 20234 (U. S. A.)
(Received June 20th, 1969; in revised form August, 7th, 1969)

ABSTRACT

The diastereoisomers of 1,2-*O*-isopropylidene-3,5-*O*-(methoxymethylidene)-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose have been prepared by treatment of 1,2-*O*-isopropylidene-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose with trimethyl orthoformate in acidified *N*,*N*-dimethylformamide. Treatment of an equilibrated mixture of these diastereoisomers with acetone gave 1,2:3,5-di-*O*-isopropylidene-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose and methyl formate. Equilibration reactions of the diastereoisomers in various solvents, at various temperatures, have been studied quantitatively by n.m.r. spectroscopy. The resulting thermodynamic data allow a decision as to the diastereoisomer that is stabilized by intramolecular dipole–dipole interactions, which, when used in conjunction with conformational evidence from vicinal coupling-constants obtained by a computed-spectrum analysis indicated the configuration of the new asymmetric carbon atom. The configurational assignments made for these diastereoisomers were supported by chemical-shift data and by the observation of a spin-coupling over five bonds. Evidence for a favored orientation of the methoxyl group in one of the diastereoisomers was obtained from nuclear Overhauser experiments in which a 25% enhancement of the signal intensity of the neighboring methylidene proton was observed. Van der Waals interaction energies have been calculated for the conformations assigned to the diastereoisomers and for the possible conformations of the closely related 1,2:3,5-di-*O*-benzylidene-α-D-glucofuranose derivatives. The nonbonded interactions in these conformations are discussed and compared.

INTRODUCTION

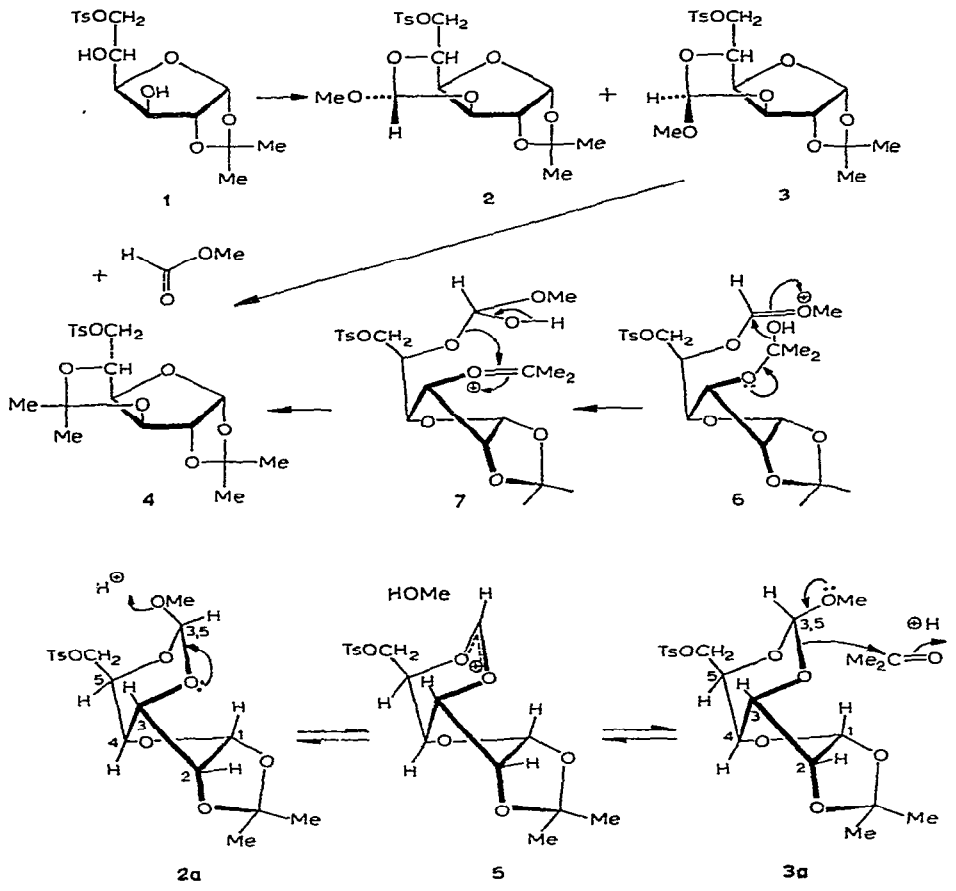
A previous investigation of the conformations of a series of 1,2:3,5-di-*O*-benzylidene-α-D-glucofuranose derivatives by p.m.r. spectroscopy[1] unexpectedly revealed that the *m*-dioxane ring in these molecules tends to assume that chair conformation having C-6* of the sugar axially attached. In continuation of these

*Throughout this paper, the numbering of hydrogen (or proton), carbon, and oxygen atoms in carbohydrate derivatives is based on the carbon chain of the sugar, not on the numbering of the *m*-dioxane ring.

studies, diastereoisomeric methoxymethylidene derivatives[2-4] (**2** and **3**) of 1,2-*O*-isopropylidene-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose have been prepared. Several preparations of diastereoisomeric 1, 2-orthoester derivatives of aldohexoses have been described in recent years[5-8], but few quantitative studies of their equilibria have appeared. The amounts of each diastereoisomer isolated by solution washing procedures may, or may not, reflect their equilibrium constant, depending on the distribution coefficients of the diastereoisomers. In this work, variation of the reaction conditions (temperature, solvent) gave some control over the yield of each diastereoisomer isolated. The relative importance of steric and intramolecular, dipole–dipole interactions in **2** and **3** and in closely related analogs is discussed.

DISCUSSION

Acid-catalyzed reactions of 1,2-*O*-isopropylidene-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose (**1**) with trimethyl orthoformate in *N*,*N*-dimethylformamide gave syrups containing two major components in approximately equal proportions. By chromatography on silicic acid, the mixtures were separated into crystalline (**2**) and syrupy (**3**) components which gave very similar p.m.r. spectra (Figs. 1a and 1b, respectively)



that each contained new methine and methoxyl signals. Elementary analysis and i.r. spectroscopy of **2** and **3** suggested that they were isomeric *O*-isopropylidene-*O*-(methoxymethylidene)-*O*-*p*-tolylsulfonylhexoses.

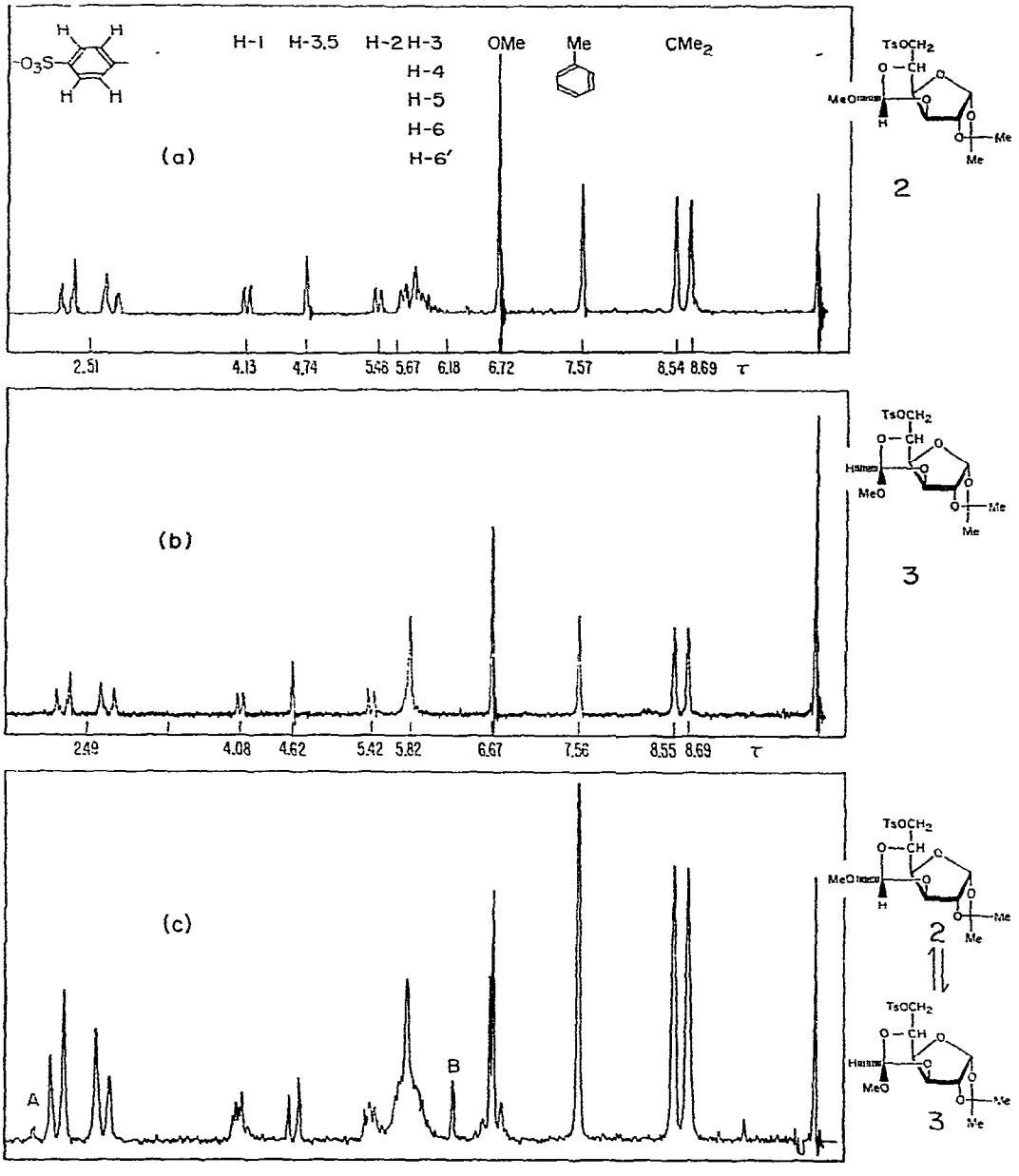


Fig. 1. P.m.r. spectra in chloroform-*d* at 60 MHz: (*a*) 1,2-*O*-isopropylidene-3,5-*O*-[(*endo*-methoxy)-methylidene]-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose (**2**), (*b*) 1,2-*O*-isopropylidene-3,5-*O*-[(*exo*-methoxy)methylidene]-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose (**3**), (*c*) **2** and **3** in equilibrium. Methyl formate signals appear at A and B.

Treatment of either **2** or **3** with a mixture of trimethyl orthoformate, *N,N*-dimethylformamide, and sulfuric acid led, in each case, to a 1.2:1 mixture of **2** and **3**. Compounds **2** and **3** could also be equilibrated rapidly (2–3 min) by treatment of their solutions in chloroform-*d* with a trace of *p*-toluenesulfonic acid. Under these conditions, other (slow) degradative reactions occurred, as evidenced by the gradual appearance of the characteristic signals of methyl formate in the p.m.r. spectrum. A spectrum of an equilibrated solution is shown in Fig. 1c. Derivatives **2** and **3** could also be equilibrated in *N,N*-dimethylformamide. A quantitative study of these equilibria is described later in this paper.

For further structural characterization of **2** and **3**, their equilibrated mixture in *p*-toluenesulfonic acid–chloroform-*d* was treated with one molecular proportion of acetone. Studies of p.m.r. spectra of the mixture, and isolation from it of 1,2:3,5-di-*O*-isopropylidene-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose (**4**) demonstrated that methoxymethylidene substituents in both **2** and **3** were replaced by isopropylidene groups, with liberation of methyl formate[9].

The foregoing evidence indicates that **2** and **3** are diastereoisomeric 1,2-*O*-isopropylidene-3,5-*O*-(methoxymethylidene)-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranoses.

The problem of the configuration of the methylidene carbon atom (C-3,5) remains to be discussed. The acid-catalyzed exchange of the 3,5-*O*-(methoxymethylidene) substituent for an isopropylidene group may be rationalized in terms of the mechanism **2a** + **3a** → **6** → **7** → **4**, in which the acetone conjugate-acid suffers nucleophilic attack by either O-3 or O-5 of the cyclic acetal, with scission of the O-3–C-3,5, or O-5–C-3,5 bond assisted by the electromeric effect of the oxygen atom of the methoxyl group. Migration of the hydroxyl group in the resulting oxo-carbonium ion (*e.g.* **6**) to C-3,5 would then give a rearranged oxo-carbonium ion (*e.g.* **7**) which would undergo cyclization, and elimination of a proton and methyl formate, to give the 3,5-isopropylidene acetal **4**. Alternative mechanisms involving the fission of the C-3–O-3, or C-5–O-5 bonds seem much less likely, because the resulting carbonium ion (at C-3, or C-5) would exhibit no great tendency toward intramolecular stabilization, unless this arose from participation (at C-3 or C-5) of an oxygen atom of the *p*-tolylsulfonyl group.

The most plausible mechanism for acid-catalyzed interconversion of the diastereoisomers **2** and **3** involves protonation of the oxygen atom of the methoxyl group (*e.g.* in **2a**), and loss of methanol promoted by the electromeric effects of both O-3 and O-5. The stabilized, intermediate, dioxo-carbonium ion **5** may then react with methanol to give either **2a** or **3a**.

CONFORMATIONS

To obtain reasonably accurate values of the vicinal coupling-constants, a 100-MHz spectrum (Fig. 2a) of **2** was subjected to an iterative computer-analysis as a seven-spin sub-system. [The methylidene proton (H-3,5) was, in fact, weakly coupled ($J/\delta = 0.42/117.3 = 0.004$) to one of the other ring protons*]. However,

*The results of a double-resonance experiment showed that this proton is H-4.

owing to the limitations of the computer program employed, an eight-spin system could not be handled, and so, initially, H-3,5 was excluded. The initial and refined parameters from the iterative analysis are shown in Table I, and the resulting theoretical spectrum (see Fig. 2b) is in good agreement with Fig. 2a.

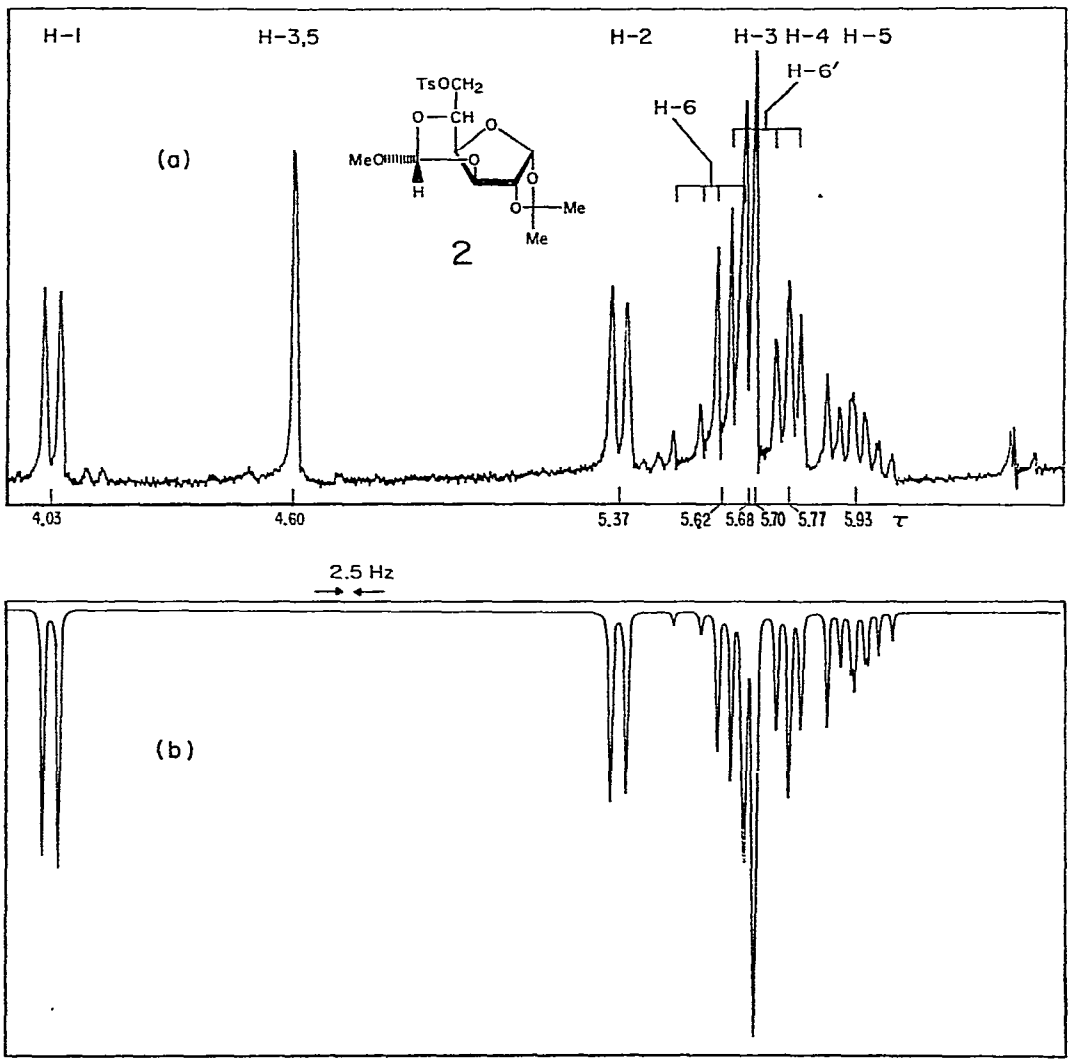


Fig. 2. (*a*) Partial p.m.r. spectrum of 1,2-*O*-isopropylidene-3,5-*O*-[(*endo*-methoxy)methylidene]-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose (**2**) in 6:1 (v/v) acetone-d_6–pyridine-d_5 at 100 MHz. (*b*) Computed spectrum of same compound, from iterative analysis.

By analogy with previous work[1,10], the near-equality of $J_{1,2}$ and $J_{3,4}$ and the small value of $J_{2,3}$, for **2** (see Table I) indicate that the shape of its furanoid ring is quite close to that of the $^3T_2$ conformation, as in conformation **2a** (or **3a**).

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TABLE I

SPECTRAL PARAMETERS (Hz) OF 1,2-*O*-ISOPROPYLIDENE-3,5-*O*-[(*endo*-METHOXY)METHYLIDENE]-6-*O*-*p*-TOLYLSULFONYL-α-D-GLUCOFURANOSE (2) AT 100 MHz

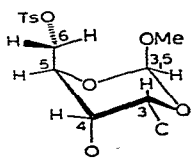
Parameter	*Initial*	*Refined (6 iterations)*	*Parameter*	*Initial*	*Refined (6 iterations)*
ν_1	596.97	596.93±0.01[a]	$J_{2,5}$	0	−0.10±0.02
ν_2	462.80	462.83±0.02	$J_{2,6}$	0	0.38±0.04
ν_3	432.20	431.98±0.02	$J_{2,6'}$	0	−0.26±0.05
ν_4	422.30	422.55±0.02	$J_{3,4}$	2.95	2.87±0.02
ν_5	407.77	406.69±0.01	$J_{3,5}$	0	−0.06±0.02
ν_6	438.84	438.26±0.02	$J_{3,6}$	0	−0.02±0.03
$\nu_{6'}$	430.55	430.40±0.03	$J_{3,6'}$	0	0.16±0.04
$J_{1,2}$	3.75	3.69±0.02	$J_{4,5}$	3.00	3.31±0.02
$J_{1,3}$	0	0.03±0.02	$J_{4,6}$	0	0.12±0.04
$J_{1,4}$	0	−0.06±0.02	$J_{4,6'}$	0	0.23±0.03
$J_{1,5}$	0	−0.04±0.02	$J_{5,6}$	6.97	6.02±0.02
$J_{1,6}$	0	−0.06±0.03	$J_{5,6'}$	5.03	6.60±0.03
$J_{1,6'}$	0	0.03±0.03	$J_{6,6'}$	−10.50	−10.64±0.02
$J_{2,3}$	0.30	0.39±0.02	$\nu_{3,5}$	539.6	
$J_{2,4}$	0	0.01±0.02	$J_{3,5:4}$	0.42	

[a]Computed probable error.

The fact that $J_{1,2}$ and $J_{3,4}$ are more nearly equal to each other than are those for a series of 1,2:3,5-di-*O*-benzylidene-α-D-glucofuranose derivatives[1] implies that, in **2**, there is less distortion of the furanoid ring toward the $^{V}3$ conformation. In this respect, the conformation of **2** closely resembles that of the parent 1,2-*O*-isopropylidene-α-D-*xylo*-hexofuranose derivatives[10]. According to the explicit form[1] of the Karplus equation[11], the value $J_{2,3}$ 0.39 Hz implies a proton dihedral angle ($\phi_{2,3}$) of ~72°, or ~104°, either of which values is consistent with a conformation such as **2a**, depending on the degree of twist in the furanoid ring.

The relatively small value of $J_{4,5}$, namely, 3.31 Hz, shows that H-4 and H-5 are disposed mainly in the *gauche* orientation, as in conformation **2a** (or **3a**), in which the *m*-dioxane ring assumes that chair conformation having both of these protons equatorially attached but with C-6 of the sugar moiety axially attached.

The comparatively large values of $J_{5,6}$ and $J_{5,6'}$, (6.02 and 6.6 Hz, respectively) suggest that each of these coupling constants contains a considerable contribution from a rotamer in which H-5 and H-6, or H-5 and H-6', are true *trans*. These are precisely those rotamers (**8** and **9**) in which the *p*-tolylsulfonyloxy group is directed away from the *syn*-axial substituents at C-3 and C-3,5.



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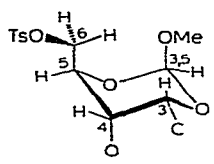

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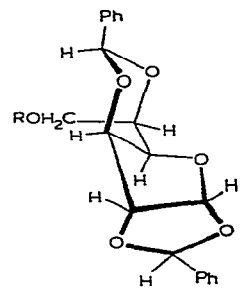

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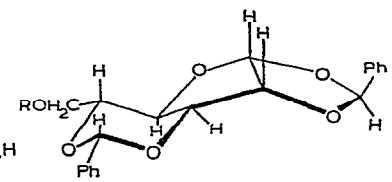

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Full analysis of the p.m.r. spectra at 100 MHz of the diastereoisomer **3** was unexpectedly difficult, because, in each of three solvents examined, H-3, H-4, H-5, H-6, and H-6' formed a complex ABCDE sub-system. Nevertheless, the first-order coupling constants $J_{1,2}$ 3.7 Hz and $J_{2,3}$ <0.4 Hz for the furanoid ring of **3** were very similar to those for its isomer **2**; hence, it appears likely that **3** has fundamentally the same type of conformation (**3a**) as has **2**. In previous work[1,12], it was demonstrated that inversion of the chair conformation of the *m*-dioxane ring (as in **3a**) to the alternative chair conformation (having C-6 of the sugar equatorially attached), requires inversion of the shape of the furanoid ring, also, to a form in which $\phi_{2,3}$ is approximately 160°, and for which a large value of $J_{2,3}$ would be expected. No such evidence was found for **3**. The foregoing results resemble those obtained for the 1,2:3,5-di-*O*-benzylidene-α-D-xylofuranose[12] and -α-D-glucofuranose[1] analogs.

DETERMINATION OF CONFIGURATION

Evidence for the configuration of C-3,5 in **2** and **3** was obtained from three sources.

In derivatives containing pyranose rings (and in other rings), intramolecular dipole–dipole interactions often exert a controlling influence on conformation[13-18] and configuration[19]. Such interactions, commonly known as anomeric effects[19b], or *reverse* anomeric effects[3], depending on whether the non-hydrogen substituent atom attached to C-1 is electron-rich, or electron-deficient, have generally been ascribed to repulsions or attractions between the dipoles associated with this atom and with the ring-oxygen atom[19]. On this basis, it might be anticipated that the methoxyl substituent in **2** and **3** would show a doubled "anomeric effect", due to the presence of two hetero oxygen atoms in the attached *m*-dioxane ring. Little information is as yet available on "anomeric effects" in *m*-dioxane derivatives. However, it has recently been reported[20] that, in the simple derivatives 2-methoxy- and 2-ethoxy-1,3-dioxane, the alkoxyl group favors the axial orientation by 0.35 kcal/mole at 25°. If this value is taken as the difference between the anomeric effect of the alkoxyl group and its conformational free-energy difference (A-value), then, if the A-value is large, so also will be the value of the anomeric effect. It is not appropriate to attempt here the calculation of this anomeric effect by using the A-value of a methoxyl substituent on a cyclohexane ring[21a], as recent work has indicated that 2-methyl-1,3-dioxane favors the equatorial conformation to a much greater extent than does methylcyclohexane [$-\Delta G = \geqslant 3.55$ (Ref. 20) and 1.7 kcal/mole (Ref. 21), respectively]. Such a procedure has, however, been applied to several 2-methoxytetrahydropyran derivatives, and, depending on the solvent employed, has yielded values of the anomeric effect of the methoxyl group ranging from 0.9 to 1.4 kcal/mole[22,23]. Similar values have been obtained (by other means) from anomeric pairs of methyl glycopyranosides[24,25]. The increased conformational free-energy of an axially attached 2-substituent on *m*-dioxane has been attributed to diminished *syn*-axial distances caused by puckering of the ring, and by C–O bond lengths shorter than that of C–C[20].

Other studies of pyranoid derivatives have shown that the anomeric effects of 1-substituents decrease on passing from a nonpolar to a polar solvent, due evidently to solvation of the intramolecular dipoles[22,26,27]. Accordingly, it was expected for the diastereoisomers **2** and **3** that the diastereoisomer having its methoxyl group in the axial orientation on the *m*-dioxane ring would be destabilized with respect to its equatorial isomer on passing from equilibria in a nonpolar to those in a polar solvent. For a study of the equilibria of **2** and **3**, chloroform-*d* and *N*,*N*-dimethylformamide were selected as suitable, aprotic solvents, of differing polarity. Equilibrium constants (K = [**2**]/[**3**]; see Table II) were determined by integration of the H-3,5 (or OMe) singlets of **2** and **3** in the p.m.r. spectra (*e.g.*, Fig. 1c) of their equilibrated mixtures.

Since the methoxyl substituent is not spherically symmetrical, it cannot be assumed that the entropies of **2** and **3** are exactly the same, particularly because, in one of their conformations (**2a**), the rotational freedom of this substituent is restricted by interaction with a *syn*-axial methylene group. Therefore, in order to ascertain which of the two (**2** and **3**) is stabilized by an "anomeric effect", it was necessary to establish their enthalpy differences in the two solvents; this was done by measuring the equilibrium constants at different temperatures. The resulting thermodynamic

parameters are given in Table II, from which it may be seen that the crystalline diastereoisomer **2** is more stable than the syrupy isomer **3** both in chloroform-*d* and *N*,*N*-dimethylformamide (for example, at 35°, $\Delta G = -0.265$, and -0.16 kcal/mole, respectively). Because of the negative change in entropy, the enthalpy differences (ΔH) are larger than the free-energy differences. Moreover, the fact that ΔH changes from -0.55 kcal/mole in chloroform-*d* to -0.39 kcal/mole in *N*,*N*-dimethylformamide strongly suggests that it is the crystalline isomer **2** that is stabilized by the "anomeric effect", and which therefore contains the methoxyl group in the axial orientation. It is assumed here that the relative magnitudes of the steric interactions in **2** and **3** are influenced less by the change of solvent polarity than are the intramolecular dipole–dipole interactions.

TABLE II

EQUILIBRATION PARAMETERS OF DIASTEREOISOMERIC 1,2-*O*-ISOPROPYLIDENE-3,5-*O*-(METHOXYMETHYLIDENE)-6-*O*-*p*-TOLYLSULFONYL-α-D-GLUCOFURANOSES (**2** AND **3**)

Solvent	*Temperature*, °*K*	K = [2]/[3]	n[a]	$-\Delta G$, *kcal.mole*$^{-1}$	$-\Delta S$, *cal.deg.*$^{-1}$*mole*$^{-1}$	$-\Delta H$, *kcal.mole*$^{-1}$
Chloroform-*d*	308.6 ±0.1	1.540 ±0.005	107	0.265 ±0.002	0.93 ±0.08	0.55 ±0.02
	263.7 ±0.3	1.795 ±0.010	100	0.306 ±0.003		
N,*N*-Dimethyl-	308.6 ±0.2	1.299 ±0.006	46	0.160 ±0.003	0.75 ±0.08	0.39 ±0.02
formamide	264.0 ±0.3	1.447 ±0.006	108	0.193 ±0.002		

[a]Number of determinations of *K*.

The configurations and conformations of **2** and **3** may, therefore, be represented as shown in **2a**, and **3a**, respectively. The negative changes in entropy, namely, $\Delta S = -0.93$ and -0.75 cal.deg.$^{-1}$mole^{-1} in chloroform-*d* and *N*,*N*-dimethylformamide, respectively, are entirely consistent with this assignment, as epimerization of **3a** to **2a** involves movement of the methoxyl substituent from an unhindered, *exo*-equatorial position to a hindered, *endo*-axial position. On this basis, crystalline **2** may be described as 1,2-*O*-isopropylidene-3,5-*O*-[(*endo*-methoxy)methylidene]-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose, and its syrupy isomer **3**, as 1,2-*O*-isopropylidene-3,5-*O*-[(*exo*-methoxy)methylidene]-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose.

The assignment of an equatorial position to H-3,5 of **2** (as in **2a**) is also supported by the observation of its long-range coupling (0.42 Hz) with H-4, which is equatorially oriented with respect to the *m*-dioxane ring (see formula **8** or **9**). The results of a survey of the literature[28–33] suggested that a favored arrangement for ^5J coupling in saturated, six-membered, oxygen heterocycles occurs when the protons in the 1,4 relationship are both equatorially attached. Examples are given in Table III. The observation of this type of long-range coupling obviously does not depend on attachment of the 1,4 diequatorial protons to a rigid, six-membered ring.

The chemical shifts of H-3,5 in **2** and **3** provided further evidence for the configuration of C-3,5 in these compounds. In *m*-dioxane derivatives lacking an

TABLE III

EXAMPLES OF $^5J_{e,e}$ IN SATURATED, SIX-MEMBERED, OXYGEN HETEROCYCLES

Compound	*References*	$^5J_{e,e}$, *Hz*	
4-Phenyl-1,3-dioxane	28	$J_{2e,5e}$	0.9
cis-5-Deuterio-4-phenyl-1,3-dioxane	29	$J_{2e,5e}$	~0.9
		($J_{2e,5a}$	~0
		in *trans* isomer)	
4-Methyl-1,3-dioxane	28	$J_{2e,5e}$	0.7
cis-4,6-Dimethyl-1,3-dioxane	30	$J_{2e,5e}$	~0.6
4-*t*-Butyl-4-methyl-1,3-dioxane	30	$J_{2e,5e}$	~0.7
α-D-Gulopyranose pentaacetate	31	$J_{1e,4e}$	~0.7
1,6-Anhydro-β-D-glucopyranose triacetate	32	$J_{1e,4e}$	0.1
		$J_{2e,5e}$	0.5
α-D-Idopyranose pentaacetate	33	$J_{1e,4e}$	0.6
1,2-*O*-Isopropylidene-3,5-*O*-[(*endo*-methoxy)methylidene]-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose (**2**)	Present work	$J_{3,5;4}$	0.4

axially attached substituent on C-4 or C-6, H-2*e* generally resonates some 0.3–0.4 p.p.m. to lower field than does[20,29,30] H-2*a*. However, the axial erection of a methyl group at either of these carbon atoms has been shown to cause a downfield shift (~0.2–0.3 p.p.m.) of the H-2*a* signal[20,30], together with a shift (~0.2 p.p.m.) of the H-2*e* signal to *higher* field[30]. The net result is that, when H-2*a* and the methyl group are *syn*-axial, H-2*a* resonates at lower field than H-2*e* by ~0.1 p.p.m.

Each of the conformations assigned, namely, **2a** and **3a**, has an axial methylene substituent in a 1,3 relationship with H-3,5. Furthermore, it has been shown that the *p*-tolylsulfonyloxy group is mainly directed away from C-3,5 and its substituents. It may be expected, therefore, that the axial methylene group will have much the same influence on the chemical shifts of H-3,5 in its two orientations as does an axial methyl group. Experimentally, H-3,5 in **3** (see Fig. 1b) resonates at lower field by 0.12 p.p.m. than does H-3,5 in **2** (see Fig. 1a). In view of the similar chemical shift in the simple *m*-dioxane derivatives, this is consistent with the assignment of an axial orientation to H-3,5 in **3**, and an equatorial orientation to H-3,5 in **2** (conformations **3a** and **2a**, respectively).

NONBONDED INTERACTIONS

There is now much evidence available that suggests that repelling 1,3-diaxial interactions in carbohydrate derivatives are not of such great importance as was at one time supposed[34]. Many of these derivatives have been found by n.m.r.spectroscopy to favor assumption of conformations that contain a majority of axially attached substituents[13–15,17,18,33,35]. Often, intramolecular dipole–dipole interactions exert a greater influence than steric effects on conformation. A further example of such influences is furnished by the present work. Since the enthalpy change **3** → **2** is negative in both chloroform-*d* and *N*,*N*-dimethylformamide (Table II),

it is apparent that the stabilization of **2** provided by the anomeric effect exceeds the destabilization from repulsive steric interactions. The most important of the latter interactions might be expected to arise from the *syn*-axial oxygen atom of the methoxyl group and methylene group, which, in Dreiding molecular models of conformation **2a**, are quite close to each other (~2.3 Å).

A similar situation had been encountered with the 1,2:3,5-di-*O*-benzylidene derivatives[1], where the favored conformation **10** contains the C-6 methylene group, H-3 and H-3,5 in a *syn*-axial relationship, and the furanoid ring a form intermediate between the $^3T_2$ and V^3 shapes. In an effort to explain why **10** is favored over its alternative (**11**), approximate, theoretical, steric interactions have been calculated from the Hill equation[36] for those pairs of atoms, which, in Dreiding models appear to have internuclear distances close to, or less than, the sum of the van der Waals radii of the two atoms; that is, for those atom pairs wherein the repulsive part of the van der Waals interaction might be expected to be of some importance. Since p.m.r.

TABLE IV

SELECTED, THEORETICAL, VAN DER WAALS INTERACTION-ENERGIES IN CONFORMATIONS OF 1,2:3,5-DI-*O*-BENZYLIDENE-α-D-GLUCOFURANOSE DERIVATIVES

Conformation	*Interacting atoms*	*Interatomic distance (Å, from Dreiding models)*		*Interaction kcal.mole*$^{-1}$
10	H_2C-6:H-3,5	2.4		+2.16
	H_2C-6:H-3	3.3		−0.14
	O-4:O-5	3.2		−0.10
	O-3:O-4	3.2		−0.10
	C-1:O-3	3.1		−0.13
	C-1:O-5	3.9		−0.06
			Total	+1.63
11	H-3,5:H-2	1.9		+0.36
	H-3,5:C-2	2.5		+0.08
	C-1:C-5	3.1		−0.05
	C-3,5:H-2	2.6		−0.01
	C-3,5:C-2	3.0		+0.04
			Total	+0.42
10	H_2C-6:H-3,5	2.5		+1.22
		2.6		+0.64
		2.7		+0.29
		2.8		+0.08
		(2.859)		G
		2.9		−0.04
		3.0		−0.10
		3.1		−0.13
		3.2		−0.14
		3.3[a]		−0.14

[a] *m*-Dioxane ring in that half chair form that has C-3, C-4, C-5, and O-5 coplanar.

evidence[1] had indicated that the O-6 in **10** is directed mainly away from H-3,5 and H-3, the interaction of the C-6 methylene group with these hydrogen atoms could be calculated approximately as being that of a methyl group, by using suitable parameters derived previously for ring D of steroids[37,38]. The results of some of these calculations are shown in Table IV.

For conformation **10**, several "O-inside" interactions[39] were considered, in addition to the *syn*-axial interactions of H_2C-6 with H-3 and H-3,5. On the other hand, several "H-inside" and "C-inside" interactions were calculated for conformation **11**. In both instances, interatomic distances measured on undistorted Dreiding models were used. It may be seen that the major interaction in **10** is a repulsive interaction between H_2C-6 and H-3,5. However, the small attractive interactions exert a cumulative effect. In contrast, the most significant interaction in **11** appears to be an "H-inside" type of interaction between H-3,5 and H-2. On the basis of this limited number of steric interactions that are thought to be important in an undistorted Dreiding model, the interaction totals predict that **11** should be more stable than **10** by 1.2 kcal/mole; this is in fact, the converse of the experimental observation[1]. Further calculations for **10** (see Table IV, lower half) showed that the H_2C-6–H-3,5 repulsive interaction is very rapidly relieved by small increments ($\sim$0.1 Å) to the interatomic distance, so that recession of H_2C-6 and H-3,5 by less than 0.5 Å is sufficient to lower their *syn*-axial interaction to zero. On further increase of the distance, the attractive forces become predominant, and the minimum on the potential-energy curve is reached at that point where the *m*-dioxane ring has assumed that half-chair shape having C-3, C-4, C-5, and O-5 in a plane. Such conformational distortion is, of course, counterbalanced by bond-length, bond-angle, and torsional strains[21b].

A comparison of the nonbonded interactions in **2a** and **3a** was made by restricting the calculations to those atoms and groups whose interactions are directly affected by inversion of the configuration of C-3,5. In this case, also, the orientation of the C-6 methylene group defined by the coupling-constant evidence allowed this substituent to be treated as a methyl group having a van der Waals radius of 2.0 Å. It was also assumed that minimization of strain energy would occur by a direction of the methyl group of the methoxyl group away from C-6 and H-3. Experimental evidence for this assumption was obtained from an intramolecular nuclear Overhauser experiment. Saturation of the methoxyl protons of **2** resulted in a 25% enhancement of the integrated intensity of its methylidene proton resonance signal when compared with a similar experiment in which the irradiating frequency was applied off-resonance. However, a similar irradiation of the methyl protons of the *p*-tolylsulfonyl group in **2** produced only a 7% enhancement of the signal intensity of the methylidene proton. In view of previous work[40] in which irradiation of methyl protons in 3,3-dimethylacrylic acid and in *N*,*N*-dimethylformamide resulted in a 17–18% enhancement of the signal intensity of a *cis* methine proton, but no significant effect when the methine proton was *trans* to the irradiated methyl group, the results from **2** suggest that the methoxyl protons are in *cis* relationship with H-3,5, that is,

with the *O*-methyl group oriented away from *syn*-axial H-3 and H_2C-6 (as in **2a**, **8**, and **9**). Conversely, the small nuclear Overhauser effect observed on irradiation of the aromatic methyl protons indicates that, for the most part, they are spatially remote from H-3,5. The relevant steric interactions were, therefore, those of O-3,5 (in **2a**), or H-3,5 (in **3a**), with H-3, H_2C-6, C-3, or C-5. From the results given in Table V, it may be seen that calculations based on inspection of an undistorted Dreiding model of **2a** produce a H_2C-6:O-3,5 interaction energy ($\sim$17 kcal/mole) which is obviously too large [by comparison with the corresponding interaction (+2.16 kcal/mole) for the diastereoisomer **3a**]. Because the magnitude of even a doubled anomeric effect would not suffice to counteract a relative instability of 15.2 kcal/mole (the total interactions in **2a** less those in **3a**), it is apparent that the large interactions of H_2C-6 with O-3,5 and H-3,5 in **2a** and **3a**, respectively, must be relieved by conformational distortion. Table V (lower half) shows how the H_2C-6:O-3,5 repulsion decreases rapidly as the interatomic distance is increased slightly. Again, appreciable flattening of the 1,3-dioxane ring appears to be necessary for this interaction to reach the minimum (~ -0.35 kcal/mole, attractive) on the potential energy curve. Consideration of the small magnitudes of such attractive forces suggests that it is unlikely that anomeric effects could actually *originate* in this way, instead of by dipole–dipole interaction. If it is assumed (somewhat arbitrarily) that the increase of the H_2C-6:O-3,5 and H_2C-6:H-3,5 distances in **2a** and **3a**, respectively, is about the same as that ($\sim$0.4 Å) which must occur in conformation **10**, and that the other relevant distances change in the way indicated by distortion of Dreiding models, a modified set of interactions may be calculated (see Table V). For the four steric interactions considered, the net difference in energy of similarly distorted **2a** and **3a**, is now only 2.14 kcal/mole, in favor of **3a**. If, for comparative purposes, it is assumed that the anomeric effect of a 2-methoxy group on a *m*-dioxane ring is 2.6 kcal/mole, that is, twice that of the methoxyl group in 2-methoxy-4-methyl-tetrahydropyran in a nonpolar solvent (*p*-dioxane)[22], the enthalpy difference of *distorted* **2a** and **3a** should be $2.14-2.6 = -0.46$ kcal/mole in favor of **2a** (Found: -0.55 kcal/mole in chloroform-*d*). This illustrative calculation ignores the other strain energies which would undoubtedly be introduced by distortion of **2a** and **3a**. However, for the same degree of distortion, these energies should be similar, and, hence, should cancel out in any comparison of the relative stabilities of **2** and **3**.

The formulation of a general computation of minimization of strain energy for complex, carbohydrate derivatives is a difficult problem, owing to the many rotational modes possible for typical substituents on carbohydrates, and also to uncertainty in, or to unavailability of, suitable potential functions for the four main types of strain energy that must be considered in a general calculation. For the free aldopentopyranoses and aldohexopyranoses, partial success has recently[41] been achieved by iterative calculations of the van der Waals interaction-energies in hypothetical models in which the geometry of the pyranose ring and its directly bonded substituent atoms was fixed. Energy minimization was achieved by successive rotation of each remaining atom about an appropriate bond between a carbon atom and its

TABLE V

SELECTED, THEORETICAL, VAN DER WAALS INTERACTION-ENERGIES IN 1,2-*O*-ISOPROPYLIDENE-3,5-*O*-(METHOXYMETHYLIDENE)-6-*O*-*p*-TOLYLSULFONYL-α-D-GLUCOFURANOSES (2 AND 3)

Conformation	*Interacting atoms*	*Interatomic distance (Å, from Dreiding models)*			*Interaction kcal.mole*$^{-1}$		
		Undistorted		*Distorted*	*Undistorted*		*Distorted*
2a	H-3:O-3,5	2.6	→	2.6	−0.08	→	−0.08
	H_2C-6:O-3,5	2.3	→	2.7	+17.42	→	+2.32
	C-3:O-3,5	2.8	→	2.8	−0.03	→	−0.03
	C-5:O-3,5	2.7	→	2.9	+0.09	→	−0.10
				Totals	+17.40	→	+2.11
3a	H-3:H-3,5	2.6	→	2.5	−0.04	→	−0.05
	H_2C-6:H-3,5	2.4	→	2.8	+2.16	→	+0.08
	C-3:H-3,5	2.6	→	2.6	−0.01	→	−0.01
	C-5:H-3,5	2.5	→	2.7	+0.09	→	−0.05
				Totals	+2.20	→	−0.03
2a	H_2C-6:O-3,5	2.3			+17.42		
		2.5			+6.78		
		2.7			+2.32		
		2.9			+0.52		
		3.1			−0.13		
		3.3			−0.33		
		3.4			−0.35		
		3.5			−0.34		

directly bonded substituent atom. However, a more general calculation for the free sugars would also necessitate inclusion of intramolecular dipole–dipole interactions and hydrogen-bonding possibilities, and suitable potential-energy functions thereof.

EXPERIMENTAL*

General. — Melting points are uncorrected. Solutions were evaporated under diminished pressure (bath temperature $<40°$). T.l.c. was conducted on unactivated layers of Silica Gel G (E. Merck, Darmstadt) which after spotting and development, were sprayed with 5% sulfuric acid, and heated. For t.l.c. and column chromatography, 0.1% of triethylamine in dichloromethane (solvent mixture *A*) was used as a developing solvent, unless stated otherwise. I.r. spectra were recorded on Perkin–Elmer 137B and 257 spectrometers; only selected absorption maxima are listed (in cm^{-1}) with intensities strong (s), moderate (m), and weak (w), and probable assignments. P.m.r. spectra were recorded on commercial spectrometers operating at 60 and 100 MHz with tetramethylsilane (τ 10.00) as the internal reference standard, or an internal lock-signal. Spectra for analysis were recorded at sweep widths of 50 or 250 Hz.

Spectral assignments and calculations. — At a radiofrequency of 100 MHz, the spectra of solutions of either **2** or **3** in a variety of solvents were usually complex. However, the spectrum (Fig. 2a) of a solution of **2** in 6:1 (v/v) acetone-d_6–pyridine-d_5 was amenable to full analysis as a seven-spin system. Most of the assignments (Fig. 2a) for protons H-1 to H-6′ were made by analogy with previous work[1]. The origin of the long-range coupling (0.4 Hz) in the H-3,5 doublet was determined by means of a frequency-swept, spin-decoupling experiment. Irradiation of H-3,5 (τ 4.60) caused the H-4 multiplet to collapse to a sharp triplet. However, as H-3,5 and H-4 were only weakly coupled, H-3,5 was excluded initially from the analysis, so that protons H-1 to H-6′ could be analyzed as a seven-spin system. As a starting point for the computer analysis, the six lines of H-6 and H-6′ that could be clearly observed were subjected to analysis as part of the AB portion of an ABX sub-system composed of H-6, H-6′, and H-5. The experimental and recalculated line-positions agreed within ± 0.05 Hz. From the ABX analysis, values (see Table I) of $J_{5,6}$, $J_{5,6'}$, $J_{6,6'}$, ν_6, and $\nu_{6'}$ were obtained; these were combined with first-order spacings and mean chemical-shifts weighted, according to peak height, from H-1 to H-4. A trial spectrum was computed from these initial parameters (see Table I) by using the LAOCN 3 program, and a digital, incremental, x–y plotter (Lorentzian and Gaussian line-widths, 0.5 Hz). An iterative computation was then made in which 66 of the 3003 theoretical transitions were assigned to 27 experimental line-positions (precision, ± 0.1 Hz), some allowance being made for the line width of the H-3 signal because of its small coupling (~ 0.3 Hz) with H-2. All 28 of the chemical-shift and spin-coupling

*Mention of a commercial instrument in this paper does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the equipment identified is necessarily the best available for the purpose.

parameters were allowed to vary. In its original form, the computer program proceeded for excessive times in the iterative mode, and it was therefore modified so that for the largest Hamiltonian matrix (35×35), only the diagonal terms were differentiated with respect to each of the 28 parameters, thus lessening the computational time considerably (for all smaller matrices, off-diagonal terms were included). After two iterations, the frequencies of seven of the theoretical transitions were found to differ from their assigned experimental frequencies by more than 0.1 Hz. These experimental frequencies were, therefore, re-assigned to other nearby theoretical lines, so as to minimize the frequency error. Four more iterations were performed, during which the root mean-square error of the frequencies of the assigned lines decreased to a constant value. For iterations 1–6, these errors were, respectively, 0.689, 0.135, 0.114, 0.044, 0.043, and 0.043. The maximal deviation of any assigned pair of theoretical and observed lines was 0.15 Hz. However, the remaining frequency errors were appreciably less.

Further confirmation of the validity of the analysis was obtained by computation of trial spectra by two other methods. (*a*) The chemical-shifts calculated after 6 iterations were multiplied by 0.6, and the calculation for the seven spin system was repeated. The resulting theoretical spectrum was in excellent agreement with the complex spectrum of **2** recorded at 60 MHz. (*b*) As H-2 and H-3 were only weakly coupled, a computation was also performed for the six-spin sub-system consisting of H-3,5, H-3, H-4, H-5, H-6, and H-6′. The appropriate, best-fitting parameters from the iterations were used, together with the chemical shift (539.6 Hz) and long-range coupling ($J_{3,5:4}$ 0.42 Hz) of H-3,5, its remaining coupling-constant parameters being set to zero. Plotting of the resulting partial, theoretical spectrum again provided a good fit with the 100-MHz spectrum of **2**.

Intramolecular nuclear Overhauser effect. — A solution of **2** (150 mg) in 7:1 (v/v) acetone-d_6–pyridine-d_5 (0.4 ml) was freed of oxygen by saturation with dry nitrogen, tetramethylsilane being added just prior to termination of the flow of nitrogen. At a spectrometer frequency of 100 MHz, an irradiating frequency was applied in turn to the methoxyl protons, to the aromatic methyl protons, and to an off-resonance position 0.62 p.p.m. upfield from the aromatic methyl proton signal, with an on-load oscillator power level of 200 mV, and a somewhat diminished detector input. In each case, the methylidene proton signal was observed, and integrated 10 times, in the frequency-sweep mode. For irradiation at the methoxyl, aromatic methyl, and off-resonance positions, the corresponding mean integrated relative intensities were 124.8 $\pm$1.5, 106.7 $\pm$1.2, and 100.0 $\pm$1.1; the quoted error is the standard deviation of the mean.

Thermochemical measurements. — Equilibration reactions of the diastereoisomers **2** and **3** in solution were studied by periodic observation and integration of their p.m.r. spectra at 60 MHz, and by t.l.c. Occasionally, crystalline **2** equilibrated spontaneously with **3** on dissolution in apparently neutral chloroform-*d*. However, normally, a pure diastereoisomer or an enriched mixture (67–104 mg) was dissolved in chloroform-*d* (0.35 ml) or dry *N*,*N*-dimethylformamide (0.4 ml), and the equili-

bration was catalyzed by the addition of anhydrous *p*-toluenesulfonic acid (1.4–2.5 mg) to the solution. Integration of the H-3,5 singlets of **2** and **3** at a sweep width of 50 Hz was the most generally useful method for determining when equilibrium had been attained, because unfavorable effects of solvent and temperature often caused the methoxyl singlets of **2** and **3** to be insufficiently resolved; nevertheless, where these signals were resolved, concordant results were obtained. Equilibrium constants $K(=[2]/[3])$ were calculated as the ratio of the integrals of the H-3,5 (or OMe) singlets at higher and at lower field. Between 36 and 100 spectrometric integrals were taken, and the ratios obtained were checked by 4–20 planimetric measurements of the n.m.r. absorption spectra recorded at high amplitude and at a sweep-width of 50 Hz.

In the calculations of mean equilibrium constants, equal weighting was given to the two types of integral; thus, the values (see Table II) are derived mainly from integration by use of the spectrometer. Equilibria were approached from both sides; firstly, at the normal probe-temperature ($T_1 = 308.6°K$) of the spectrometer, and, secondly, at $\sim 264°K$. Re-equilibration experiments at the alternative temperature were also conducted. At the lower temperature (T_2) equilibration of **2** and **3** in *N*,*N*-dimethylformamide was slow (~ 32 days), due, apparently, to the basicity of the solvent. Further experiments were therefore conducted in which an increased quantity (10 mg) of *p*-toluenesulfonic acid was used; similar results were obtained. The temperatures of the samples in the variable-temperature probe of the spectrometer were determined by using a copper–constantan thermocouple as a primary standard and the chemical shift (6–10 determinations) of the hydroxyl and methyl multiplets of absolute methanol as a secondary standard. For 264°K, comparison of the two techniques indicated the necessity of making a correction of +3.8°K to the temperatures derived from a standard calibration-chart[42]. This correction for systematic error was very similar to that from a modified calibration-chart kindly supplied by Dr. Regitze Shoup. No correction was necessary at the normal probe-temperature. For calibration of the 500-Hz sweep-width, audio-sideband modulation and the chemical shift (δ 436 Hz) of 2% of benzene in carbon tetrachloride[42] were used as primary and secondary standards, respectively.

From the equilibrium constants K_1 and K_2 (corresponding to T_1 and T_2) were calculated the entropy change $\Delta S = RT_1 \ln K_1 - RT_2 \ln K_2)/\Delta T$, the enthalpy change $\Delta H = RT_1 T_2 \ln(K_1/K_2)/\Delta T$, and the associated free-energy changes (see Table II) ($\Delta T = T_1 - T_2$).

Probable errors ($p_{\bar{x}}$) of mean experimental parameters ($\bar{X} = K_1, K_2, T_1$, or T_2) were calculated from $fS_x/\sqrt{n}$, where $S_{\bar{x}}$ is the standard deviation of X, n is the number of determinations, and $f = 0.6745$, 0.703, 0.718, or 0.728, for $n > 10$, 10, 7 or 8, or 6, respectively[43].

Propagated errors in ΔS were calculated from $p^2_{\Delta S} = \sum[\partial(\Delta S)/\partial\bar{X}]^2 \cdot p_{\bar{x}}^2$, where
$\partial(\Delta S)/\partial\bar{X} = RT_1/K_1\,\Delta T, -RT_2/K_2\,\Delta T, RT_2 \ln(K_2/K_1)/\Delta T^2$, or $RT_1 \ln(K_1/K_2)/\Delta T^2$, and for ΔH these errors were calculated from $p^2_{\Delta H} = \sum[\partial(\Delta H)/\partial\bar{X}]^2 \cdot p_{\bar{x}}^2$, where

$\partial(\Delta H)/\partial\bar{X} = RT_1T_2/K_1\Delta T$, $-RT_1T_2/K_2\Delta T$, $RT_2^2\ln(K_2/K_1)/\Delta T^2$, or $RT_1^2\ln(K_1/K_2)/\Delta T^2$, and where $\bar{X} = K_1$, K_2, T_1, or T_2.

Theoretical, steric interactions. — Van der Waals interaction-energies (E_v) were calculated from the Hill equation[36] $E_v = \varepsilon\{-2.25[r/(r_1+r_2)]^{-6}+8.28\times10^5 \exp[-r/0.0736(r_1+r_2)]\}$, where the energy-coordinate parameter ε was obtained from previously published tables[38,44], and r, the interatomic distance (in Å), was estimated from study of Dreiding molecular models. For the van der Waals radii (r_1 and r_2) of the interacting atoms or groups, the values H, 1.2; C, 1.7; O, 1.4; and H_2C-6, 2.0 Å were used[38,44].

Diastereoisomeric 1,2-O-isopropylidene-3,5-O-(methoxymethylidene)-6-O-p-tolylsulfonyl-α-D-glucofuranoses (**2** and **3**). — To dry *N,N*-dimethylformamide (20 ml) was added concentrated sulfuric acid (0.6 ml, 10 mmole), followed by trimethylorthoformate (22 ml, 200 mmoles) and 1,2-*O*-isopropylidene-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose (3.74 g, 10 mmoles). The clear solution was rotated at 80 torr for 7 days. Its initial volume was restored after 2.75 days, and 4 days, by the addition of further portions of trimethylorthoformate (10 ml, and 5 ml). The mixture was then made neutral by addition of powdered calcium oxide, and filtered through Filter Cel, and the solids were washed with 5:1 (v/v) dichloromethane–trimethylorthoformate (120 ml). The filtrate and washings were combined, washed four times with water, "dried" by filtration through paper wetted with dichloromethane, and evaporated (bath temperature <45°) to a pale-yellow syrup that was dried at room temperature/0.05 torr to give 3.582 g (86%). T.l.c. in 1% of methanol in dichloromethane, and a p.m.r. spectrum (60 MHz) of a solution of the product in chloroform-*d*, indicated that it consisted of two major components, present in approximately equal proportions.

A solution of the syrup (3.285 g) in solvent *A* was chromatographed on a column of silicic acid (Mallinckrodt, 250 g; 100 mesh). Evaporation of the first fraction gave a syrup (0.841 g) which, on being dried in *vacuo*, crystallized as clusters of rods. Recrystallization from aqueous methanol containing a trace of triethylamine gave colorless rods (0.631 g) of 1,2-*O*-isopropylidene-3,5-*O*-[(*endo*-methoxy)methylidene]-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose (**2**), m.p. 73–75°. After three further recrystallizations from aqueous methanol and two from methanol, followed by drying for 40 min at room temperature/0.1 torr, it had m.p. 82–83°, $[\alpha]_D^{26}$ +54.5° (*c* 1.51, dichloromethane); i.r. data (as a film from chloroform-*d*): ν_{max} 2988m, 2950m, and 2842w (CH and OMe), 1597m and 1490w cm^{-1} (Ar); p.m.r. data in chloroform-*d* at 60 MHz: τ 2.51 (AA'BB' system, four aromatic protons of *p*-tolylsulfonyl), 4.13 (one-proton doublet, $J_{1,2}$ 3.7 Hz, H-1), 4.74 (one-proton singlet, methylidene H-3,5), 5.48 (one-proton doublet, $J_{1,2}$ 3.7 Hz, H-2), 5.67–6.18 (five-proton, complex multiplet, H-3, H-4, H-5, and two H-6), 6.72 (three-proton singlet, OMe), 7.57 (three-proton singlet, aromatic OMe), 8.54 and 8.69 (two three-proton singlets, two isopropylidene Me).

Anal. Calc. for $C_{18}H_{24}O_9S$: C, 51.9; H, 5.8; S, 7.7. Found: C, 51.9; H, 6.0; S, 7.7.

Trimethyl orthoformate showed ν_{max}^{neat} 2994s, 2945s, and 2840s cm^{-1} (CH and OCH_3), and p.m.r. in chloroform-*d*: τ 4.92 (one-proton singlet, CH); 6.61 (nine-proton singlet, three OMe). It has been reported[4,5] that the methylidyne proton in the related 1,2-*O*-isopropylidene-3,5,6-*O*-methylidyne-α-D-glucofuranose resonates at τ 3.99.

Evaporation of the second fraction gave a chromatographically homogeneous syrup (0.463 g) of 1,2-*O*-isopropylidene-3,5-*O*-[(*exo*-methoxy)methylidene]-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose (**3**), which has thus far resisted attempts at crystallization. It had $[\alpha]_D^{26}$ +30.7° (*c* 1.74, dichloromethane); i.r. data: ν_{max} 2986m, 2938m, and 2948w (CH and OMe), 1593m and 1490w cm^{-1} (Ar.); p.m.r. in chloroform-*d* at 60 MHz: τ 2.49 (AA'BB' system, four aromatic protons of *p*-tolylsulfonyl), 4.08 (one-proton doublet, $J_{1,2}$ 3.7 Hz, H-1), 4.62 (one-proton singlet, H-3,5), 5.82 (five-proton, complex multiplet, H-3, H-4, H-5, and two H-6), 6.67 (three-proton singlet, OMe), 7.56 (three-proton singlet, aromatic Me), 8.55 and 8.69 (two three-proton singlets, two isopropylidene Me).

Anal. Calc. for $C_{18}H_{24}O_9S$: C, 51.9; H, 5.8; S, 7.7. Found: C, 52.05; H, 5.9; S, 7.8.

Compounds **2** and **3** were unstable, and were therefore either stored at −13°, or were manipulated in solutions containing a trace of triethylamine or pyridine-d_5. Isomerization was inhibited by the latter procedure.

Equilibration of the diastereoisomeric 1,2-O-isopropylidene-3,5-O-(methoxymethylidene)-6-O-p-tolylsulfonyl-α-D-glucofuranoses (**2** and **3**). — (*a*) *With trimethyl orthoformate* in *N,N-dimethylformamide*. Each pure diastereoisomer (152 mg) was dissolved in a mixture of trimethyl orthoformate (2.2 ml), dry *N,N*-dimethylformamide (2 ml), and concentrated sulfuric acid (0.06 ml). The solution was rotated under a pressure of 80 torr for 3 days at 24°, and then neutralized with powdered calcium oxide, diluted with dichloromethane, and filtered. The solids were washed with dichloromethane, and the filtrate and washings were combined, washed with water (4 × 100 ml), "dried" by filtration through a dichloromethane-wetted filter paper, and, after addition of triethylamine (1 drop), evaporated to a syrup which was freed of residual solvents by addition and distillation of dichloromethane followed by drying at room temperature/50–100 mtorr (135 mg from **2**, and 118 mg from **3**). T.l.c. of each equilibrated mixture showed two major components having the same mobilities as **2** and **3**. A solution of each mixture (130 mg, and 118 mg) in benzene-d_6 (0.35 ml) was examined by p.m.r. spectroscopy at 60 MHz. In each case, the spectrum obtained was identical with that of the mixture of **2** and **3** already described. The relative proportions of **2** and **3** were determined (by integration of the H-3,5 and OMe singlets) to be 1.2±0.1:1 in the mixture prepared from **2**, and 1.1±0.1:1 in that from **3**.

(*b*) *In chloroform*-d. (*i*) *Equilibration of* **2**. After 1 min under catalyzed conditions, the H-3,5 singlet of the diastereoisomer **3** had appeared at τ 4.62; it had about half the intensity of the corresponding signal of **2**. After 2 min, the intensities of these singlets had reached a constant ratio (by visual estimation). Inspection of a complete

spectrum recorded after 20 min revealed two H-1 doublets, two H-3,5 singlets, two H-2 doublets, and two OMe singlets, and also the characteristic quartet (τ 2.03) and doublet (τ 6.3) of methyl formate. Equilibration of **2** and **3** was confirmed by integration of their H-3,5 and OMe singlets after 1 h and 19 h. From the beginning, the intensities of the methyl formate signals increased.

The solution was then re-equilibrated for 3 days at −9°. T.l.c. (0.1% of triethylamine in dichloromethane) at this point suggested that the solution still contained mainly a mixture of **2** and **3**, with **2** preponderating.

Occasional, spontaneous epimerizations of crystalline **2** in chloroform-*d* solutions were noted by p.m.r. spectroscopy. Typically, after 1.5 h at 35°, the intensity of the H-3,5 resonance of **3** was about a fifth of that of **2**. Integration of the H-3,5 and OMe resonances after 28 h and 6 days revealed that equilibrium had been attained within 28 h (K_{35} 1.52). During storage for a further 27 days at room temperature, the solution became highly acidic, and the disappearance of the H-3,5 and OMe signals from its spectrum evinced the complete decomposition of **2** and **3**. The appearance of a quartet (τ 2.02) and a doublet (τ 6.29) (1.5 h), and finally (33 days) of a sharp singlet (τ 7.87), suggested that both of the cyclic acetal groups were being hydrolyzed. The solution was then diluted with chloroform-*d* (0.5 ml), and the volatile components were distilled off at <60°/80 torr into an n.m.r. sample-tube cooled in Dry Ice–dichloromethane. The chemical shifts in a p.m.r. spectrum of the distillate served to identify the volatile products of the reaction as methyl formate (quartet at τ 2.05 and doublet at τ 6.30), and acetone (singlet at τ 7.87). The solution also showed ν_{max} 1713s cm^{-1} (C=O).

(*ii*) *Equilibration of* **3** *and isolation of* **2**. After 30 sec, the methylidene H singlet of the stereoisomer **2** was just visible at τ 4.72. Its intensity then increased rapidly until, after 3 min, a constant ratio to the corresponding signal of **3** had been reached (visual inspection). The signals of methyl formate were observed after 4 min, and their intensities increased slowly with time. Integration of the pairs of H-3,5 and OMe singlets after 0.5, 3.5, and 23 h, and also t.l.c., confirmed that equilibration had been achieved.

After re-equilibration and further n.m.r. measurements at −9°, the solution was diluted with dry dichloromethane (0.65 ml), kept for 22 h at −80°, and then poured into a stirred suspension of powdered calcium oxide in dichloromethane, precooled to −80°. The mixture was allowed to warm to room temperature, and filtered through Filter-Cel, and the solid matter was washed thoroughly with dichloromethane. The filtrate and washings were combined, treated with pyridine-d_5 (5 drops), and evaporated to a colorless syrup (65 mg), which was dissolved in chloroform-*d* (0.35 ml) containing a trace of pyridine-d_5. Integration of H-3,5 and OMe singlets in the p.m.r. spectrum of the solution disclosed that the ratio of **2**:**3** was 1.73:1. The solution was evaporated to dryness, and the residue dissolved in solvent *A* (5 ml) and chromatographed on silicic acid (Mallinckrodt, 100 mesh, 7 g). A solution of the first fraction in cold methanol deposited clusters of prisms on nucleation with **2**; 20 mg, m.p. 79–81°. The i.r. spectrum (film), p.m.r. spectrum (in chloroform-*d*

containing one drop of pyridine-d_5), and t.l.c. of this product were identical with those of authentic **2**.

(*c*) *In* N,N-*dimethylformamide.* — Under catalysis with 2 mg of *p*-toluenesulfonic acid per 0.4 ml of solvent at 35°, integration of the H-3,5 singlets at τ 4.48 (**3**) and τ 4.57 (**2**) revealed that equilibrium was attained within 17 h.

*Conversion of the diastereoisomeric 1,2-*O*-isopropylidene-3,5-*O*-(methoxymethylidene)-6-*O*-p-tolylsulfonyl-α-*D*-glucofuranoses* (**2** and **3**) *into 1,2:3,5-di-*O*-isopropylidene-6-*O*-p-tolylsulfonyl-α-*D*-glucofuranose* (**4**). A solution of a mixture of **2** and **3** in chloroform-*d* that had been prepared, as already described, by equilibration of (**3**) (85 mg) with *p*-toluenesulfonic acid (1.4 mg) was treated with dry acetone (16 μl, 1.1 molar equivalents). Periodic observation of the p.m.r. spectrum (60 MHz) showed that the intensities of each of the H-3,5 and OMe signals of **2** and **3** decreased gradually, at the same rate, until, after 4 h, these signals had disappeared from the spectrum. Concurrently, the intensities of the methyl formate signals and a singlet at τ 8.75 (isopropylidene Me) increased, and the pairs of H-1 and H-2 doublets of **2** and **3** were replaced by single doublets. The spectrum was unchanged after 22 h, and, except for the presence of methyl formate and some unreacted acetone, was identical with that of authentic **4**.

After 3 days, the solution was poured into a stirred suspension of calcium oxide powder in dry dichloromethane, and the suspension was filtered by use of a layer of Filter-Cel. The filtrate was treated with triethylamine (1 drop) and evaporated to a syrup (70 mg) which, on nucleation with **4**, crystallized from aqueous ethanol; 41 mg, m.p. 81–85°. Recrystallization from the same solvent gave clusters of colorless rods, 31 mg, m.p. 84–85°, mixed m.p. 86–87°, having i.r. ν_{max}^{film} 1600m and 1480w cm^{-1}, Ar) and p.m.r. (chloroform-*d*) spectra identical with those of authentic **4**.

ACKNOWLEDGMENTS

Thanks are due Drs. H. M. Fales and R. J. Highet for providing access to a 100-MHz n.m.r. spectrometer at the National Institutes of Health, Dr. R. B. Johannesen for his help, and Dr. R. Schaffer for his support.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

PRÉPARATION ET COUPLAGE SUR DES POLYPEPTIDES DE 5'-*O*-CARBOXYMÉTHYL-RIBONUCLÉOSIDES

J. P. COAT ET S. DAVID

Laboratoire de Recherches sur la Chimie des Composés Biologiques, Faculté des Sciences, 91 Orsay, (France)

(Reçu le 5 août 1969)

ABSTRACT

Condensation of ethyl iodoacetate with the sodium salt of methyl 2,3-*O*-isopropylidene-α,β-D-ribofuranoside gave the ester **1** which was reduced with lithium aluminium hydride to methyl 5-*O*-(2-hydroxyethyl)-2,3-*O*-isopropylidene-α,β-D-ribofuranoside (**3**). Removal of the protecting group gave a substituted ribose derivative which was either peracetylated, or used for the synthesis of 5'-*O*-(2-hydroxyethyl)-adenosine (**16**), or glycosidated to give 5-*O*-(2-hydroxyethyl)-D-ribofuranoside (**5**). The tribenzoate of **5** was converted into 1-*O*-acetyl-2,3-di-*O*-benzoyl-5-*O*-(2-benzoyloxyethyl)-D-ribofuranose (**6**), which gave a glycosyl halide used for the syntheses of the 5'-*O*-(2-hydroxyethyl) derivatives of uridine (**9**), cytosine (**12**), and guanosine (**14**). These four substituted ribonucleotides were catalytically oxidized to give the 5'-*O*-carboxymethylnucleotides **10**, **13**, **15**, and **17**, which were condensed with polyalanine–polylysine in the presence of carbodiimides to give potential antigens. The synthesis of 2'3'-*O*-benzylidene-5'-*O*-carboxymethyluridine is also described.

SOMMAIRE

La condensation de l'iodoacétate d'éthyle sur le dérivé sodé du méthyl 2,3-*O*-isopropylidène-α,β-D-ribofuranoside donne un ester **1** réduit par l'aluminohydrure de lithium en méthyl 5-*O*-(2-hydroxyéthyl)-2,3-*O*-isopropylidène-α,β-D-ribofuranoside, **2**. Après déblocage, on obtient un ribose substitué qui a été, soit converti en tétraacétate, utilisé directement pour la synthèse de la 5'-*O*-(2-hydroxyéthyl)adénosine **16**, soit glycosidé en méthyl 5-*O*-(2-hydroxyéthyl)-α,β-D-ribofuranoside (**5**). Le tribenzoate correspondant à **5** est transformé, par l'intermédiaire du 1-*O*-acétyl-2,3-di-*O*-benzoyl-5-*O*-(2-benzoyloxyéthyl)-α,β-D-ribofuranose **7**, en halogénose, utilisé pour la synthèse des dérivés 5'-*O*-(2-hydroxyéthyl) de l'uridine, **9**, de la cytosine, **12**, et de la guanosine, **14**. Ces quatre ribonucléosides substitués sont oxydés catalytiquement en 5'-*O*-carboxyméthylnucléosides, **10**, **13**, **15**, et **17**, qui sont condensés sur la polyalanine–polylysine par les carbodiimides, pour donner des antigènes potentiels. On décrit aussi la synthèse de la 2',3'-*O*-benzylidène-5'-*O*-carboxyméthyluridine.

INTRODUCTION

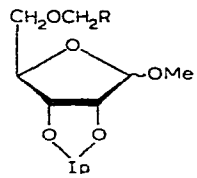
Diverses synthèses d'antigènes artificiels à déterminant nucléoside ont été récemment proposées. Ainsi, le dialdéhyde provenant de l'oxydation periodique des

ribonucléosides est cyclisé sur le groupe ε-aminé de la lysine de la sérumalbumine, et cet intermédiaire est stabilisé par réduction avec le borohydrure de sodium. On a traité ainsi les acides adényliques et uridyliques, la guanosine, la cytidine, et le thymine β-D-riboside[1]. Mais le furanose est devenu une morpholine. Sela *et coll.* ont couplé à la polyalanine–polylysine, par liaison amide, des nucléosides oxydés où la fonction alcool primaire a été remplacée par un groupe carboxyle. La méthode, décrite d'abord pour l'uridine[2], puis la cytidine, l'adénosine, la thymidine et la désoxyuridine[3] a été étendue à la pseudo- et à la dihydro-uridine[4]. La combinaison de mononucléotides sur la sérum-albumine humaine, à l'aide de carbodiimides, semble d'application limitée à l'acide thymidylique[5,6]. Le produit de dégradation de la guanosine, photooxydée par le rayonnement dans le visible en présence de bleu de méthylène, se condense sur les groupes ε-aminés de la lysine d'un polypeptide[7]. On a pu fixer ainsi la structure intacte des acides thymidylique et adénylique, à partir de leurs esters de guanosine. La structure d'ancrage reste inconnue[7]. Nous décrivons ici la synthèse des dérivés 5′-*O*-carboxyméthyles des quatre principaux ribonucléosides, et leur emploi à la place des nucléosides oxydés dans la condensation sur la polyalanine–polylysine.

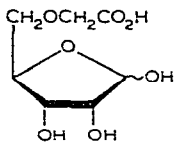
RÉSULTATS ET DISCUSSION

Le méthyl 2,3-*O*-isopropylidène-α,β-D-riboside[8] est considéré ici comme un mélange α,β, bien qu'il paraisse homogène en chromatographie en phase gazeuse, et qu'on observe seulement en r.m.n. un singulet δ 5,89 (dans le sulfoxyde de méthyle-d_6) attribué à H-1 de l'anomère β. Ceci est aussi vrai des composés **1** et **2**. Son dérivé sodé donne avec l'iodoacétate d'éthyle l'éther **1** (75%), qui est hydrolysé par traitement basique, puis acide, en 5-*O*-carboxyméthyl-D-ribose **4**, isolable à l'état de sel de baryum. Nous n'avons pas pu hydrolyser sélectivement les trois groupements protecteurs de **1**, et nous l'avons réduit en alcool **2** par l'aluminohydrure de lithium, avec l'intention de revenir à l'acide à l'étape finale. L'hydrolyse acide de **2** donne un sucre sirupeux, acétylé en **6**, ou bien converti en méthyl D-riboside substitué **5** par le gaz chlorhydrique méthanolique (*cf.* ref. 9). On obtient aussi **5** par traitement prolongé du sucre protégé **2** par le même réactif. Le spectre de r.m.n. semble indiquer la présence prépondérante de l'anomère β. Ce même riboside, **5**, a été transformé en 1-*O*-acétyl-2,3-di-*O*-benzoyl-5-*O*-(2-benzoyloxyéthyl)-α,β-D-ribofuranose **7**, sirupeux, en suivant la méthode décrite pour le méthyl D-riboside[10]. Toutes les propriétés examinées s'accordent avec la structure proposée **7**, avec forte prépondérance de l'anomère β. On passe à l'halogénose correspondant **8** par le gaz chlorhydrique dans l'éther. Nous n'avons obtenu que des mélanges inutilisables après les essais d'oxydation de l'éther allylique, **3**, préparé avec 89 % de rendement.

Pour les nucléosides synthétisés, nous avons considéré que la dispersion rotatoire entre 365 et 589 nm, de même allure que celle de l'analogue naturel, indiquait l'orientation β (*cf.* ref. 11, 12). L'halogénose **8** a été condensé avec la 2,4-dibenzyloxypyrimidine[13] en présence de tamis moléculaire[14], pour donner, après déblocage,



1 R=CO_2Et
2 R=CH_2OH
3 R=CH=CH_2



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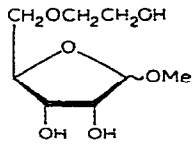
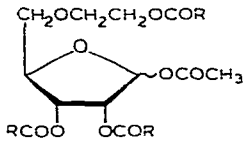
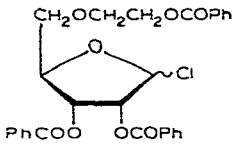


5



6 R=Me
7 R=Ph



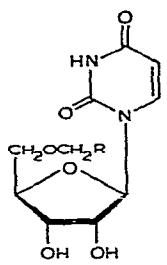
8

comme premier intermédiaire cristallisé, la 5'-*O*-(2-hydroxyéthyl)uridine **9**. Elle est oxydée quantitativement par voie catalytique[15], au pH 9, en 5'-*O*-carboxyméthyluridine amorphe, **10**, qui a les propriétés physiques attendues. La réaction sur le sel de mercure de la *N*-acétylcytosine de deux equivalents de **8**, selon Codington et Fox[16], donne la 4-*N*-acétyl-2',3'-di-*O*-benzoyl-5'-*O*-(2-benzoyloxyéthyl)cytidine (**11**). Le produit débloqué **12** donne un picrate cristallin, et il est oxyde catalytiquement en 5'-*O*-carboxyméthylcytidine (**13**), dont les cristaux sont très peu solubles dans l'eau et les solvants organiques.

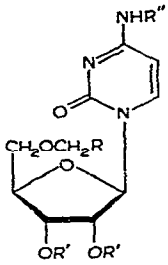
L'extension à notre série de la synthèse de la guanosine de Davoll et Lowy[17], qui part du sel de mercure de la 2,6-di-*N*-acétylpurine, a été difficile, car, entre nos mains, aucun produit n'a cristallisé, et il a fallu deux fois purifier par l'intermédiaire de sels de plomb. On obtient finalement **14**, après isolement chromatographique sur papier épais, à l'état de mousse incolore qui a la composition et le spectre u.v. attendus. Le produit d'oxydation **15** paraît pur par chromatographie sur papier, et le spectre u.v. est très voisin de celui de la guanosine, de même que le spectre r.m.n., sur lequel on voit en plus le singulet du groupe méthylène du groupe carboxyméthyle.

On a préparé l'adénosine substituée **16** par réaction du peracétate **6**, dissous dans le 1,2-dichloréthane, avec le sel de mercure de la 6-benzamidopurine, en présence de tétrachlorure de titane, suivant la technique publiée d'abord par Baker[18], qui peut d'ailleurs donner une quantité appréciable de nucléoside α-D. Les cristaux obtenus après déblocage paraissent purs en chromatographie sur papier, et, après triméthylsilylation, en phase gazeuse. En r.m.n. dans le sulfoxyde de méthyle-d_6, le doublet du proton anomère (δ 5,95) est plus voisin de celui de l'adénosine (δ 6,02) que de celui de 1' anomère[19] α de l'adénosine (δ 6,38). La constante de couplage est intermédiaire. La 5'-*O*-carboxyméthyladénosine **17** est également cristalline.

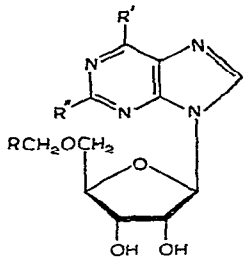
Par action du sel disodique de la 2',3'-*O*-isopropylidèneuridine sur l'iodoacétate d'éthyle dans le tétrahydrofurane bouillant, nous n'avons observé qu'une



9 $R = CH_2OH$
10 $R = CO_2H$



11 $R = CH_2OCOPh$
$R' = COPh$
$R'' = COMe$
12 $R = CH_2OH$
$R' = R'' = H$
13 $R = CO_2H$
$R' = R'' = H$



14 $R = CH_2OH$
$R' = OH$; $R'' = NH_2$
15 $R = CO_2H$
$R' = OH$; $R'' = NH_2$
16 $R = CH_2OH$
$R' = NH_2$; $R'' = H$
17 $R = CO_2H$
$R' = NH_2$; $R'' = H$

substitution sur N-3 : le spectre de r.m.n. dans le sulfoxyde de méthyle-d_6 du produit amorphe, purifié sur couche de silice de 5 mm, présente encore le triplet δ 5,00, J 5,1 du groupe hydroxyle, tandis que le doublet de H-3, δ 11,21, $J_{3,5}$ 1,8 a disparu. Il y a une bande i.r. ν 3490 cm^{-1} (OH). On peut aussi remarquer que le groupe hydroxyle n'est pas substitué sur les trois produits majeurs (sur huit en tout) que donne le sel monosodique de la 4-*N*-benzoyl-2′,3′-*O*-isopropylidènecytidine dans les mêmes conditions. Nous avons alors eu connaissance de la technique particulière de synthèse de **10** de Halford et Jones[20], avec le chloroacétate de sodium, sur le sel disodique de la 2′,3′-*O*-isopropylidèneuridine. En l'appliquant à la 2′,3′-*O*-benzylidèneuridine, nous avons pu éviter la séparation à contre-courant, et obtenir d'emblée par cristallisation le dérivé 5′-*O*-substitué, pur sur couche mince (35%). L'hydrolyse acide donne **10**.

Le couplage des composés **10**, **13**, **15**, et **17** par formation d'amides avec les groupes aminés de la polyalanine–polylysine, à l'aide de carbodiimides[2,3] a conduit aux taux de fixation rassemblés dans le Tableau I. On a fait les mesures par spectrophotométrie au maximum d'absorption proche de 260 nm du nucléoside.

TABLEAU I

TAUX DE FIXATION DES RIBONUCLÉOSIDES SUR LA POLYALANINE–POLYLYSINE[a]

Ribonucléoside	*Proportion (% en poids)*	*Composition en nucléoside–lysine–alanine*
Uridine	7,6	1–4,2–43,7
Cytidine	5,8	1–5,2–54
Adénosine	6,8	1–5–52
Guanosine	10,0	1–3,5–36,4

[a]Poids mol. 42.000; rapport alanine à lysine, 10,4.

PARTIE EXPÉRIMENTALE

Conditions générales. — Tous les solvants ont été éliminés à l'évaporateur rotatif, à une température inférieure à 40°. Les déterminations suivantes ont été effectuées: les points de fusion, corrigés, sur platine Köfler; les spectres de résonance magnétique nucléaire (r.m.n.), exprimés en p.p.m. à partir de la bande du (tétraméthyl)silane, à 60 MHz, à 35°, dans le solvant entre parenthèses, avec l'appareil JEOL; les spectres i.r. avec l'Infracord 237 Perkin–Elmer; les spectres u.v. avec l'appareil Shimadzu, sur des solutions voisines de 5×10^{-5} M dans le méthanol, sauf indication contraire; les pouvoirs rotatoires avec le polarimètre Perkin–Elmer, et les temps de rétention R_T en chromatographie en phase gazeuse (c.p.g.) avec l'appareil Aerograph-600 C, colonne SE 30 à 5% sur Chromosorb W, à 210°, dans l'azote et à pression de 1.7 atm.

Les chromatographies sur papier (c.p.) ont été faites avec les irrigants A, 2-propanol–ammoniaque–eau (7:1:2) ou B, butanol–acide acétique–eau (2:1:1). Pour les chromatographies en couches minces (c.c.m.), les conditions sont : alumine (irrigant : benzène–chloroforme, 1:1), gel de silice Prolabo (benzène–acétate d'éthyle, 17:3) et cellulose MN 300 G (butanol–acide acétique–eau, 2:1:1).

Méthyl 5-O-allyl-2,3-O-isopropylidène-α,β-D-ribofuranoside (**3**). — On ajoute à une solution agitée vigoureusement de méthyl 2,3-*O*-isopropylidène-α,β-D-ribofuranoside (15 g, 73,5 mmoles) dans le tétrahydrofurane sec (150 ml) une suspension (4,1 g, 85 mmoles) d'hydrure de sodium à 50% dans l'huile minérale. Après 4 h de chauffage à reflux, on refroidit et ajoute goutte à goutte le bromure d'allyle (12 g, 100 mmoles) en modérant constamment par refroidissement la réaction vive. Après 2 h de chauffage à reflux, on filtre le bromure de sodium, évapore à sec et distille le résidu : liquide (16 g, 89%), p.éb. 69°/0,07 mm; i.r. : pas d'absorption à 3460 cm^{-1} (OH).

Anal. Calc. pour $C_{12}H_{20}O_5$: C, 59,01; H, 8,19; O, 32,78. Trouvé : C, 59,16; H, 8,10; O, 32,47.

Méthyl 5-O-carbéthoxyméthyl-2,3-O-isopropylidène-α,β-D-ribofuranoside (**1**) — On verse goutte à goutte le méthyl 2,3-*O*-isopropylidène-α,β-D-ribofuranoside (23,4 g, 114 mmoles) dans le tétrahydrofurane sec (50 ml) sur une suspension à 50% d'hydrure de sodium dans l'huile minérale (8,6 g, 179 mmoles). Après 2 h de chauffage à reflux, on ajoute goutte à goutte à 10° l'iodoacétate d'éthyle (50 ml). Après 4 h d'agitation à 20°, on neutralise à l'acide acétique la solution rouge brique, et on évapore au maximum sous 0,1 mm. On reprend à l'eau et l'éther, et on évapore sous 0,1 mm la phase éthérée. Le résidu se sépare en deux couches, on distille la couche inférieure, et recueille **1**, liquide incolore (24,8 g, 75%), p.éb. 104–112°/0,06 mm; $[\alpha]_{589}^{26}$ −51,6°, $[\alpha]_{365}^{26}$ −150° (*c* 1,4, chloroforme); c.c.m. : R_F 0,91 (alumine); c.p.g. : 1,75 min; i.r. : 1775 cm^{-1} (C=O), pas de bande OH (liquide pur); r.m.n. : δ 4,00 ($OCH_2C{=}O$), 4,18 (quadruplet, *J* 7,2 Hz), 1,35 (triplet, *J* 7,2 Hz) (OCH_2CH_3) et 4,8 (H-1) (CCl_4).

Anal. Calc. pour $C_{13}H_{22}O_7$: C, 53,78; H, 7,64; O, 38,58. Trouvé : C, 53,78; H, 7,51; O, 38,77.

Sel de baryum du 5-O-carboxyméthyl-α,β-D-ribose (**4**). — Après chauffage pendant 2 h à reflux de **1** (2,06 g, 7,1 mmoles) dans une solution d'hydroxyde de sodium (14,2 mmoles) dans l'éthanol à 90% (20 ml), on déplace l'éthanol par addition d'eau suivie d'évaporation, acidifie au pH 2,5 avec l'acide sulfurique 0,5M, et extrait à l'éther : sirop jaune clair (1,8 g, 98%) dont on hydrolyse une fraction (1,26 g) par chauffage pendant 3 h à reflux dans un mélange de méthanol (7 ml) et d'acide sulfurique 0,2M (3 ml). On remplace le méthanol par addition d'eau, suivie d'évaporation, neutralise avec la baryte aqueuse (0,6 mmole), centrifuge et évapore à sec le surnageant pour obtenir le 5-*O*-carboxyméthyl-α,β-D-ribose, sirop (0,92 g); c.c.m. : R_F 0,53 (cellulose; révélable par le nitrate d'argent ou le bleu de bromophénol). Le sucre est dissous dans l'eau (10 ml), la solution est neutralisée par une solution de baryte et **4** est précipité par addition d'éthanol (100%) : $[\alpha]_{589}^{22}$ −29° (*c* 1,2, eau); r.m.n. : δ 4,98 (singulet, H-1, anomère β), 5,10 (doublet, $J_{1,2}$ 4 Hz, H-1, anomère α) (D_2O, réf. HOD δ 4,5).

Anal. Calc. pour $C_{14}H_{22}O_{14}Ba$: C, 30,48; H, 4.02. Trouvé : C, 31,14; H, 4,57.

Méthyl 5-O-(2-hydroxyéthyl)-2,3-O-isopropylidène-α,β-D-ribofuranoside (**2**). — On ajoute goutte à goutte **1** (21,9 g, 76 mmoles) dans l'éther anhydre (80 ml) à une solution d'aluminohydrure de lithium (4 g) dans l'éther (280 ml). Après chauffage pendant 1 h à reflux, on traite à l'acétate d'éthyle, puis avec la quantité stricte d'eau pour convertir le réactif en aluminate. On filtre, lave à l'éther le précipité, et distille les phases éthérées réunies, pour obtenir **2**, (13,5 g, 72%), liquide, p.éb. 105°/0,2 mm; c.p.g. : R_T 1.33 min; $[\alpha]_{589}^{22}$ −67°; i.r. : 3470 cm^{-1} (OH), liquide pur.

Anal. Calc. pour $C_{11}H_{20}O_6$: C, 53,21; H, 8,12; O, 38,67. Trouvé : C, 53,63; H, 8,29; O, 38,02.

5-O-(2-Hydroxyéthyl)-α,β-D-ribose. — On chauffe à 100° le composé **2** (6,75 g) dans l'acide chlorhydrique 0,04M (100 ml) jusqu'à sa disparition (c.p.g.), soit 2,5 h, on fait passer la solution sur une colonne de Dowex-1 (HCO_2^-), et on évapore à sec : sirop (5,25 g, 99%); c.c.m. : R_F 0.54 (cellulose, révélateur nitrate d'argent); r.m.n. : δ 5,20 (doublet, *J* 1,5 Hz, H-1, anomère β), et 5,30 (doublet, *J* 4,5 Hz, H-1, anomère α) (D_2O, réf. HOD, δ 3,66).

5-O-(2-Acétoxyéthyl)-1,2,3,-tri-O-acétyl-α,β-D-ribofuranose (**6**). — On laisse pendant 24 h à 20° le composé précédent dans la pyridine (10 ml) et l'anhydride acétique (10 ml). On évapore à sec et distille : liquide visqueux (3,5 g, 85%); p.éb. 163°/0,07 mm; c.p. : R_F 0,64 (papier imprégné de sulfoxyde de méthyle; irrigant : éther; révélateur : hydroxyde de sodium dans éthanol, puis nitrate d'argent dans l'acétone); i.r. : 1750 cm^{-1} (C = O); r.m.n. : δ 6,03 (doublet, *J* 1,5 Hz, H-1), δ 5,38 (doublet, *J* 5 Hz, H-3), δ 5,19 (quadruplet, *J* 1,5 Hz, *J* 5 Hz, H-2), δ 3,65 (multiplet, 2 H-5, OCH_2CH_2), δ 4,15 (H-4, CH_2OCO) et δ 2 (3 pics, Ac).

Anal. Calc. pour $C_{15}H_{22}O_{10}$: C, 49,72; H, 6,12; O, 44,16. Trouvé : C, 49,52; H, 5,97; O, 44,29.

Méthyl 5-O-(2-hydroxyéthyl)-α,β-D-ribofuranoside (**5**). — On laisse pendant

45 min à 20° le 5-*O*-(2-hydroxyéthyl)-D-ribose (3 g, 15,4 mmoles) dans le méthanol contenant 2% de gaz chlorhydrique sec (70 ml). On agite pendant 2 h avec le carbonate d'argent (4 g), filtre sur noir animal–Célite, et distille le filtrat, pour obtenir **5**, huile (2,85 g, 88%), p.éb. 174°/0,2 mm; c.c.m. : R_F 0,75 (cellulose, révélateur : permanganate de potassium–iodure de potassium); c.p.g. après (triméthyl)silylation : 2,40 min; r.m.n. : δ 4,84 (H-1), 3,68 (2 H-5, OCH_2CH_2O), 3,37 (OMe) (chloroforme-*d*).

Anal. Calc. pour $C_8H_{18}O_6$: C, 49,72; H, 6,12; O, 44,16. Trouvé : C, 49,52; H, 5,97; O, 44,29.

Méthyl 2,3-di-O-benzoyl-5-O-(2-benzoyloxyéthyl)-α,β-D-ribofuranoside. — On ajoute goutte à goutte avec agitation du chlorure de benzoyle (48,2 ml, 420 mmoles) à une solution de **5** (29,08 g, 139 mmoles) dans la pyridine sèche refroidie (100 ml). On laisse 12 h, verse dans l'eau glacée, extrait au chloroforme (3 × 100 ml). On lave la phase organique par l'hydrogénosulfate de potassium M, puis l'hydrogénocarbonate de sodium saturé, puis à l'eau, sèche avec le sulfate de sodium et évapore (64 g).

1-O-Acétyl-2,3-di-O-benzoyl-5-O-(2-benzoyloxyéthyl)-α,β-D-ribofuranose (**7**). — On dissout par 30 min d'agitation le produit précédent (35 g) dans une solution de gaz bromhydrique dans l'acide acétique à 35%. On ajoute de l'acide acétique (70 ml), on refroidit à 8° et on ajoute, goutte à goutte, de l'eau (50 ml), en 40 min, en maintenant entre 7° et 11°. Puis on sort le mélange du bain et on agite 20 min. On verse dans l'eau (800 ml), on extrait au chloroforme (3 × 200 ml), et lave la couche organique avec l'hydrogénocarbonate de sodium aqueux saturé, puis à l'eau. On sèche l'extrait par deux entraînements à la pyridine sèche, et on ajoute de la pyridine (60 ml) et de l'anhydride acétique (20 ml). Après 48 h, on verse dans l'eau, et on extrait au chloroforme. L'huile rouge (32 g) obtenue après les traitements habituels, en solution dans le benzène, est adsorbée sur l'alumine (100 g). L'huile jaune éluée par le mélange benzène–acétate d'éthyle (17:3) est séchée pendant 48 h à 50° sous vide de 0.1 mm (28,8 g); c.c.m. : un constituant fortement prépondérant, R_F 0,60 (gel de silice); i.r. : 1740, 1220 (CO acétyle) et 1720, 1270 cm^{-1} (CO benzoyle) (liquide pur); r.m.n. : δ 8–7 (Ph), 6,26 (doublet $J_{1,2}$ 1,5 Hz, H-1), 5,55 (quadruplet, $J_{1,2}$ 1,5 Hz, $J_{2,3}$ 5,2 Hz, H-2), 5,76 (doublet $J_{2,3}$ 5,2 Hz, H-3), 4,45 (H-4, CH_2OCO), 3,81 (2 H-5, OCH_2CH_2O), 2,01 (Me) (CCl_4).

Préparation de l'halogénose **8**. — On sature par le gaz chlorhydrique une solution de **6** (5 g) dans l'éther sec (150 ml) à 0°. Après 4 jours à −3°, on évapore à sec et on chasse les traces de gaz chlorhydrique par trois entraînements au benzène sec.

5'-O-(2-Hydroxyéthyl)uridine (**9**). — On garde pendant 5 jours une solution de **8** (32 g, 5,9 mmoles) et de 2,4-dibenzyloxypyrimidine (1,7 g, 5,9 mmoles) dans le cyanure de méthyle (36 ml), en présence de tamis moléculaire 3 Å (6 g). On filtre, évapore à sec et chromatographie le résidu sur couche de 1 mm de gel de silice (irrigant : éthanol). La gomme isolée (0,95 g, 23%) est considérée comme la 1-*N*-[2'3'-di-*O*-benzoyl-5'-*O*-(2-benzoyloxyéthyl)-β-D-ribofuranosyl]-4-benzyloxy-2-pyrimidinone; u.v. : λ_{max} 274 (ε 8000), 226 (ε 35,000), λ_{min} 252 (ε 4600) nm.

On la dissout dans le chloroforme saturé par du gaz chlorhydrique sec, et

après 7 h on évapore à sec, et reprend par une solution méthanolique 0,04M de méthylate de sodium (50 ml). Après 10 h à 20°, on ajoute 10 g de Dowex-50 (H^+), on filtre, évapore à sec, reprend à l'eau et extrait le benzoate de méthyle à l'éther. La phase aqueuse est évaporée à sec et le résidu recristallisé dans le méthanol (331 mg, 83%), p.f. 132°; $[\alpha]_{589}^{20}$ +6,9°, $[\alpha]_{365}^{20}$ +69,2°; u.v. : λ_{max} : 261 (ε 9590), 231 (ε 2500) nm; r.m.n. : δ 11,25 (doublet $J_{3,5}$ 2 Hz, H-3), 5,80 (doublet $J_{1',2'}$ 4,6 Hz, H-1') (sulfoxyde de méthyle-d_6).

Anal. Calc. pour $C_{11}H_{16}N_2O_7$: C, 45,83; H, 5,6; N, 9,72. Trouvé : C, 45,13; H, 5,8; N, 9,72.

5'-O-Carboxyméthyluridine (**10**). — On fait barbotter pendant 24 h de l'oxygène dans une solution maintenue à 80° de **9** (288 mg, 1 mmole) dans 36 ml de tampon pH 9 (84 mg d'hydrogénocarbonate de sodium et 40 mg de carbonate de sodium·10 H_2O) en présence de platine d'Adams (30 mg). On filtre, élimine les ions Na^+ par l'Amberlite IR-50 (H^+) et on évapore à sec : verre jaune clair (295 mg); c.p. : R_F 0,23 (irrigant A, tache visible en u.v., ou révélable par le réactif permanganate de potassium–periodate de potassium); u.v. : comme **9**; $[\alpha]_{589}^{25}$ +7,2°, $[\alpha]_{365}^{25}$ +68° (*c* 1,5, eau); neutralisation : pK_a 3,8 et 9,5; équivalent : calc. 302, trouvé : 310.

4-N-Acétyl-2',3'-di-O-benzoyl-5'-O-(2-benzoyloxyéthyl)cytidine (**11**). — On distille 50 ml d'une suspension du sel de mercure de la *N*-acetylcytosine (3,18 g, 9 mmoles) dans le xylène sec (400 ml), puis on ajoute **8** (10,35 g, 19,2 mmoles). Après 45 min de chauffage à reflux avec agitation, on refroidit et verse dans l'éther de pétrole (1 l). On dissout tout ce qui est insoluble par le chloroforme, lave la solution par l'iodure de potassium aqueux à 30%, sèche et évapore à sec. On purifie le résidu sur couche épaisse de silice (irrigant : acétate d'éthyle) en retenant la zone R_F 0,5 visible en u.v. (2.1 g, 18%). On recristallise dans l'alcool, p.f. 136°; u.v. : λ_{max} : 299 (ε 6300), 281 (ε 7000), et 230 (ε 40,000) nm; λ_{min} 277 et 289 nm.

Anal. Calc. pour $C_{34}H_{31}N_3O_{10}$: C, 63,64; H, 4,87; N, 6,55; O, 24,94. Trouvé : C, 62,89; H, 4,93; N, 6,79; O, 24,75.

5'-O-(2-Hydroxyéthyl)cytidine (**12**). — Une solution de **11** (700 mg) dans l'éthanol (70 ml), saturée en gaz ammoniaque à 0°, est ensuite chauffée pendant 16 h à 100° en tube scellé. On évapore à sec, reprend à l'eau et purifie par extraction au chloroforme la phase aqueuse, qui, évaporée à sec, donne 480 mg de sirop, principalement **12**. On en purifie 35 mg sur papier épais, R_F 0,41 (irrigant B). Mousse blanche par lyophilisation (22 mg); u.v. : λ_{max} 270 (ε 7000), λ_{min} 250 (ε 5000) nm (*c* 7×10^{-5}M dans tampon phosphate pH 7).

Le picrate est recristallisé dans le méthanol (30%), p.f. 150–153°; u.v. presque identique à celui du picrate de cytidine dans le tampon pH 7.

Anal. Calc. pour $C_{17}H_{20}N_6O_{13}$: C, 38,72; H, 3,64; N, 16,48. Trouvé : C, 38,41; H, 3,83; N, 16,74.

5'-O-Carboxyméthylcytidine (**13**). — On fait barbotter l'oxygène pendant 20 h dans une solution de **12** brut (446 mg, 1,625 mmole) dans un tampon de pH 9 (60 ml), maintenue à 80°, en présence de platine d'Adams fraîchement réduit (250 mg). On filtre, ajuste la solution au pH 7, et la fait passer sur une colonne de cellulose DEAE

(40 g) équilibrée dans un tampon phosphate de pH 7. On lave à l'eau, puis on élue **13** avec l'ammoniaque 0,5M. On concentre à 5 ml, et amène au pH 4 par l'acide sulfurique 0,5M. Des cristaux blancs se déposent, p.f. 255–258°; c.p. : R_F 0,29 (irrigant A); u.v. : λ_{max} 270 (ε 8840), λ_{min} 243 (ε 6200) nm (tampon phosphate pH 7); $[\alpha]_{589}^{25}$ +30,6°, $[\alpha]_{365}^{25}$ +205° (*c* 0.77, ammoniaque 0,28M).

Anal. Calc. pour $C_{11}H_{15}N_3O_7$: C, 43,85; H, 5,02; N, 19,95. Trouvé : C, 43,76; H, 5,00; N, 19,64.

5'-O-(2-Hydroxyéthyl)adénosine (**16**). — Dans une solution agitée du composé **6** (8,3 g; 23,5 mmoles) dans le 1,2-dichloroéthane sec (850 ml), on met en suspension le mélange de Célite (14 g) et de sel de mercure de la 6-benzamidopurine (13,43 g, 28,5 mmoles). On ajoute le tétrachlorure de titane (28,5 mmoles, 5,5 g). Après avoir chauffé pendant 14 h à reflux, on refroidit, on ajoute 50 ml d'acide chlorhydrique M, et on agite pendant 1 h. On filtre et lave 3 fois l'insoluble par le chloroforme chaud (100 ml). Filtrats et eaux de lavage réunis sont lavés par 3 × 300 ml d'iodure de potassium aqueux à 30%, puis séchés avec le sulfate de sodium, et évaporés à sec (14,3 g). Le résidu est purifié sur couche de 4,5 mm de silice, en recueillant la fraction R_F 0,23–0,46 (irrigant acétate d'éthyle), qui est éluée à l'éthanol chaud : huile (7 g, 54%) que l'on dissout dans l'éthanol absolu (100 ml) contenant 3,6 mmoles de méthylate de sodium. Après avoir chauffé pendant 30 min à reflux, on filtre le précipité (2,5 g, 35%) qui est recristallisé dans l'éthanol, p.f. 144°; u.v. : λ_{max} 258 (ε 14,400), λ_{min} 226 (ε 2300) nm; $[\alpha]_{589}^{20}$ −27,5°, $[\alpha]_{365}^{20}$ −101° (*c* 0,9, eau); r.m.n. : δ 7,25 (NH_2), 5,95 (doublet $J_{1',2'}$ 5,2 Hz, H-1') (sulfoxyde de méthyle-d_6).

Anal. Calc. pour $C_{12}H_{17}N_5O_5$: N, 22,5. Trouvé : N, 22,8.

Le picrate est préparé par chauffage pendant 10 min à reflux dans le méthanol avec un équivalent d'acide picrique. Cristaux par refroidissement (30%), p.f. 181,2°.

Anal. Calc. pour $C_{18}H_8N_{20}O_{12}$: C, 40,00; H, 3,73; N, 20,74. Trouvé : C, 39,60; H, 4,00; N, 20,61.

5'-O-Carboxyméthyladénosine (**17**). — On fait barbotter l'oxygène pendant 40 h dans une solution à 90° de **16** (933 mg) dans un tampon à pH 3 (120 ml), en présence de platine d'Adams fraîchement réduit (880 mg). On amène au pH 7 avec l'acide sulfurique dilué, concentre à 20 ml et fait passer sur une colonne de cellulose DEAE équilibrée au pH 7 par un tampon phosphate 0,01M. On élue à l'eau **16** non transformée, et par l'ammoniaque 0,05M le composé **17**. On évapore à sec, reprend à l'eau (15 ml), ajuste au pH 2,3 par l'acide sulfurique 0,05M; on centrifuge et lave à l'eau par centrifugation le précipité (50%), p.f. 215–218°; c.p. : une seule tache sur papier, R_F 0.32 (irrigant A); $[\alpha]_{589}^{25}$ −30,9°, $[\alpha]_{365}^{25}$ −111° (*c* 0,82 dans l'ammoniaque dilué); u.v. : λ_{max} 259 (ε 14,500), λ_{min} 227 (ε 2400) nm (*c* 7×10^{-5} dans tampon pH 7).

Anal. Calc. pour $C_{12}H_{15}N_5O_6$: C, 44,31; H, 4,65; N, 21,53. Trouvé : C, 43,80; H, 4,60, N, 21,74.

5'-O-(2-Hydroxyéthyl)guanosine (**14**). — On met en suspension dans le xylène sec (400 ml) un mélange de Célite (7 g) et de sel de mercure de la 2,6-diacétamidopurine (6 g, 10,6 mmoles). Avec agitation, on distille une fraction du xylène, puis on ajoute l'halogénose préparé à partir de **7** (7,33 g, 13,6 mmoles), et on maintient le chauffage

à reflux pendant 3 h. On filtre la solution refroidie, et lave l'insoluble avec 3 × 100 ml de chloroforme. On concentre le xylène à 100 ml et verse dans 1 litre d'éther de pétrole. Le précipité est repris au chloroforme, et les phases chloroformiques réunies, lavées par 3 × 100 ml d'iodure de potassium aqueux à 30%, séchées et évaporées à sec donnent 7 g d'un verre jaune clair. On en dissout 1 g dans le 2-méthoxyéthanol anhydre (10 ml), on refroidit à 0°, et verse dans le méthanol saturé à 0° en gaz ammoniaque (20 ml). Après avoir gardé la solution pendant 16 h à 3°, on l'évapore, on reprend le résidu par 20 ml d'eau et 20 ml de chloroforme. On évapore à sec la phase aqueuse après lavage avec le chloroforme. On obtient 220 mg de verre incolore, principalement la 2-acétamido-6-amino-9-[5-*O*-(2-hydroxyéthyl)-β-D-ribofuranosyl]purine. Ce dernier produit (1,34 g) dans l'eau chaude (9,2 ml), est traité par le nitrite de sodium (2,9 g). On refroidit, ajoute 2,9 ml d'acide acétique, et agite jusqu'à ce que la solution soit limpide. Après 1 h, on dilue par l'eau (12 ml), laisse pendant 12 h, puis amène le volume à 70 ml. On ajoute 2,6 g d'acétate de plomb, alcalinise à l'ammoniaque, centrifuge et lave à l'ammoniaque le sel de plomb précipité. On le redissout dans l'acide acétique à 20%, puis le décompose avec l'hydrogène sulfuré. On filtre, et par évaporation à sec du filtrat, on obtient 920 mg, principalement la 2-acétamido-6-hydroxy-9-*N*[5-*O*-(2-hydroxyéthyl)-β-D-ribofuranosyl]purine.

On fait bouillir pendant 1 h 730 mg de ce composé dans le méthanol (50 ml) contenant 13 mmoles de méthylate de sodium. On ajoute 10 ml d'eau et on fait bouillir pendant encore 10 min. Puis on fait passer sur l'Amberlite IR-50 (H^+) (4 g) et on évapore à sec (528 mg). On purifie une partie (150 mg) par le sel de plomb comme précédemment, et on termine par une chromatographie sur papier Whatman 3MM (butanol–acide acétique–eau, 2:1:1) en recueillant la bande R_F 0,30–0,50 : mousse blanche après lyophilisation (92 mg); u.v. : λ_{max} 253 (ε 11,900), λ_{min} 224 (ε 3100) nm; épaulement à 270 nm (*c* 5×10^{-5} dans tampon pH 7); $[\alpha]_{589}^{20}$ −38,6°, $[\alpha]_{365}^{20}$ −229° (*c* 3,7 dans l'hydroxyde de sodium M).

Anal. Calc. pour $C_{12}H_{17}N_5O_6$: C, 44,03; H, 5,24; N, 21,40. Trouvé : C, 44,35; H, 5,31; N, 21,50.

5'-O-Carboxyméthylguanosine (**15**). — On part de **14** avant purification par le sel de plomb (327 mg, 1 mmole) dissous dans 40 ml de tampon pH 9. On fait barbotter l'oxygène dans la solution maintenue à 90°, en présence de platine d'Adams fraîchement réduit (300 mg). La réaction suivie chromatographiquement est complète après 20 h. On filtre la solution, on la fait passer sur Amberlite IR-50 (H^+) et on lyophilise l'éluat : poudre blanche (320 mg; 90%); c.p. : une seule tache R_F 0,11 (irrigant A); u.v. : λ_{max} 253,5 (ε 10,700), λ_{min} 225 (ε 2600) nm (*c* 10^{-5} dans tampon phosphate pH 7); r.m.n. : δ 5,65 (doublet, $J_{1',2'}$ 5,5 Hz, H-1') ($NaOD–D_2O$; réf. HOD δ 4,69).

2',3'-O-Benzylidène-5'-O-carboxyméthyluridine. — On lyophilise une solution de 2',3'-*O*-benzylidèneuridine (1,726 g; 5,2 mmoles) dans l'hydroxyde de sodium M (10 ml). On ajoute le chloroacétate de sodium (0,62 g; 5,3 mmoles) et on sèche le mélange pendant 4 h à 60° sous vide. On reprend le solide par le sulfoxyde de méthyle fraîchement distillé (25 ml) et garde pendant 24 h. On évapore le solvant sous 0,1 mm,

on reprend avec l'eau (20 ml) et on ajuste au pH 2. Il précipite une gomme que l'on recristallise dans le cyanure de méthyle (710 mg, 35%), p.f. 175°; u.v. : λ_{max} 260 nm (ε 10,300), λ_{min} 229 nm (ε 2000); r.m.n. : δ 11,27 (doublet, *J* 1,7 Hz, H-3).

Anal. Calc. pour $C_{18}H_{18}N_2O_7$: C, 55,38; H, 4,65; N, 7,18. Trouvé : C, 55,21; H, 4,61; N, 7,30.

On fait bouillir pendant 3 h à reflux 500 mg du composé ci-dessus dans l'acide acétique à 60% (50 ml). Après le traitement habituel, on obtient un sirop (375 mg; 96%), homogène en c.c.m. sur silice, R_F 0.45 et en c.p., R_F 0.23 (A).

Condensations avec la polyalanine–polylysine. — (*a*) *5'-O-Carboxyméthyluridine.* On ajoute le chlorhydrate de 3'-diméthylamino-3-éthyl-1-propylcarbodiimide (EDP) (14,3 mg) à une solution de **10** (24,6 mg) et de polyalanine–polylysine (PAPL) (37,5 mg) dans l'eau (2 ml). Après 18 h à 20°, on dialyse contre 5 × 500 ml d'eau distillée, et on lyophilise (28 mg).

(*b*) *5'-O-Carboxyméthylcytidine.* — On dissout **13** (0,03 mmole) dans l'eau (2 ml) contenant 0,0225 mmole d'hydroxyde de sodium. Après addition de PAPL (15 mg) et de EDP (11,5 mg), on laisse 20 h à 20°, on dialyse contre 3 × 500 ml d'hydrogénocarbonate de sodium aqueux à 5%, puis contre 3 × 500 ml d'eau distillée et on lyophilise. La mousse obtenue (14 mg, 4,8% de cytidine combinée) est recyclée pour donner 12 mg de produit final.

(*c*) *5'-O-Carboxyméthylguanosine.* — Par addition de EDP (11,5 mg) à une solution de **15** (20,4 mg) et de PAPL (30,8 mg) dans l'eau (2 ml). On garde pendant 18 h à 20°, dialyse contre 5 × 500 ml d'eau distillée, et lyophilise (22 mg).

(*d*) *5'-O-Carboxyméthyladénosine.* — A une solution de PAPL (15 mg) dans l'eau (0,1 ml) on ajoute 1,9 ml de *N,N*-diméthylformamide fraîchement distillée, puis 9,2 mg de **17** et 8 mg de *N,N'*-dicyclohexylcarbodiimide. On agite 20 h à 20°, puis on dialyse la solution trouble contre 3 × 500 ml d'hydrogénocarbonate de sodium aqueux à 5%, puis contre 3 × 500 ml d'eau distillée, et lyophilise (11 mg, 3,5% d'adénosine combinée). On recycle ce produit.

REMERCIEMENTS

Les auteurs remercient le Professeur M. Sela pour le don de la polyalanine–polylysine utilisée dans ce travail. La collaboration technique partielle de Madame Gisèle Coat a été appréciée.

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Carbohyd. Res., 12 (1970) 335–346

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

OXIRANE RING-OPENING REACTIONS OF 3-*O*-(2,3-EPOXYPROPYL)-1,2:5,6-DI-*O*-ISOPROPYLIDENE-α-D-GLUCOFURANOSE

R. E. WING, W. M. DOANE, AND C. E. RIST
Northern Regional Research Laboratory, Peoria, Illinois 61604 (U. S. A.)*
(Received August 15th, 1969)

ABSTRACT

Reactions are reported of the oxirane ring of 3-*O*-(2,3-epoxypropyl)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose with water, primary and secondary alcohols, lithium aluminum hydride, ammonia, primary and secondary amines, and dipotassium hydrogen phosphate. In most reactions, the expected products were obtained in quantitative yields.

INTRODUCTION

Investigations are being conducted at this Laboratory on methods for substituting starch with side chains that contain reactive functional groups. Because these reactive centers provide sites for attachment of various monomeric and polymeric materials, starch derivatives might be prepared having a broad spectrum of properties.

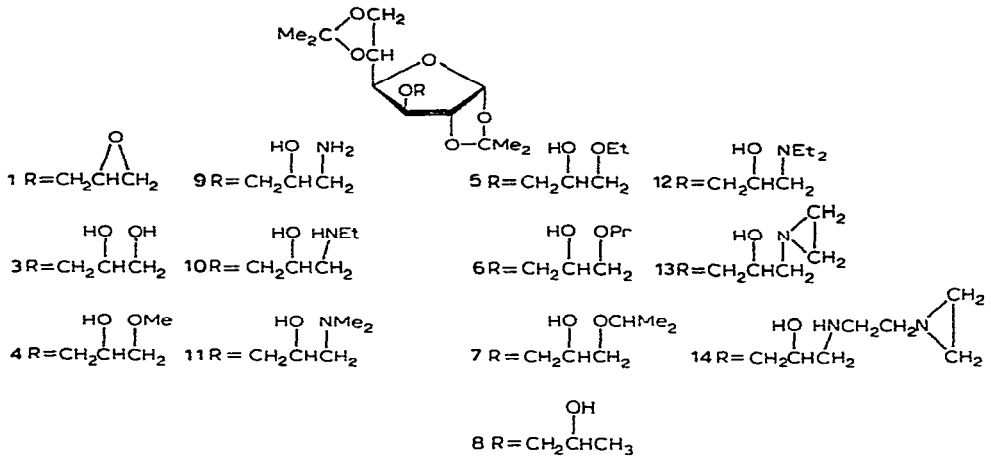
Recently we described methods whereby an alkyl group containing an oxirane or thiirane ring could be appended to the model compound 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose[1]. Now we report several reactions that the oxirane ring of 3-*O*-(2,3-epoxypropyl)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**1**) undergoes.

DISCUSSION

Oxiranes constitute one of the most reactive classes of organic compounds with respect to attack by nucleophilic reagents[2-4]. The oxirane group reacts with water under acidic or basic catalysis to form glycols[5-7]. Treatment of **1** with dilute sulfuric acid opens the oxirane ring, and simultaneously cleaves both isopropylidene acetal linkages, to form 3-*O*-(2,3-dihydroxypropyl)-D-glucose (**2**). Treatment of **1** with aqueous sodium hydroxide gives 3-*O*-(2,3-dihydroxypropyl)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**3**). In the absence of acid or base, the oxirane ring of **1** is stable to distilled water at 70° under diminished pressure.

The oxirane ring is opened by simple alcohols to give glycol ethers[8-13]. Treating **1** with methanol, ethanol, propyl alcohol, and isopropyl alcohol, under

*This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.



basic catalysis, gives the corresponding 3-alkoxy-2-hydroxy derivatives (**4–7**). The stereochemistry of this reaction is well known[2,3,14], and proof that nucleophilic attack takes place at the least-substituted carbon atom, with the concurrent formation of a secondary hydroxyl group, is shown by n.m.r. spectroscopy (doublet τ 5.3) for products **4–7**. Use of an acid catalyst in these reactions would afford the isomeric 2-alkoxy-3-hydroxy derivatives (formation of the primary hydroxyl group would result, because the secondary carbon atom would form the more stable carbonium ion)[2–3]. Acid hydrolysis of **6** and **7** with 0.5M sulfuric acid gives 3-*O*-[2-hydroxy-3-(propoxy)propyl]-D-glucose (**15**) and 3-*O*-[2-hydroxy-3-(isopropoxy)propyl]-D-glucose (**16**), respectively.

Compound **1** was treated also with alcohols of higher molecular weight (octyl alcohol, dodecyl alcohol, and 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose). The reaction with octyl alcohol proceeded to completion but more slowly than for the lower alcohols; however, dodecyl alcohol and 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose gave no reaction (80° and 1% base). Micheel and Schiller[15] reported that 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose reacts with simple oxiranes, but under more strenuous conditions (130–150° and 3–5% base).

The reduction of oxiranes is a convenient method to prepare secondary alcohols. The direction of cleavage of oxirane rings is the same as that for other nucleophiles[16,17]. The use of complex metal hydrides (lithium aluminum hydride) results in the reaction ceasing at the alcohol stage and also ensures selectivity of the attack[18]. When **1** was treated with an ethereal solution of lithium aluminum hydride, the product was 3-*O*-(2-hydroxypropyl)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**8**).

Reaction of ammonia or amines with oxiranes produces ethanolamines[11,12,19,20]. When **1** was treated with ammonia, a primary amine (ethylamine), and several secondary amines (dimethylamine, diethylamine, and ethylenimine), the corresponding ethanolamines were formed (**9–13**). Little or no aminolysis occurred in the absence of a hydroxyl-containing solvent; however, the inclusion of small amounts

of water or methanol[21,22] resulted in a complete reaction within a few h, even at room temperature. When **1** was treated with *N*-(2-aminoethyl)aziridine, aminolysis by the primary amine formed **14**, which contains both secondary and tertiary nitrogen. The treatment of **1** with a tertiary amine (triethylamine) gave no reaction, even in the presence of water at 50°. But, when the reaction was performed in the presence of methanol, the triethylamine acted as a basic catalyst and **4** was the product. Literature is available which describes the reaction of triethylamine with oxiranes to form quaternary compounds[21,23].

Lampson and Lardy[24] reported the reaction of 1,2-epoxypropane and 5,6-anhydro-1,2-*O*-isopropylidene-D-glucofuranose with dipotassium hydrogen phosphate to form the corresponding phosphate esters. When **1** was treated under similar conditions, the only product formed was **3**. The dibasic phosphate, being only slightly alkaline and moderately nucleophilic, probably was sterically hindered by the isopropylidene blocking groups. The smaller hydroxyl ions, even though present in low concentration, reacted preferentially to form **3**.

The conditions used for these ring-opening reactions and the steric hindrance of the isopropylidene groups near the reaction site prevent the formation of dimers or polymers similar to those reported with smaller oxirane compounds[2,3]. Numerous reports[2-4,21,24-27] show these reactions with anhydro sugars.

Compounds **2–16** were recovered as syrups. Each was homogeneous by t.l.c. and they gave elemental analyses and spectral data consistent with their proposed structures. Compounds **3–14** were soluble in most organic solvents, whereas **2**, **15**, and **16** were soluble only in water and alcohol.

EXPERIMENTAL

General. — Optical rotations were determined with a Model 80 Rudolph* polarimeter in 1-dm tubes. I.r. spectra were recorded on a Perkin–Elmer 137 Infracord spectrophotometer. N.m.r. spectra were recorded on a Varian HA-100 spectrometer with methyl sulfoxide-d_6 as the solvent. For column chromatography, Silica Gel Adsorbosil (Applied Science Laboratories, Inc., P. O. Box 140, State College, Pennsylvania) 60–100 mesh, neutralized to pH 7.0 with ammonium hydroxide, was used with a graded hexane–chloroform eluant. T.l.c. was conducted on 20 × 20 cm plates of Silica Gel G (Brinkmann Instruments, Inc., Cantiague Road, Westbury, New York) with irrigants (*a*) 2:1 hexane–acetone[1,28] and (*b*) 9:6:3:1 butyl alcohol–acetic acid–ether–water[29]. The plates were developed by spraying with a solution of 20% sulfuric acid in ethanol and heating for 20 min at 140°. Colored solutions were decolorized with Darco G-60 carbon.

Reaction of **1** *with water.* — (*a*) *3-O-(2,3-Dihydroxypropyl)-D-glucose* (**2**). Compound **1** (0.656 g, R_F^a 0.50) was dissolved in *p*-dioxane (25 ml) and 0.5M sulfuric

*Mention of firm names or trade products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture over other firms or similar products not named.

acid (25 ml) was added. After stirring for 6 h at 50°, the reaction mixture was diluted with water (50 ml) and stirred to neutrality with Rexyn 201 (OH^-). Filtration and evaporation under diminished pressure afforded **2** as a colorless syrup (0.520 g, 100%); $[\alpha]_D^{24}$ +39° (*c* 1, water); R_G^b 0.85.

Anal. Calc. for $C_9H_{18}O_8$: C, 42.52; H, 7.09. Found: C, 42.30; H, 7.30.

(b) 3-O-(2,3-Dihydroxypropyl)-1,2:5,6-di-O-isopropylidene-α-D-glucofuranose (**3**). Compound **1** (0.67 g) was dissolved in *p*-dioxane (25 ml) and 0.50M sodium hydroxide (25 ml) was added. After the reaction mixture had been stirred for 12 h at 50°, t.l.c. showed 80% **3** and 20% degraded material (ether cleavage). The solution was diluted to 150 ml with water and neutralized by stirring with Rexyn 101 (H^+). Filtration and evaporation followed by column chromatography gave **3** as a colorless syrup (0.49 g, 70%); $[\alpha]_D^{24}$ −44.5° (*c* 2, chloroform); R_F^a 0.13.

Anal. Calc. for $C_{15}H_{26}O_8$: C, 53.89 ; H, 7.78. Found: C, 53.70; H, 7.76.

Reaction of **1** *with alcohols. 3-O-(3-Alkoxy-2-hydroxypropyl)-1,2:5,6-di-O-isopropylidene-α-D-glucofuranoses* (**4**–**7**). — Compound **1** (1.00 g, 3.16 mmoles) was dissolved in the appropriate alcohol (1.58 moles) and powdered sodium hydroxide (1% of alcohol) was added. The mixture was heated for 3 h under reflux, at which time t.l.c. showed complete reaction. The solution was stirred with a small amount of Rexyn 101 (H^+) to adjust the pH to 7. Filtration and evaporation left a residue which was extracted into chloroform and evaporated to a colorless syrup.

Alcohol (ml)	*NaOH (g)*	*Product*	*Yield (g)*
MeOH (64.0)	0.37	**4**	1.10
EtOH (92.2)	0.43	**5**	1.14
PrOH (121.6)	0.46	**6**	1.19
isoPrOH (121.6)	0.46	**7**	1.19

3-*O*-(2-Hydroxy-3-methoxypropyl)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**4**); $[\alpha]_D^{24}$ −42.5° (*c* 2, chloroform); R_F^a 0.33; n.m.r. data: τ 5.26 (doublet, secondary hydroxyl group).

Anal. Calc. for $C_{16}H_{28}O_8$: C, 55.17; H, 8.05. Found: C, 55.02; H, 8.22.

3-*O*-(3-Ethoxy-2-hydroxypropyl)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**5**); $[\alpha]_D^{24}$ −40.0° (*c* 2, chloroform); R_F^a 0.40; n.m.r. data: τ 5.30 (doublet, secondary hydroxyl group).

Anal. Calc. for $C_{17}H_{30}O_8$: C, 56.35; H, 8.29. Found: C, 56.31; H, 8.31.

3-*O*-[2-Hydroxy-3-(propoxy)propyl]-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**6**); $[\alpha]_D^{24}$ −37.5° (*c* 2, chloroform); R_F^a 0.45; n.m.r. data: τ 5.30 (doublet, secondary hydroxyl group).

Anal. Calc. for $C_{18}H_{32}O_8$: C, 57.45; H, 8.51. Found: C, 57.34; H, 8.56.

3-*O*-[2-Hydroxy-3-(isopropoxy)propyl]-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**7**); $[\alpha]_D^{24}$ −37.5° (*c* 2, chloroform); R_F^a 0.45; n.m.r. data: τ 5.34 (doublet, secondary hydroxyl group).

Anal. Calc. for $C_{18}H_{32}O_8$: C, 57.45; H, 8.51. Found: C, 57.74; H, 8.75.

Reduction of **1** *with lithium aluminum hydride. 3-O-(2-Hydroxypropyl)-1,2:5,6-*

di-O-isopropylidene-α-D-glucofuranose (**8**). — A solution of **1** (0.700 g) in ether (10 ml) was added dropwise to an ethereal solution of lithium aluminum hydride (0.107 g in 20 ml of ether). After the reaction mixture had been stirred for 15 min at 25° and for 1 h under reflux, t.l.c. showed complete conversion into **8**. A few drops of ethanol were slowly added to decompose the excess hydride. The volume was brought to 150 ml with ether and the solution was extracted with several 150-ml portions of water. The ethereal layer was dried with sodium sulfate and was evaporated to leave **8** as a colorless syrup (0.705 g, 100%); $[\alpha]_D^{24}$ −52.0° (*c* 2, chloroform); R_F^a 0.42; n.m.r. data: τ 5.54 (doublet, secondary hydroxyl group).

Anal. Calc. for $C_{15}H_{26}O_7$: C, 56.61; H, 8.18. Found: C, 56.56; H, 8.52.

Reaction of **1** *with ammonia. 3-O-(3-Amino-2-hydroxypropyl)-1,2:5,6-di-O-isopropylidene-α-D-glucofuranose* (**9**). — When **1** (0.95 g) was dissolved in liquid ammonia and kept for 3 h at −50°, no reaction occurred. Addition of methanol (5 ml) did not promote the reaction during an additional 3 h at −50°. However, when the solution was kept overnight at 25°, t.l.c. showed complete conversion into **9**. Evaporation of the reaction mixture afforded **9** as a colorless syrup (1.00 g, 100%); $[\alpha]_D^{24}$ −44.0° (*c* 2, chloroform); R_G^b 0.82.

Anal. Calc. for $C_{15}H_{27}NO_7$: C, 54.05; H, 8.11; N, 4.21. Found: C, 53.90; H, 8.18; N, 3.93.

In a separate experiment **1** (1.00 g) was dissolved in *p*-dioxane (25 ml) and concentrated ammonium hydroxide (25 ml) was added. The reaction mixture was stirred for 12 h at 25°, at which time t.l.c. showed complete conversion of **1** into **9**. Some decomposition of **9** occurred, as evidenced by t.l.c. and by carbonyl absorption at 1700 and 1575 cm^{-1} in an i.r. spectrum, during evaporation of the solution under diminished pressure at 60°.

Reaction of **1** *with amines. 3-O-[(3-Alkylamino-2-hydroxy)propyl]-1,2:5,6-di-O-isopropylidene-α-D-glucofuranoses* (**10–14**). — Compound **1** was first dissolved in the solvent and then the amine was added. The mixture was stirred until the reaction was complete. Evaporation of the solvent afforded a colorless syrup.

1, *g*	*Solvent (ml)*	*Amine (ml)*	*Temp.*	*Time(h)*	*Product*	*Yield(g)*
0.97	*p*-Dioxane (25)	33% Ethylamine (25)	25°	2	**10**	1.11
1.00	Ether (25)–MeOH (2)	Dimethylamine (10)	0°	24	**11**	1.14
1.06	MeOH (2)	Diethylamine (11.5)	50°	24	**12**	1.31
1.05	Ether (25)	Ethylenimine (15)	30°	24	**13**	1.19
1.04	—	*N*-(2-Aminoethyl)-aziridine (10)	25°	24	**14**	1.32

3-*O*-[(3-Ethylamino-2-hydroxy)propyl]-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**10**); $[\alpha]_D^{24}$ −42.0° (*c* 2, chloroform); R_G^b 0.69.

Anal. Calc. for $C_{17}H_{31}NO_7$: C, 56.51; H, 8.59, N, 3.88. Found: C, 56.12; H, 8.68; N, 3.79.

3-*O*-[(3-Dimethylamino-2-hydroxy)propyl]-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**11**); $[\alpha]_D^{24}$ −37.5° (*c* 2, chloroform); R_G^b 0.38.

Anal. Calc. for $C_{17}H_{31}NO_7$: C, 56.51; H, 8.59; N, 3.88. Found: C, 56.26; H, 8.67; N, 3.72.

3-*O*-[(3-Diethylamino-2-hydroxy)propyl]-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**12**); $[\alpha]_D^{24}$ −29.5° (*c* 2, chloroform), R_G^b 0.49.

Anal. Calc. for $C_{19}H_{35}NO_7$: C, 58.61; H, 9.00; N, 3.60. Found: C, 58.80; H, 8.90; N, 3.49.

3-*O*-[(3-Aziridinyl-2-hydroxy)propyl]-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**13**); $[\alpha]_D^{24}$ −39.5° (*c* 2, chloroform); R_G^b 0.44; i.r. data[30]: 818 cm^{-1} (*C*-aziridinyl).

Anal. Calc. for $C_{17}H_{29}NO_7$: C, 57.14; H, 8.12; N, 3.92. Found: C, 57.28; H, 8.06; N, 4.14.

3-*O*-{[3-(2-aziridinylethylamino-2-hydroxy]propyl}-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**14**); $[\alpha]_D^{24}$ −33.0° (*c* 2, chloroform), R_G^b 0.27.

Anal. Calc. for $C_{19}H_{34}N_2O_7$: C, 56.72; H, 8.46; N, 6.94. Found: C, 56.56; H, 8.68; N, 7.08.

ACKNOWLEDGMENTS

Mrs. Bonita Heaton and Mrs. Karen Jones performed the microanalyses, and Larry Tjarks the n.m.r. spectra. The Dow Chemical Company, Midland, Michigan, supplied the sample of *N*-(2-aminoethyl)aziridine.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

STRUCTURAL AND IMMUNOLOGICAL STUDIES OF CHICK, ALLANTOIC ANTIGEN

PART I. ANALYTICAL STUDIES

M. J. How* AND J. D. Higginbotham
Department of Chemistry, The University, Edgbaston, Birmingham 15 (Great Britain)

(Received August 15th, 1969)

ABSTRACT

Analyses for sugars and amino acids are reported for an antigenic, sulphated glycopeptide from chick, allantoic fluid. Galactose in the antigen is identified as the D-enantiomer. Preliminary comparisons are made with other sulphated glycopeptides.

INTRODUCTION

Previous investigations have shown[1,2] that chick, allantoic antigen combines with antibody to influenza-virus host material and inhibits the agglutination of red blood-cells by influenza virus. The antigen is a sulphated glycopeptide which contains galactose, fucose, 2-amino-2-deoxyglucose, 2-amino-2-deoxygalactose, and acetyl groups[3]. The present paper describes more-detailed, analytical studies of the antigen.

MATERIALS AND METHODS

Purified allantoic glycopeptide[4], which was homogeneous on gel chromatography, analytical ultracentrifugation, and free-boundary electrophoresis, was a generous gift from Dr. A. Harboe.

Analytical methods. — I.r. spectra were determined on discs containing antigen (1.5 mg) and KBr (300 mg).

Neutral and basic components in antigen hydrolysates were separated by elution from Dowex-50 (H^+) resin with water and 2N hydrochloric acid, respectively. Neutral components were converted into *O*-trimethylsilyl (TMS) derivatives[5,6] or into alditol acetates[7]. Basic components were converted into TMS derivatives. Derivatives were analysed by using a temperature-programmed, Pye 104 gas chromatograph and a column packing of 10% SE-30 on siliconised Celite (100–200 mesh, Pye Unicam Ltd.) at 180°, for TMS derivatives, or 2% ECNSS-M on Gas Chrom Q (60–80 mesh, Applied Science Lab. Inc., State College, Pa., U. S. A.) heated from 150–200° at 1.5°/min, for alditol acetates.

*Present address: Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford, Great Britain.

Total 2-amino-2-deoxyhexose was determined by the method of Gatt and Berman[8]. Galactose and 6-deoxygalactose (fucose) were determined simultaneously by using essentially the cysteine–sulphuric acid assay[9]. To determine the enantiomeric nature of galactose, antigen (460 μg) was hydrolysed (2N hydrochloric acid, 6 h, 100°) in a sealed tube, and the neutral and basic components of the hydrolysate were separated by using Dowex-50 (H^+) resin. Neutral components were eluted with water, and the aqueous eluate was passed through a column of Amberlite IR-4B (OH^-) resin and analysed for galactose by the cysteine–sulphuric acid method, and for D-galactose by using D-galactose oxidase[10]. Authentic D-galactose was recovered quantitatively after passage through the two ion-exchange resins. Basic components were eluted from the Dowex-50 resin with 2N hydrochloric acid, and the acid was removed by rotary evaporation with the addition of water. The product was analysed for 2-amino-2-deoxyhexoses by g.l.c.

Sialic acid was liberated from the antigen by hydrolysis (0.2N sulphuric acid, 1 h, 80°), separated from neutral and basic components of the hydrolysate by ion-exchange chromatography[11], and determined[12] as *N*-acetylneuraminic acid.

Amino acid residues present after hydrolysis of the antigen with 6N hydrochloric acid (for 20 h at 110° under nitrogen in a sealed tube) were determined by using a Technicon autoanalyser. Prior to acid hydrolysis, a known amount of norleucine was added to the sample as an internal standard. In preliminary investigations of the amino acid composition of the antigen, samples containing norleucine were treated[13] with performic acid to oxidise cysteine and methionine residues to cysteic acid and methionine sulphone, respectively.

Acetyl group[14], sulphate[15], and phosphorus[16] analyses were based on literature procedures. Ash (as sodium and potassium) was determined by flame photometry.

RESULTS

Infrared analysis. — The spectrum of the antigen showed absorption bands at 1240 (S=O stretching, characteristic of sulphate), and 1640 and 1540 cm^{-1} (strong, acetamido groups). Absorption bands at 850 and 920 cm^{-1} may be diagnostic of axial, rather than equatorial, sulphate residues.

Chemical analyses. — Quantitative, analytical data for the antigen are summarised in Table I. Maximal release of 2-amino-2-deoxyhexose was obtained by hydrolysis of the antigen with 1.67N hydrochloric acid for 7.75 h at 100°. The molar ratio of 2-amino-2-deoxyglucose to 2-amino-2-deoxygalactose liberated under these conditions was 2.3:1 (g.l.c. analysis). The antigen contained equimolar amounts of acetyl and 2-amino-2-deoxyhexose residues. Analysis, using D-galactose oxidase, of the neutral sugars liberated on hydrolysis (and subsequently separated from basic components by ion-exchange chromatography) showed that galactose was present exclusively as the D-enantiomer.

DISCUSSION

Quantitative, analytical data for the immunologically active, apparently

TABLE I

CHEMICAL ANALYSES OF CHICK, ALLANTOIC ANTIGEN[a]

Component	*μmoles/mg*[b]	%[b]	*Component*	*μmoles/mg*[b]	%[b]
D-Galactose	1.45	26.0	Proline	0.11	1.2
6-Deoxyhexose (as fucose)	0.26	4.3	Alanine	0.14	1.0
Total hexosamine	1.43	25.6	Isoleucine	0.07	0.8
2-Amino-2-deoxyglucose	1.00	17.9	Aspartic acid	0.05	0.6
2-Amino-2-deoxygalactose	0.43	7.7	Glycine	0.09	0.5
Sialic acid (as *N*-acetylneuraminic acid)	—	1.0	Leucine	0.03	0.4
Acetyl	1.50	6.5	Valine	0.03	0.3
Sulphate	1.20	12.0	Cysteine	0.01	0.2
Ash (as Na)	1.30	3.0	Lysine	0.01	0.1
Ash (as K)	0.10	0.3	Tyrosine	Trace	—
Phosphorus	0.00	0.0	Histidine	Trace	—
Threonine	0.33	3.4	Arginine	Trace	—
Serine	0.32	2.8	Peptide	—	13.0
Glutamic acid	0.10	1.3			

[a]92% of the antigen was accounted for by these analyses. [b]Values are not corrected for ash content.

homogeneous, chick, allantoic antigen presented in this paper agree with, and supplement, the previous data[3]. All of the galactose in the antigen is identified as the D-enantiomer, and the peptide content is higher than that reported previously[3]. This reflects the relative inaccuracy of the colorimetric method used in earlier work to estimate peptide.

The analytical data confirm other workers' opinions[17,18] that the allantoic antigen is clearly distinct from the keratan sulphates and other sulphated glycosaminoglycans of ground substance. The presence of approximately equimolar amounts of 2-amino-2-deoxygalactose and hydroxyamino acids (serine and threonine) indicates that the antigen may contain *O*-glycosyl linkages between 2-acetamido-2-deoxygalactose and serine and/or threonine. The structural importance of *O*-glycosylated hydroxyamino acids in glycoproteins and sulphated glycosaminoglycans is well known[19].

A so-called 'chick allantoic fluid saccharide', isolated by Meyer *et al.*[17] from papain-digested allantoic fluid, was shown to contain the same sugar components as, but significantly larger amounts of sialic acid(s) (*ca.* 13%) and less sulphate (*ca.* 7%) than, those reported in the present paper. The peptide contents of the two materials, however, were similar. The material isolated by Meyer *et al.* was shown to be a major component of the allantoic fluid and was immunologically related to, but much less active than, the antigen used in the present work. These two materials will be compared in more detail in subsequent papers in this series.

ACKNOWLEDGMENTS

We thank Dr. A. Harboe and his colleagues of the National Institute of Public Health, Virus Department B, Oslo, Norway, for valuable discussions and provision of purified, chick, allantoic antigen; Professor M. Stacey, C.B.E., F.R.S., and Dr. S. A. Barker for their interest; and the Science Research Council for financial support.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

BENZYL 2,3,4-TRI-*O*-BENZYL-β-D-GLUCOPYRANOSIDURONIC ACID AND SOME RELATED COMPOUNDS

EMMANUEL ZISSIS AND HEWITT G. FLETCHER, JR.

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, Maryland 20014 (U. S. A.)

(Received August 27th, 1969)

ABSTRACT

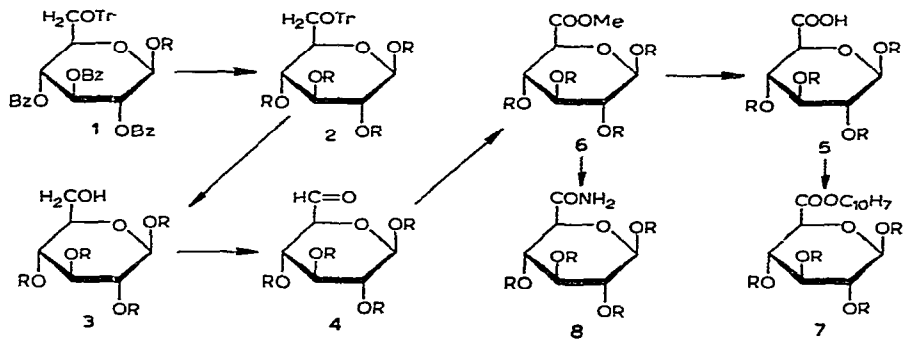
A D-glucuronic acid derivative fully protected with hydrogenolyzable groups except at C-6 has been synthesized. Successive tritylation and benzoylation of benzyl β-D-glucopyranoside gave benzyl 2,3,4-tri-*O*-benzoyl-6-*O*-trityl-β-D-glucopyranoside (**1**). The benzoyl groups in **1** were replaced with benzyl groups to give **2**, and the trityl group was then removed by hydrolysis, giving benzyl 2,3,4-tri-*O*-benzyl-β-D-glucopyranoside (**3**). Oxidation of **3** with the Pfitzner–Moffatt reagent afforded the corresponding aldehyde (**4**), which was further oxidized, with iodine and methanolic potassium hydroxide, to the methyl ester **6**. Alkaline hydrolysis of **6** gave the desired D-glucuronic acid derivative, benzyl 2,3,4-tri-*O*-benzyl-β-D-glucopyranosiduronic acid (**5**). The conversion of **3** into **5** was also achieved, in a one-step process, through the use of chromium trioxide and dilute sulfuric acid in acetone. The ester, **6**, was further characterized through the corresponding amide (**8**), and the steric accessibility of the carboxyl group in **5** was demonstrated through its conversion into the 2-naphthyl ester (**7**).

INTRODUCTION

The synthesis of alkali-labile derivatives of such polyfunctional molecules as the uronic acids presents unusual difficulties, inasmuch as the choice of protecting groups is, of necessity, restricted to those that may be removed under neutral or mildly alkaline conditions. For such syntheses, the benzyl ether group appears to be well suited and, indeed, Pravdić and Keglević[1] synthesized benzyl 2,3,4-tri-*O*-benzyl-D-glucopyranuronate, and it was later shown[2] that various 1-*O*-acyl derivatives of this compound could, through catalytic hydrogenolysis, serve as intermediates in the synthesis of 1-*O*-acyl-D-glucopyranuronic acids. We now report the synthesis of another type of partially protected D-glucuronic acid, namely, benzyl 2,3,4-tri-*O*-benzyl-β-D-glucopyranosiduronic acid (**5**), a compound of potential utility for the synthesis of D-glucuronic acid derivatives variously substituted at C-6. Some of these, such as the esters of D-glucuronic acid with alcohols, have been but little studied[3], and its esters with phenols have never, as far as we are aware, been prepared.

Benzyl β-D-glucopyranoside, a well-known and readily accessible substance[4], served as the starting point for the synthesis. The primary hydroxyl group in this

compound was masked through the action of trityl chloride but, as is frequently found in such "selective" tritylations, the immediate product was amorphous and required preparative, thin-layer chromatography for its purification. Direct benzoylation of the material in the tritylation mixture, however, afforded crystalline benzyl 2,3,4-tri-*O*-benzoyl-6-*O*-trityl-β-D-glucopyranoside (**1**) in an overall yield of 81%. The benzoyl groups in **1** were replaced by benzyl groups through conventional debenzoylation–benzylation with benzyl chloride and potassium hydroxide, and the product (**2**) was detritylated by heating with aqueous acetic acid. The partially substituted D-glucoside thus formed, namely, benzyl 2,3,4-tri-*O*-benzyl-β-D-glucopyranoside (**3**), was accompanied by a small proportion of a monoacetate which is, presumably, benzyl 6-*O*-acetyl-2,3,4-tri-*O*-benzyl-β-D-glucopyranoside. Acetylation by warm aqueous acetic acid has been observed by others[5].



Tr = Ph_3C , Bz = PhCO and R = $PhCH_2$

The conversion of the hydroxymethyl group on C-5 of **3** into a carboxyl group was initially attempted through the Heyns oxidation, a solution of the compound in aqueous *p*-dioxane being treated with oxygen in the presence of a platinum catalyst at temperatures approaching 90°. However, in our hands, this procedure was unsuccessful. It may be noted that variable results have been reported in the application of the Heyns oxidation to partially benzylated carbohydrate derivatives. Thus, whereas methyl 2,3,4-tri-*O*-benzyl-β-D-glucopyranoside[1] and methyl 2,3-di-*O*-benzyl-α-D-glucopyranoside[6] were oxidized to the corresponding uronic acids, an attempt to oxidize the primary hydroxyl group in 1,2,3,5-tetra-*O*-benzyl-D-glucitol was unsuccessful[7].

As a second approach, we examined a two-step oxidation of **3**. The Pfitzner–Moffatt reagent[8] (methyl sulfoxide, pyridine, phosphoric acid, and *N*,*N*′-dicyclohexylcarbodiimide) readily converted **3** into the aldehyde, namely, benzyl 2,3,4-tri-*O*-benzyl-β-D-*gluco*-hexodialdo-1,5-pyranoside (**4**). Although this compound gave a crystalline (2,5-dichlorophenyl)hydrazone having the expected composition, purification of **4**, either through recrystallization or by chromatography, failed to give a product having an acceptable elemental analysis. Following a procedure devised by Inch, Ley, and Rich[9], a methanolic solution containing the aldehyde **4** and iodine

was treated with methanolic potassium hydroxide, and methyl (benzyl 2,3,4-tri-*O*-benzyl-β-D-glucopyranosid)uronate (**6**) was obtained in high yield. Both **6** and benzyl 2,3,4-tri-*O*-benzyl-β-D-glucopyranosiduronamide (**8**), which was readily prepared from **6**, were readily crystallizable compounds. Treatment of the methyl ester **6** with dilute alkali gave the desired benzyl 2,3,4-tri-*O*-benzyl-β-D-glucopyranosiduronic acid (**5**). Although **5** was crystalline and chromatographically homogeneous, and gave a satisfactory elemental analysis, its melting point was highly variable.

After the work just described had been completed, Matsui and coworkers[7] showed that 3-*O*-benzoyl-2,4,5,6-tetra-*O*-benzyl-L-gulitol could be oxidized to 3-*O*-benzoyl-2,4,5,6-tetra-*O*-benzyl-L-gulonic acid through the action of a solution of chromium trioxide and dilute sulfuric acid in acetone. Under these experimental conditions, we found **3** to be readily oxidized to **5**, the product being conveniently identified through conversion into its methyl ester (**6**). No attempt was made to maximize the yield of **5** (58%) in this one-step oxidation, but, as a preparative method, the procedure is unquestionably superior to the multi-step conversion developed earlier.

In order to demonstrate the steric accessibility of the carboxyl group in **5** for ester formation, the acid was condensed with 2-naphthol in the presence of *N,N'*-dicyclohexylcarbodiimide and pyridine. The product, namely, 2-naphthyl (benzyl 2,3,4-tri-*O*-benzyl-β-D-glucopyranosid)uronate (**7**), was obtained in 89% yield; it was crystalline, and gave a satisfactory elemental analysis.

EXPERIMENTAL

General methods. — Melting points are corrected values. Thin-layer chromatography was conducted on Silica Gel G (E. Merck AG, Darmstadt) by use of the solvent systems specified, the components being detected (unless otherwise noted) by spraying with 10% sulfuric acid and heating. Column chromatography was performed on No. 7734 Silica Gel (0.05–0.20 mm, E. Merck AG). N.m.r. spectra were recorded, for solutions in chloroform-*d*, with a Varian A-60 spectrometer and tetramethylsilane as the internal standard.

Benzyl 2,3,4-tri-O-benzoyl-6-O-trityl-β-D-glucopyranoside (**1**). — To a solution of benzyl β-D-glucopyranoside[4] (15.8 g) in pyridine (75 ml) was added trityl chloride (18.5 g), and the mixture was kept for 1 week at room temperature. Benzoyl chloride (27 ml) was then added and, after a further 18 h at room temperature, the excess of benzoyl chloride was decomposed by the addition of water (1 ml). The mixture was poured onto crushed ice, and the syrup that was precipitated was thoroughly washed with cold water; on storage under water overnight at +5°, the syrup crystallized; after decantation of the water, the solid was triturated with ethanol, washed with fresh ethanol, and dried; yield 50.5 g. Recrystallized from ethanol (25 ml/g), the product (**1**, 39.1 g, 81%) was obtained as elongated, flat prisms, m.p. 156.5–157.5°, $[\alpha]_D^{20}$ −12.6° (*c* 1.3, chloroform).

Anal. Calc. for $C_{53}H_{44}O_9$: C, 77.17; H, 5.38. Found: C, 77.00; H, 5.49.

*Benzyl 2,3,4-tri-*O*-benzyl-6-*O*-trityl-β-*D*-glucopyranoside* (**2**). — A mixture of **1** (68.8 g), *p*-dioxane (60 ml), and powdered potassium hydroxide (75 g) was gradually warmed to the boiling point, and stirred vigorously while benzyl chloride (60 ml) was added dropwise. After the addition was complete, the mixture was boiled and stirred for 2 h; it was then cooled and steam-distilled to remove the *p*-dioxane, benzyl chloride, and benzyl alcohol. The crude product was extracted with three 300-ml portions of ether, and the extracts were combined, washed with two 400-ml portions of water, dried (magnesium sulfate), and evaporated *in vacuo* to a mobile syrup which began to crystallize spontaneously after several days. Warm ethanol was added and, when crystallization was complete, the product (32.6 g, 50%) was removed by filtration. After recrystallization from chloroform–pentane and then from ethanol, compound **2** was obtained as large, rectangular prisms: m.p. 111–112°, $[\alpha]_D^{20}$ −3.6° (*c* 1, chloroform).

Anal. Calc. for $C_{53}H_{50}O_6$: C, 81.30; H, 6.44. Found: C, 81.27; H, 6.54.

*Benzyl 2,3,4-tri-*O*-benzyl-β-*D*-glucopyranoside* (**3**). — The trityl ether **2** (10 g) was dissolved in glacial acetic acid (80 ml), and the solution was warmed to 75–85°. Water (20 ml) was slowly added, care being taken to keep the mixture homogeneous. The temperature was held at 75–85° for 1 h and then, after storage overnight at room temperature, the precipitated triphenylmethanol was removed by filtration and washed with cold, 80% acetic acid. Concentration of the combined filtrate and washings yielded a second crop of triphenylmethanol which was removed by filtration. The filtrate was diluted with warm ethanol and then with a comparatively large volume of pentane; a stream of air was directed across the surface of the solution, and crystallization was allowed to progress until a solid mass of needle-shaped crystals was obtained; the cold mixture was filtered, the crystals (5.2 g) being washed with pentane. On being chilled, the filtrate yielded further fractions (1.8 g) which were dissolved in butyl alcohol–pentane and the contaminating triphenylmethanol was removed by filtration. On concentration and cooling, the filtrate yielded 1.0 g, to make a total yield of 6.2 g (90%) of **3**, still slightly contaminated with triphenylmethanol as shown by t.l.c. (6:1 benzene–ether). The compound may be crystallized from ethanol, ethanol–pentane, acetone–water, or cyclohexane, although chromatography on a column of silica gel with 6:1 benzene–ether is more effective in removing the last traces of triphenylmethanol as it gave pure **3** as needles (from ethanol), m.p. 105–106°, $[\alpha]_D^{20}$ −9.2° (*c* 1, chloroform).

Anal. Calc. for $C_{34}H_{36}O_6$: C, 75.53; H, 6.71. Found: C, 75.61; H, 6.69.

A repetition of the preparation, with use of 20.5 g of **2**, afforded 7.2 g of **3** as the first crop and then a second crop of crystals (3.4 g) which proved to be a mixture of **3**, triphenylmethanol, and a third product. Recrystallization of the second crop of crystals from ethanol removed most of the triphenylmethanol; the remainder (1.7 g) was chromatographed on a column of silica gel (35 g) with 6:1 benzene–ether. The third product moved the most rapidly of the three, and crystallized readily from ethanol to give 0.7 g (4.6%). Recrystallized from chloroform–pentane, benzyl 6-*O*-acetyl-2,3,4-tri-*O*-benzyl-β-D-glucopyranoside was obtained as needles, m.p.

116–117°, $[\alpha]_D^{20}$ −2.9° (*c* 1, chloroform); its n.m.r. spectrum included a three-proton singlet at τ 7.94 (OAc).

Anal. Calc. for $C_{36}H_{38}O_7$: C, 74.20; H, 6.57. Found: C, 74.33; H, 6.38.

*Benzyl 2,3,4-tri-*O*-benzyl-β-*D*-gluco-hexodialdo-1,5-pyranoside* (**4**). — The oxidation technique devised by Pfitzner and Moffatt[8] was used as described by Horton, Nakadate, and Tronchet[10]. To a solution of compound **3** (1 g) in methyl sulfoxide (4 ml) and benzene (1 ml) were added anhydrous pyridine (0.2 ml), 85% phosphoric acid (0.1 ml), and, finally, *N,N'*-dicyclohexylcarbodiimide (2 g). The mixture was stirred for 7 h at room temperature and kept overnight, the *N,N'*-dicyclohexylurea then being removed by filtration. A solution of oxalic acid monohydrate (2 g) in methanol (6 ml) was added to the filtrate, the mixture was stirred for 1 h, and filtered, and the filtrate was diluted with benzene and washed with aqueous sodium hydrogen carbonate solution (5%). The washings were combined, and extracted with ethyl acetate, and the extract was added to the benzene solution; the solution was dried (Drierite), and evaporated *in vacuo*. Benzene was added to the residue, the suspension was filtered, and the filtrate was evaporated *in vacuo* to yield a mobile syrup. This syrup was dissolved in a small volume of methanol, and the solution was kept for several days at +5°, to give a crystalline product that was removed by filtration at +5°, and washed with cold methanol. The crude **4** (0.8 g, 80% yield) thus obtained had m.p. ~65–75°, and could be recrystallized from methanol as prismatic needles, and from chloroform–pentane as radial clusters of needles, but these operations failed to yield chromatographically homogeneous material. Recrystallization from hot hexane caused partial conversion of the product into another substance, detected by t.l.c.; similarly, an attempt at preparative t.l.c. (5:1 benzene–ether) caused the product to become heterogeneous. With the fuchsin aldehyde reagent, the aldehyde gave a violet color, and, on t.l.c., spraying the compound with aniline hydrogen phthalate solution and then heating gave a brown spot.

A mixture of **4** (255 mg), (2,5-dichlorophenyl)hydrazine (100 mg), and methanol (5 ml) was heated on a steam bath until an almost dry residue remained. This was dissolved in a small volume of warm methanol; crystallization occurred spontaneously, to give the (2,5-dichlorophenyl)hydrazone of **4** (240 mg, 73%). The compound was successively recrystallized from methanol (once), cyclohexane (thrice), and methanol, to give needles, m.p. 138.5–139.5°, $[\alpha]_D^{20}$ −53.7° (*c* 1, chloroform). The (2,5-dichlorophenyl)hydrazone is somewhat unstable, both in solution and when exposed to the air on a t.l.c. plate.

Anal. Calc. for $C_{40}H_{38}Cl_2N_2O_5$: C, 68.86; H, 5.49; Cl, 10.16; N, 4.02. Found: C, 68.72; H, 5.73; Cl, 10.02; N, 3.97.

*Methyl (benzyl 2,3,4-tri-*O*-benzyl-β-*D*-glucopyranosid)uronate* (**6**). — An oxidation procedure similar to one used by Inch, Ley, and Rich[9] was employed. A stirred solution of **4** (2.3 g) and iodine (1.4 g) in methanol (23 ml) was heated to 55–60°, and was stirred while methanolic potassium hydroxide (4%, w/v) was added dropwise. After 45 min, the product began to crystallize from the hot solution; addition of the methanolic alkali was continued for another hour (until the color

of the iodine had disappeared; 20 ml of the alkaline solution was used). The mixture was allowed to cool to room temperature, and a few drops of water were added to incipient turbidity. Needle-like crystals of **6** were removed by filtration and washed with 50% aqueous methanol; wt. 2.1 g (86%). The product thus isolated contained an impurity that moved more slowly than the major part on t.l.c. with 20:1 benzene–ether, and this impurity was not removed by recrystallization from methanol, cyclohexane, acetone–water, or chloroform–pentane. Purification of a 200-mg sample was effected by preparative t.l.c. with 20:1 benzene–ether, and 175 mg of chromatographically homogeneous product was obtained; recrystallized twice from methanol, it had m.p. 109.5–110.5°, $[\alpha]_D^{20}$ −26.0° (*c* 1, chloroform).

Anal. Calc. for $C_{35}H_{36}O_7$: C, 73.92; H, 6.38. Found: C, 73.83; H, 6.33.

The largest fragment detected by mass spectroscopy was at 478, equivalent to *m/e* $PhCH_2$. The n.m.r. spectrum of the compound included a sharp, three-proton singlet at τ 6.25, very close to a similar signal at τ 6.28 assigned by Roy and Glaudemans[11] to the methyl group in 1,2,3,4-tetra-*O*-acetyl-6-*O*-(methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate)-β-D-glucopyranose.

*Benzyl 2,3,4-tri-*O*-benzyl-β-*D*-glucopyranosiduronamide* (**8**). — A solution of the methyl ester **6** (200 mg) in acetone (12 ml) was treated with ammonium hydroxide (6 ml) and kept at room temperature. After 1.5 h, an additional quantity (1 ml) of ammonium hydroxide could be added without causing precipitation of **6**. During the next 4 days, the mixture deposited a mass of silky needles. Water (1 ml) was added and, after 1 h, the crystals were removed by filtration and washed with aqueous acetone; wt. 103 mg. T.l.c. of the filtrate showed that a very small proportion of **6** was present; concentration of the solution afforded a second crop of crystals (84 mg), making a total yield of crude product of 187 mg (96%). Recrystallized from acetone and then from aqueous acetone, the amide had m.p. 193–194°, $[\alpha]_D^{20}$ −39.3° (*c* 1, chloroform).

Anal. Calc. for $C_{34}H_{35}NO_6$: C, 73.76; H, 6.37; N, 2.53. Found: C, 73.61; H, 6.11; N, 2.62.

*Benzyl 2,3,4-tri-*O*-benzyl-β-*D*-glucopyranosiduronic acid* (**5**). — (*A*) *From methyl (benzyl 2,3,4-tri-*O*-benzyl-β-*D*-glucopyranosid)uronate* (**6**). — A solution of pure **6** (164 mg) in acetone (15 ml) was cooled to 5° and treated dropwise with 0.1M potassium hydroxide (5.7 ml). After 2.5 h, the mixture was stirred with Amberlite IR-120 (H^+) ion-exchange resin, and the resin was removed by filtration and thoroughly washed with acetone. The filtrate and washings were combined, and evaporated to dryness, and the residue was crystallized, as needles, from warm cyclohexane, wt. 153 mg (96%), m.p. 127–131° (turbid melt). Recrystallized from acetone–cyclohexane, the product was chromatographically homogeneous (t.l.c. with 5:1 benzene–ether). Widely varying melting points were observed; an analytical sample, dried *in vacuo* at 100°, had m.p. 115–125°, $[\alpha]_D^{20}$ −34.1° (*c* 1, chloroform).

Anal. Calc. for $C_{34}H_{34}O_7$: C, 73.63; H, 6.18. Found: C, 73.33; H, 5.97.

(*B*) *From benzyl 2,3,4-tri-*O*-benzyl-β-*D*-glucopyranoside* (**3**). A procedure similar to that used by Matsui and coworkers[7] was employed. A solution of benzyl 2,3,4-tri-

O-benzyl-β-D-glucopyranoside (**3**, 0.5 g) in acetone (8 ml) was stirred while a solution of chromium trioxide (0.3 g) in 3.5M sulfuric acid (0.8 ml) was added dropwise. Stirring was continued for 1 h after the addition was complete, and the dark solution was then directly decanted from the chromium sesquioxide onto crushed ice. Acetone was used to wash the chromium sesquioxide, the washings being added to the ice. The crude product that was precipitated was removed by filtration and was washed with water; dried in the air, it weighed 0.3 g (58%).

Owing to the variability in the m.p. of the compound (described in *A*), identification was made here through conversion into the methyl ester **6**. A portion (0.1 g) of the material was treated with diazomethane in ether solution; on evaporation, a crystalline residue was obtained, and this was recrystallized from methanol to give clusters of long, prismatic needles (80 mg) which had m.p. 106–110°, alone and in admixture with compound **6** prepared from **4** as already described. The n.m.r. spectra of the two samples were identical.

*2-Naphthyl (benzyl 2,3,4-tri-*O*-benzyl-β-D-glucopyranosid)uronate* (**7**). — To a solution of **5** (0.65 g) and 2-naphthol (0.65 g) in a mixture of benzene (3 ml) and pyridine (0.3 ml) was added *N,N'*-dicyclohexylcarbodiimide (0.3 g); *N,N'*-dicyclohexylurea was promptly precipitated. After being kept overnight at room temperature, the mixture was diluted with benzene and filtered, and the residue was washed with cold benzene. The filtrate and washings were combined, concentrated, and cooled, to give 0.55 g of crystalline product. Preparative t.l.c. of the material in the filtrate, with 10:1 benzene–ether, afforded a second crop (0.16 g), and raised the yield to 89% of the theoretical. The product can be recrystallized from ether or from chloroform–pentane, and crystallizes as prismatic needles. When pure, it has m.p. 147–148° and $[\alpha]_D^{20}$ −19.4° (*c* 1, chloroform).

Anal. Calc. for $C_{44}H_{40}O_7$: C, 77.63; H, 5.92. Found: C, 77.87; H, 6.14.

ACKNOWLEDGMENTS

We thank Dr. T. D. Inch for drawing our attention to the oxidation procedure described in reference 9. We are also indebted to Mr. H. W. Diehl and Mr. E. W. Tracy for technical assistance. Elemental analyses, mass spectra, and n.m.r. spectra were provided by the staff of the Section on Microanalytical Services and Instrumentation of this Institute.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

PREPARATION AND DIFFERENTIATION OF ANOMERIC PAIRS OF STEROID TRI-*O*-ACETYL-D-GLUCOPYRANOSIDURONIC METHYL ESTERS AND D-GLUCOPYRANOSIDE TETRAACETATES

JOHN J. SCHNEIDER

Department of Medicine, Jefferson Medical College, Philadelphia, Pennsylvania 19107 (U. S. A.)

(Received July 19th, 1969; in revised form, September 3rd, 1969)

ABSTRACT

Twenty-eight steroid tri-*O*-acetyl-β-D-glucopyranosiduronic methyl esters have been prepared by a standard method, and isomerized to their α-D anomers with titanium tetrachloride. Reduction of seven such pairs with lithium aluminum hydride, followed by re-acetylation, provided the corresponding D-glucopyranoside tetraacetates. The configuration at the anomeric carbon atom assigned in each case was supported by (*a*) the anomerization increment, $M_{D_\alpha} - M_{D_\beta}$, which constituted a regular series within the limits of +704 to +985, and (*b*) the correspondence between observed and calculated M_D values. Members of the anomeric pairs, with the exception of those derived from the isosapogenins, were also differentiated by i.r. spectroscopy, chiefly by a band within the range of 1146–1140 cm^{-1} that is displayed only by the α-D anomers. Methyl (tri-*O*-acetyl-β-D-glucopyranosid)uronates were distinguished from the corresponding β-D-glucopyranoside tetraacetates both by i.r. spectroscopy and mass spectrometry.

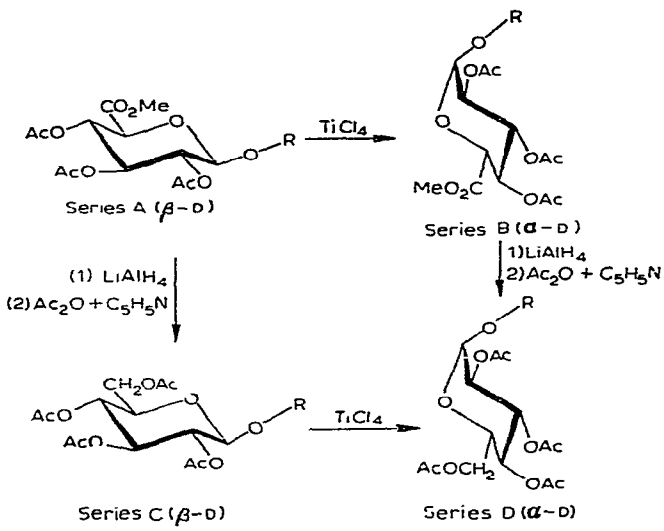
INTRODUCTION

Interest continues in the synthesis and metabolism of steroid β-D-glucopyranosiduronic acids, but it is evident from recent compilations[1] that the preparations of anomeric pairs has received little attention. We have therefore utilized experience gained in a recent synthesis of the methyl (tri-*O*-acetyl-β-D-glucopyranosid)uronate (**1**) of cholesterol[2] to prepare a total of 28 anomeric pairs of steroid D-glucopyranosiduronic acids and D-glucopyranosides, as the tri-*O*-acetyl methyl esters and tetraacetates, respectively. The steroids employed included cholesterol, the four possible stanols derived from it, and various 17-ketosteroids, pregnane derivatives, and isosapogenins. The preparation of these pairs is briefly described, their optical rotatory properties are recorded, and their differentiation by i.r. spectroscopy is discussed.

RESULTS AND DISCUSSION

Preparation of anomeric pairs. — The methyl (tri-*O*-acetyl-β-D-glucopyranosid)-uronates (Series A, Scheme 1) were prepared first, by using those conditions* that

*A benzene solution of methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucuronate (12 mmoles) and the appropriate steroid (4 mmoles) was shaken for 24 h at room temperature with silver oxide (8 mmoles). For details, see ref. 2.



Scheme 1. Generalized formulas and preparative routes (members of series A and B are listed in Table I, and of series C and D in Table II). R = aglycon moiety.

had provided the cholesterol derivative **1** in a yield of 86%. Applied to the new aglycons, this method gave the β-D anomers of the pure esters in yields ranging from 38 to 77%. For the saturated members, anomerization of each ester to its α-D anomer (Series B) was effected with titanium tetrachloride in benzene or dichloromethane at room temperature. These conditions are notably milder than those employed by Pacsu[3] and Lindberg[4], who anomerized β-D-glucopyranoside tetraacetates of simpler aglycons by treatment with this reagent in refluxing chloroform. Following failure to anomerize **1** with this reagent directly, it was assumed that the procedure would fail with other unsaturated derivatives as well. Accordingly, the derivatives of cholesterol (**1**), pregnenolone (**7**), dehydroisoandrosterone (**12**), and diosgenin (**17**) in Series A were anomerized as their dibromides (assumed to have the 5α,6β configuration, analogous to that of cholesterol dibromide), after which the α-D anomers (**1a**, **7a**, **12a**, and **17a**, respectively, in Series B) were obtained by debromination with zinc in ether–acetic acid. In the saturated series, the β-D-glucopyranoside tetraacetates **23**, **24**, **25**, **26**, and **28** (Series C) were prepared from the corresponding methyl esters of Series A by reduction with lithium aluminum hydride in ether, followed by re-acetylation of the free D-glucopyranosides thus formed. The β-D-glucopyranoside tetraacetates were then anomerized as just described, affording Series D. Conversion of the unsaturated methyl [tri-*O*-acetyl-β(and α)-D-glucopyranosid]uronates (Series A and B, respectively) into the corresponding D-glucopyranoside tetraacetates (Series C and D, respectively) was effected by reduction. This method of preparing D-glucopyranosides was useful in this connection, but has limited utility, as it is restricted to compounds that have reducible groups at C-6 only.

Some interrelationships were established by hydrogenating the unsaturated derivatives **1**, **1a**, **7**, **7a**, **12**, **12a**, **17**, **17a**, **27**, and **27a** in neutral ethanol with palladium-

on-carbon as the catalyst. The products were identical, as judged by their melting points and i.r. spectra, with the saturated 5α derivatives **3**, **3a**, **10**, **10a**, **16**, **16a**, **19**, **19a**, **28**, and **28a**, respectively.

Derivatives **1**, **7**, **10**, **12**, **14**, **15**, **16**, **22**, **22a**, **23**, **23a**, **24**, **24a**, **25**, **26**, and **27** (that included three anomeric pairs) had been prepared or isolated by other workers; the rest are new. In addition to the usefulness of anomeric pairs for intercomparisons, it is necessary only to saponify the acetylated methyl esters of Series A in order to convert them into known, or potential, excretion forms (conjugates) of steroid metabolites. Moreover, the recent demonstration that surviving potato tissue can convert dehydroisoandrosterone into its β-D-glucopyranoside[5] shows that the formation of steroid D-glucopyranosides in plants can be studied by simple, *in vitro* techniques, and justifies further efforts to prepare them by chemical means.

The preparation of the methyl (tri-*O*-acetyl-β-D-glucopyranosid)uronate (**20**) of β-cortolone merits further mention. The 20,21-isopropylidene acetal of β-cortolone can be prepared in high yield and, after the Koenigs–Knorr condensation, hydrolytic removal of the isopropylidene group can be effected in a yield approaching 90%. As this group can be introduced and removed with equal ease in 17,20α,21-glycerols and 20α(and 20β),21-glycols[6], it is clear that this approach to the preparation of the 3-D-glucopyranosiduronates of steroidal side-chain glycols and glycerols has general utility.

Optical rotatory properties of anomeric pairs. — The optical rotations of the anomeric pairs were determined in chloroform, and are given in Tables I and II as molecular rotations (M_D). The value for $M_{D_\alpha} - M_{D_\beta}$, which constitutes the anomerization increment ($\Delta_{anom.}$) for each pair, lies, in this series*, within the narrow range of +704 to +984, with an average value of +823. The configuration at the anomeric center, which is determined by the mechanism of the reaction, is confirmed in each instance by the sign of the increment. The calculated[7] M_D values are also given; their essential agreement with the corresponding observed values supports the proposed assignments.

These data were also considered in terms of Hudson's rules of isorotation[8,9]. The second of these states that the A value, which corresponds to half the anomerization increment, is dependent solely on the nature of the aglycon group. As these increments form a regular series (see Tables I and II), it follows that the contributions of the various steroids are similar, irrespective of structural differences. In addition, the magnitude of the A values (which range from +352 to +492) is consistent with the size of the aglycon groups. The first rule states that the B value, which corresponds to half the sum of the observed molecular rotations of a given α,β-pair, is characteristic of the particular carbohydrate, and is independent of the substituent at C-1. As the carbohydrate moiety remains constant within each Table, these data should provide two sets of values, each comprising a series of similar α,β sums; but, when the value

*Anomerization increments based on rotations observed at 365 nm gave a similarly regular series: $M_{365_\alpha} - M_{365_\beta}$ lay within the range of +2028 to +2917; average, +2417.

TABLE I

ANOMERIC PAIRS OF STEROID METHYL (TRI-*O*-ACETYL-D-GLUCOPYRANOSID)URONATES: OPTICAL ROTATORY PROPERTIES[a] AND PRINCIPAL BANDS IN THE 1340–880-cm^{-1} REGION

Compound no.	*Anomeric form*	*Aglycon*	M_D *(obs.)*	$\Delta_{anom.}$	M_D *(calc.)*	*Infrared bands shown at*			
						915–880 cm^{-1}	1150–1130 cm^{-1}	1185–1155 cm^{-1}	1340–1330 cm^{-1}
1	β	Cholesterol	−232	+900	−256	886		1156 (m)	
1a	α		+668		+450	892	1140 (s)	1162 (w)	1330
2	β	Cholesterol dibromide	−518	+984	−341	892		1160 (m)	
2a	α		+466		+365	892	1141 (m)	1160 (m)	1332
3	β	Cholestanol	−35	+796	−12	891		1175 (s)	
3a	α		+761		+694	895	1142 (m)	1172 (w, sh)	1331
4	β	Epicholestanol	−106	+726	−4	890		1161 (m)	
4a	α		+620		+702	895	1145 (m)	1182 (w)	1330
5	β	Coprostanol	−78	+889	0	890		1175 (br, m)	1336 (vw)
5a	α		+811		+706	895	1142 (m)	1172 (vw, sh)	1330
6	β	Epicoprostanol	0	+754	+19	890		1168 (s)	
6a	α		+754		+725	897	1145 (m)	1169 (w)	1335
7	β	Pregnenolone	+44	+854	−21	890		1170 (m)	
7a	α		+898		+685	897	1141 (s)	1168 (m)	1340 (sh)
8	β	Pregnenolone dibromide	−201	+867	−177	896		1160 (m)	
8a	α		+666		+529	896	1142 (m)	1160 (m)	1335 (w, sh)
9	β	Pregnanolone	+273	+736	+246	890		1165 (br, m)	
9a	α		+1009		+952	895	1145 (m)	1170 (vw, sh)	1336 (w, sh)
10	β	Allopregnanolone	+184	+863	+154	892		1168 (m)	
10a	α		+1047		+860	896	1148 (m)	1163(vw)	1330 (sh)

11	β	17-Hydroxypregnanolone	−91	+768	−81	890		1160 (s)		
11a	α		+677		+625	895	1142 (m)	1165 (w, sh)	1330 (vw, sh)	
12	β	Dehydroisoandrosterone	−67	+889	−89	890	1138 (w)	1170 (s)		
12a	α		+822		+617	895	1144 (s)	1165 (w)	1335	
13	β	Dehydroisoandrosterone	−306	+895	−52	895		1159 (m)		
13a	α	dibromide	+589		+654	897	1142 (m)	1160 (m)	1330 (vw)	
14	β	Androsterone	+106	+713	+177	890		1160 (m)		
14a	α		+819		+885	891	1141 (m)	1160 (vw, sh)	1328	
15	β	Etiocholanolone	+194	+704	+216	890		1168 (m)		
15a	α		+898		+922	895	1141 (m)	1165 (w)	1330	
16	β	Isoandrosterone	+146	+849	+317	890		1165 (m)		
16a	α		+995		+1023	895	1146 (m)	1165 (vw, sh)	1328	
17	β	Diosgenin	−629	+943	−599			1169 (s)		
17a	α		+314		+107		1141 (m)	1185–65 (w, sh)	1330–40 (vw)	
18	β	Diosgenin dibromide	−882	+935	−790			1180 (w, sh)		
18a	α		+53		−84		1145 (w, sh)	1185 (w)	1330–40 (vw)	
19	β	Tigogenin	−440	+865	−380		1135 (vw)	1176 (s)		
19a	α		+425		+326		1132 (w, sh)	1172 (m)	1330–40 (vw)	
20	β	β-Cortolone	+27	+765	[b]	890		1168 (w, sh)		
20a	α		+792			891	1141 (m)	1185 (w, sh)	1330	
21	β	β-Cortolone acetonide[c]	+7	+831	+62	891		1160 (s)		
21a	α		+838		+768	891	1140 (w)	1160 (m)	1330	

[a] M_D (obs.) = $[\alpha]_D$ (obs.) × mol.wt./100. $\Delta_{anom.}$ (anomerization increment) = M_{D_α} (obs.) − M_{D_β} (obs.). Bands are characterized as br, broad; m, moderate; sh, shoulder; s, strong; w, weak; and vw, very weak. [b] Calculated M_D values not determined, because of the low solubility of β-cortolone in chloroform. [c] A trivial name for 3α,17α-dihydroxy-20,21-(isopropylidenedioxy)-5β-pregnan-11-one.

TABLE II

ANOMERIC PAIRS OF STEROID D-GLUCOPYRANOSIDE TETRAACETATES: OPTICAL ROTATORY PROPERTIES AND PRINCIPAL BANDS IN THE 1340–880-CM^{-1} REGION

Compound no.	*Anomeric form*	*Aglycon*	M_D *(obs.)*	$\Delta_{anom.}$	M_D *(calc.)*	*Infrared bands shown at* 915–880 cm^{-1}	1150–1130 cm^{-1}	1185–1155 cm^{-1}	1340–1330 cm^{-1}
22	β	Cholesterol	−194	+844	−221	908	1135 (w, sh)	1171 (s)	
22a	α		+652		+319	912	1145 (s)	1169 (m)	1331
23	β	Cholestanol	+22	+740	+23	908	1130 (w, sh)	1172 (s)	
23a	α		+762		+563	895–910	1145 (m)	1170 (s)	1332
24	β	Epicholestanol	−79	+776	+31	909	1135 (w, sh)	1168 (m)	
24a	α		+697		+571	890–905	1141 (s)	1168 (m)	1335
25	β	Coprostanol	−22	+863	+35	908	1136 (w, sh)	1171 (m)	
25a	α		+841		+575	905–913	1140 (m)	1169 (m)	1330
26	β	Epicoprostanol	+79	+711	+54	907	1136 (w, sh)	1169 (m)	
26a	α		+791		+594	910	1142 (m)	1168 (s)	1330
27	β	Diosgenin	−574	+850	−564		1136 (w)	1171 (m)	
27a	α		+276		−23		1141 (m, sh)	1170 (m)	
28	β	Tigogenin	−306	+732	−345		1130 (vw)	1175 (s)	
28a	α		+426		+195		1148 (m, sh)	1172 (s)	

in the Tables were considered in these terms, it was evident that the sums actually form a generally irregular series, which includes several highly anomalous, negative values. When the B values were re-calculated after subtracting the M_D value of the appropriate free steroid from the M_D value of the intact derivative, a somewhat more regular series was obtained. Three distinctly low values were encountered (+160, +76, and +142, from pairs **4**–**4a**, **11**–**11a**, and **13**–**13a**, respectively); the remaining values ranged from +212 to +395, averaging +298. It has been shown[10] that variations in A and B values are due to interactions between the carbohydrate and steroid portions of the molecule, and it is suggested that this factor may account for some of the irregular B values in the present series. In considering the calculated M_D values (see above), it was noted that the agreement, in this series, between calculated and observed M_D values was similar to that recorded by Nitta *et al.*[11] for a number of methyl ester triacetates, but far from the close correspondence observed by Becker[12], who examined several free D-glucopyranosiduronic acids. If divergences between calculated and observed M_D values are also caused by interactions, it may be suggested that such interactions are more marked in acetates. This thesis should readily be testable by re-determining the M_D values of the present series after hydrolysis to the free acids.

Infrared spectra of anomeric pairs. — Considerable information is available on the i.r. spectra of the peracetates of the common hexoses and of simpler glycosides and glycosiduronic acids (see reviews[13], and papers by Isbell and associates[14,15] and Nitta *et al.*[16]), but interest in the spectra of steroid D-glucopyranosiduronic acids and D-glucopyranosides has developed only within the past few years. Spectra of the compounds prepared in this study were obtained for potassium bromide dispersions, and were closely compared in the region of 1350–850 cm^{-1}, the range considered most useful for distinguishing between members of anomeric pairs. These data are compiled in Tables I and II for bands observed within the following regions: 915–880, 1150–1130, 1185–1155, and 1340–1330 cm^{-1}.

All of the methyl (tri-*O*-acetyl-D-glucopyranosid)uronates exhibit a sharp band, having medium to strong intensity, in the range 897–890 cm^{-1}. This band has no value for differentiating anomeric pairs, but it distinguishes the methyl ester triacetates from the D-glucopyranoside tetraacetates, since the latter display a similar band lying *above* 900 cm^{-1} (a sharp band in the range of 912–908 cm^{-1} for **22**, **22a**, **23**, **24**, **26**, and **26a***, and broader bands, as indicated, for **23a**, **24a**, and **25a**). This differentiation cannot be applied to derivatives of diosgenin and tigogenin, which show a strong, obscuring band at 900 cm^{-1} that is characteristic of isosapogenins.

All of the derivatives examined display a band (which sometimes appears as a shoulder or is flanked by a second, weaker band) in the range 1185–1156 cm^{-1}, varying in intensity from compound to compound, from very weak to strong. This

*The β-D-glucopyranoside tetraacetates of pregnenolone, pregnanolone, allopregnanolone, 17-hydroxypregnanolone, dehydroisoandrosterone, androsterone, etiocholanolone, isoandrosterone, and β-cortolone, which we have recently prepared, but which are not formally described in this paper, show sharp bands in the range 906–909 cm^{-1}.

band is usually weaker for the α-D member of an anomeric pair, but members cannot be differentiated by this means alone. Such differentiation depends on the observation that only α-D anomers, with the inevitable exceptions provided by the isosapogenins, also display a sharp band, of moderate to strong intensity, in the range 1146–1140 cm^{-1}. One methyl ester triacetate (**12**) and all of the β-D-glucopyranoside tetraacetates show a second, usually very weak, band in the range 1138–1130 cm^{-1}; its low intensity and displacement serve to distinguish it from the band at 1146–1140 cm^{-1}. It would appear that the "α" band, in the range 1146–1140 cm^{-1}, permits the assured differentiation of anomeric pairs of steroid tri-*O*-acetyl-D-glucopyranosiduronic methyl esters and D-glucopyranoside tetraacetates.

In Tables I and II are also listed bands generally observed in the range 1335–1330 cm^{-1}; these range in intensity from moderate to weak (but well defined) bands, to weak, broad bands. With one exception (**5**), they were noted only for the α-D anomers; they were either absent or very obscure for the isosapogenins. Where clearly present, these bands serve to supplement data from the region of 1150–1130 cm^{-1}, but their usefulness is limited by their low intensity.

A general comparison of these bands with those recorded for simple methyl (tri-*O*-acetyl-D-glucopyranosid)uronates and D-glucopyranoside tetraacetates is unfortunately not possible, as most of the latter spectra were determined for chloroform solutions, rather than for potassium bromide dispersions; significantly different spectra may be obtained with the two media, as shown, for example, in Fig. 3 of the paper of Nitta *et al.*[16]. The spectral characteristics of one anomeric pair, in potassium chloride dispersion, may be mentioned, namely the methyl α(and β)-D-glucopyranoside tetraacetates[15]. Bands for the α anomer include 1180 (w), and *1145* cm^{-1} (s), whereas those for the β anomer are at *1175* (s), 1140 (m), and 1110 cm^{-1} (m). The italicized values clearly link these results with those in Tables I and II. A few i.r. spectra of steroidal β-D-glucopyranosiduronic acids and esters have been reported; these include the spectra of eleven methyl ester triacetates in the amorphous and crystalline states[17], four free acids recorded in the same way[12], and five methyl ester triacetates in a crystalline form[11]. From these data and our observations, it is evident that the most useful spectra, particularly as regards the 1185–1130-cm^{-1} region, are derived from acetylated forms in a dispersed, crystalline state.

Mass-spectral studies. — The fragmentation patterns of seven pairs of Series A and C derivatives were determined, in order to extend earlier observations[2] bearing on their differentiation. The results are summmarized in Table III, in which ion intensities are expressed as percentages of the base ion, *m/e* 43 (CH_3CO^+).

Molecular (M^+) ions were observed for all compounds except one (*e*), and were unexpectedly prominent for the *g*,*g'* pair, indicating the considerable stability of these rather complex compounds. As the free 17,20β,21-glycerol derived from *g* also provided a large M^+ ion, it follows that the noted stability is not conferred by the isopropylidene group alone.

The pyronium ions *m/e* 317 and *m/e* 331 (see Scheme 2) are derived from Series A and C, respectively. As Table III illustrates, these ions provide a reliable means for

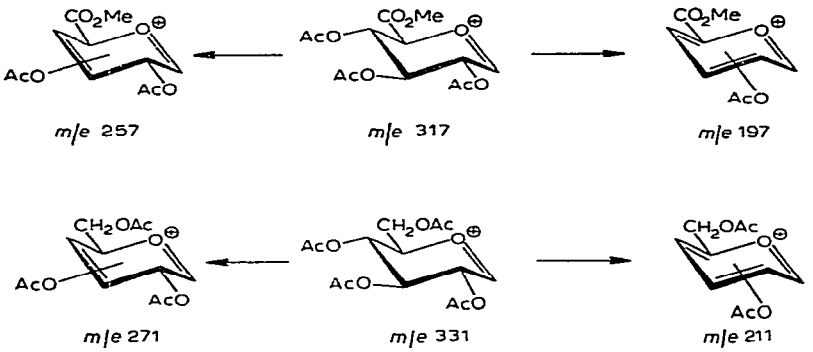
TABLE III

RELATIVE INTENSITIES OF IONS IN METHYL (TRI-*O*-ACETYL-β-D-GLUCOPYRANOSID)URONATE (SERIES A) AND β-D-GLUCOPYRANOSIDE TETRAACETATE (SERIES C) DERIVATIVES

Series	*Compound no.*	*Aglycon*	M^+	m/e					
				331	*317*	*271*	*257*	*211*	*197*
A	a (1)[a]	Cholesterol	0.03[b]		2		6		6
	b (3)	Cholestanol	0.03		3		15		3
	c (7)	Pregnenolone	0.05		3		4		5
	d (10)	Allopregnanolone	0.01		2		6		2
	e (12)	Dehydroisoandrosterone			4	40	7		6
	f (16)	Isoandrosterone	0.04		1	3	29	1	2
	g (21)	β-Cortolone acetonide	0.25	6	63	3	30	2	16
C	a′ (22)	Cholesterol	0.02	6		2		2	
	b′ (23)	Cholestanol	0.08	2		2	9		
	c′	Pregnenolone	0.02	10		2	1	3	1
	d′	Allopregnanolone	0.02	3		1	9	1	
	e′	Dehydroisoandrosterone	0.02	11		51		4	2
	f′	Isoandrosterone	0.05	2		2	4		
	g′	β-Cortolone acetonide	0.87	19		6		2	

[a]Lettered designations are for the purpose of this Table and discussion only; arabic numbers in parentheses are those generally assigned. Compounds *c′* through *g′* were prepared especially for this study, and are referred to in the i.r. section only. [b]Intensities are expressed as percent of that observed for the *m/e* 43 base ion.

distinguishing between the two classes. The apparently exceptional *m/e* 331 ion furnished by *g* is believed not to be a pyronium ion, since the required *m/e* 317 ion was also present. The free 17,20β,21-glycerol obtained from *g* provided a wholly analogous pattern, and it is therefore suggested that the *m/e* 331 ion observed for *g* was derived from a steroid residue lacking an isopropylidene group, possibly corresponding to $C_{21}H_{31}O_3$ (331.46). The presence of the isopropylidene group in *g* and *g'*, and its absence in the 17,20β,21-glycerol, was clearly shown by the characteristic *m/e* 101 ion in the spectra of the first two.



Scheme 2. Ion fragments useful in distinguishing between steroid tri-*O*-acetyl-β-D-glucopyranosiduronic methyl esters and β-D-glucopyranoside tetraacetates.

It is clear from Table III that prominent among the further reactions of the *m/e* 317 ion, is the loss of both one and two molecules of acetic acid, yielding the *m/e* 257 and *m/e* 197 ions, respectively. The further fragmentation of the *m/e* 331 ion appears to follow a similar course, furnishing, in this case, the *m/e* 271 ion and, less regularly, the well known[18,19] *m/e* 211 ion*. Our earlier suggestion[2] that the *m/e* 331 ion undergoes an initial expulsion of the $CH_3COOCH_2^+$ fragment followed by the loss of one molecule of acetic acid, successively yielding the *m/e* 257 and *m/e* 197 ions, is not supported by these data. It seems probable that the large *m/e* 271 ion derived from *e* and *e'* represents a steroid component, possibly 3,5-androstadiene-17-one (270.4).

It has been suggested[18] that, under favorable circumstances, it may be possible to distinguish anomers on the basis of their mass spectra. We have attempted to test this view with the present pairs, but differences, if present, were too small to be detected by using the direct-introduction technique.

EXPERIMENTAL

General. — Melting points were obtained with a Fisher–Johns apparatus and are uncorrected; compounds were placed on the stage preheated to a temperature 10° below the m.p. Optical rotations were determined with a Zeiss 0.005° photoelectric polarimeter at a concentration of ~1.5% and a temperature of 24 ±1°. I.r.

*The presence of the M-60, M-120, and M-180 ions of various intensities in the spectra of all derivatives indicates the successive loss of three molecules of acetic acid.

spectra were recorded with a Beckman IR-8 spectrometer; they were in accord with the assigned structures, but, as most of them displayed few bands in the 4000–1330-cm^{-1} region, except those regularly occurring at 1745–1760 and 1260–1205 (acetate), 1445–1435, and 1375–1365 cm^{-1}, they are referred to only where they provide additional information. Mass spectra were determined by Dr. Robert Schaffer, of the Morgan–Schaffer Corporation, Montreal, Canada, with a Hitachi–Perkin–Elmer RMU-6D spectrometer. T.l.c. was performed on silica gel (Camag DF-5) supported on glass plates. Columns were prepared with silica gel (Davison, grade 923); the mobile phases consisted of appropriate mixtures of ethyl acetate and 2,2,4-trimethylpentane. Yields reported are based on products that had been twice recrystallized and dried over anhydrous calcium chloride *in vacuo*. Specimens for elementary analysis (by August Peisker-Ritter, Brugg, Switzerland, and Alfred Bernhardt, Elbach über Engelskirchen, West Germany) were dried to constant weight over phosphoric anhydride under high vacuum at an appropriate temperature.

Methyl (5α,6β-dibromocholestan-3β-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (**2**) *from* **1**. — To a solution of methyl (cholest-5-en-3β-yl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid)uronate[2] (**1**, 500 mg) in ether (25 ml) was added, in one portion, a solution of bromine (75 μl) and anhydrous sodium acetate (20 mg) in acetic acid (2.5 ml). After the mixture had been stirred for about 3 min at room temperature, the product separated spontaneously in crystalline form. The flask was chilled, and the crystals were dispersed by the addition of methanol, collected by filtration, and washed with methanol. Recrystallization from ether–methanol afforded 532 mg (87%) of the pure dibromide, m.p. 173–174° (dec.); $[\alpha]_D$ −60°, $[\alpha]_{365}$ −197°.

Anal. Calc. for $C_{40}H_{62}Br_2O_{10}$: C, 55.68; H, 7.24; Br, 18.52. Found: C, 55.50; H, 7.21; Br, 18.65.

Methyl (5α,6β-dibromocholestan-3β-yl 2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate (**2a**) *from* **2**. — To a solution of **2** (466 mg) in benzene (5 ml) was added 0.1 ml of titanium tetrachloride (Fisher, purified grade). As the solution darkened rapidly, it was diluted with ethyl acetate after 30 min, washed with neutral brine, dried, and evaporated. Crystallization from ethyl acetate–methanol furnished 311 mg (67%) of needles, m.p. 185–186° (dec.); $[\alpha]_D$ +54°, $[\alpha]_{365}$ +141°.

Anal. Calc. for $C_{40}H_{62}Br_2O_{10}$: C, 55.68; H, 7.24; Br, 18.52. Found: C, 56.01; H, 7.20; Br, 18.42.

Methyl (cholest-5-en-3β-yl 2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate (**1a**) *from* **2a**. — To a solution of **2a** (573 mg) in ether (30 ml) and acetic acid (10 ml) was added zinc powder (1.5 g), and the suspension was stirred for 10 min at room temperature. The supernatant liquor was diluted with ethyl acetate, washed successively with water, cold 2M sodium hydroxide, and neutral brine, dried, and evaporated. Crystallization from ethyl acetate–ethanol provided 420 mg (90%) of needles, m.p. 191–192°; $[\alpha]_D$ +95°, $[\alpha]_{365}$ +274°.

Anal. Calc. for $C_{40}H_{62}O_{10}$: C, 68.35; H, 8.89; OCH_3, 4.41. Found: C, 68.31; H, 8.93; OCH_3, 4.46.

Methyl (5α-cholestan-3β-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (**3**). —

Compound **3** was prepared from 5α-cholestan-3β-ol (cholestanol) as described for the preparation of **1** from cholesterol[2]. Crystallization from ethyl acetate–methanol gave needles in a yield of 67%, m.p. 180–181°; $[\alpha]_D$ −5°, $[\alpha]_{365}$ −15°.

Anal. Calc. for $C_{40}H_{64}O_{10}$: C, 68.15; H, 9.15; OCH_3, 4.40. Found: C, 68.18; H, 9.14; OCH_3, 4.42.

Methyl (5α-cholestan-3β-yl 2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate (**3a**) *from* **3**. — To a solution of **3** (100 mg) in benzene (2 ml) was added 0.05 ml of titanium tetrachloride. After the yellow complex had been dispersed with glass beads, the suspension was kept for 12 h at room temperature. The product was isolated as in the preparation of **2a** from **2**, to give 78 mg (78%) of needles from acetone–methanol, m.p. 175–175.5°; $[\alpha]_D$ +108°, $[\alpha]_{365}$ +315°.

Anal. Calc. for $C_{40}H_{64}O_{10}$: C, 68.15; H, 9.15; OCH_3, 4.40. Found: C, 68.14; H, 9.30; OCH_3, 4.70.

Methyl (5α-cholestan-3α-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (**4**). — Compound **4** was prepared from 5α-cholestan-3α-ol (epicholestanol) as for the synthesis of **1**. Crystallization of the product from ethyl acetate–methanol gave long needles; yield 46%, m.p. 191–192°, $[\alpha]_D$ −15°, $[\alpha]_{365}$ −45°.

Anal. Calc. for $C_{40}H_{64}O_{10}$: C, 68.15; H, 9.15; OCH_3, 4.40. Found: C, 68.20; H, 9.11; OCH_3, 4.38.

Methyl (5α-cholestan-3α-yl 2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate (**4a**) *from* **4**. — Treatment of **4** (100 mg) with titanium tetrachloride, as in the preparation of **3a** from **3**, gave 74 mg (74%) of needles from acetone–methanol, m.p. 147–147.5°; $[\alpha]_D$ +88°, $[\alpha]_{365}$ +258°.

Anal. Calc. for $C_{40}H_{64}O_{10}$: C, 68.15; H, 9.15; OCH_3, 4.40. Found: C, 68.36; H, 9.21; OCH_3, 4.42.

Methyl (5β-cholestan-3β-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (**5**). — Compound **5** was prepared from 5β-cholestan-3β-ol (coprostanol) as in the synthesis of **1**. Crystallization from acetone furnished needles (38%), m.p. 182–183°, $[\alpha]_D$ −11°, $[\alpha]_{365}$ −29°.

Anal. Calc. for $C_{40}H_{64}O_{10}$: C, 68.15; H, 9.15; OCH_3, 4.40. Found: C, 68.19; H, 9.15; OCH_3, 4.39.

Methyl (5β-cholestan-3β-yl 2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate (**5a**) *from* **5**. — Treatment of **5** (100 mg) with titanium tetrachloride, as in the preparation of **3a** from **3**, afforded 50 mg (50%) of needles from hexane, m.p. 159–160°; $[\alpha]_D$ +115°, $[\alpha]_{365}$ +334°.

Anal. Calc. for $C_{40}H_{64}O_{10}$: C, 68.15; H, 9.15; OCH_3, 4.40. Found: C, 68.20; H, 9.19; OCH_3, 4.59.

Methyl (5β-cholestan-3α-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (**6**). — Compound **6** was obtained from 5β-cholestan-3α-ol (epicoprostanol) as in the preparation of **1**. Crystallization from ethyl acetate–methanol provided needles (56%), m.p. 176–177°; $[\alpha]_D$ 0°, $[\alpha]_{365}$ 0°.

Anal. Calc. for $C_{40}H_{64}O_{10}$: C, 68.15; H, 9.15; OCH_3, 4.40. Found: C, 68.12; H, 9.05; OCH_3, 4.48.

Methyl (5β-cholestan-3α-yl 2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate (**6a**) *from* **6**. — Anomerization of **6** (100 mg), as in the preparation of **3a** from **3**, gave 64 mg (64%) of needles from methanol, m.p. 136.5–137.5°; $[\alpha]_D$ +107°, $[\alpha]_{365}$ +311°.

Anal. Calc. for $C_{40}H_{64}O_{10}$: C, 68.15; H, 9.15; OCH_3, 4.40. Found: C, 68.07; H, 9.05; OCH_3, 4.38.

Methyl (20-oxopregn-5-en-3β-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (**7**). — Compound **7** was prepared from 3β-hydroxypregn-5-en-20-one (pregnenolone) as in the synthesis of **1**. The product was obtained as needles from methanol (54%), m.p. 184–185°; $[\alpha]_D$ +7°, $[\alpha]_{365}$ +150°; lit.[20] m.p. 183–184°, $[\alpha]_D$ +5° (chloroform).

Methyl (5α,6β-dibromo-20-oxopregnan-3β-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (**8**) *from* **7**. — To a solution of **7** (633 mg; 1 mmole) in chloroform was added, in one portion, a solution of bromine (61 μl; 1.2 mmoles) in 0.15 ml of acetic acid. As soon as decolorization was complete (~3 min), the solution was successively washed with cold, dilute sodium hydroxide and water, dried, and evaporated. Crystallization of the product from dichloromethane–methanol provided 525 mg (66%) of needles, m.p. 171–172° (dec.); $[\alpha]_D$ −38°, $[\alpha]_{365}$ −18°.

Anal. Calc. for $C_{34}H_{48}Br_2O_{11}$: C, 51.52; H, 6.10; Br, 20.17. Found: C, 51.40; H, 6.08; Br, 20.31.

Methyl (5α, 6β-dibromo-20-oxopregnan-3β-yl 2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate (**8a**) *from* **8**. — To a solution of **8** (400 mg) in dichloromethane (20 ml) was added titanium tetrachloride (0.20 ml). After 2 h at room temperature, the solution was successively washed with dilute, cold sodium hydroxide and water, dried, and evaporated. Crystallization of the product from dichloromethane–methanol provided 305 mg (75%) of needles, m.p. 196.5–197.5° (dec.); $[\alpha]_D$ +84°, $[\alpha]_{365}$ +346°.

Anal. Calc. for $C_{34}H_{48}Br_2O_{11}$: C, 51.52; H, 6.10; Br, 20.17. Found: C, 51.44; H, 6.00; Br, 19.98.

Methyl (20-oxopregn-5-en-3β-yl 2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate (**7a**) *from* **8a**. — To a stirred solution of **8a** (150 mg) in ether (10 ml) and acetic acid (3 ml) was added powdered zinc (500 mg) during 10 min. Isolation of the product as in the preparation of **1a** from **2a**, followed by crystallization from dichloromethane–methanol, gave 120 mg (80%) of needles, m.p. 197–198°; $[\alpha]_D$ +142°, $[\alpha]_{365}$ +564°.

Anal. Calc. for $C_{34}H_{48}O_{11}$: C, 64.54; H, 7.65; OCH_3, 4.90. Found: C, 64.31; H, 7.70; OCH_3, 4.79.

Methyl (20-oxo-5β-pregnan-3α-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (**9**). — Compound **9** was prepared from 3α-hydroxy-5β-pregnan-20-one (pregnanolone) as in the synthesis of **1**. The product crystallized as needles from ethyl acetate–methanol; yield 44%, m.p. 197.5–198.5°; $[\alpha]_D$ +43°, $[\alpha]_{365}$ +270°.

Anal. Calc. for $C_{34}H_{50}O_{11}$: C, 64.33; H, 7.94; OCH_3, 4.98. Found: C, 64.64; H, 8.02; OCH_3, 5.15.

Methyl (20-oxo-5β-pregnan-3α-yl 2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate (**9a**) *from* **9**. — To a solution of **9** (100 mg) in benzene (3 ml) was added 0.05 ml of titanium tetrachloride. After 5 h at room temperature, the product was isolated;

it furnished, from ethyl acetate–methanol, 80 mg (80%) of plates, m.p. 222–223°; $[\alpha]_D$ +159°, $[\alpha]_{365}$ +610°.

Anal. Calc. for $C_{34}H_{50}O_{11}$: C, 64.33; H, 7.94; OCH_3, 4.98. Found: C, 64.21; H. 7.74; OCH_3, 4.93.

Methyl (20-oxo-5α-pregnan-3β-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (**10**). — Compound **10** was obtained from 3β-hydroxy-5α-pregnan-20-one (allopregnanolone) as in the synthesis of **1**. Crystallization of the product from ethyl acetate–methanol gave needles; yield 51%, m.p. 203–204°; $[\alpha]_D$ +29°, $[\alpha]_{365}$ +244°; lit.[21] m.p. 201–202°.

Methyl (20-oxo-5α-pregnan-3β-yl 2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate (**10a**) *from* **10**. — Anomerization of **10** (100 mg), as in the preparation of **9a** from **9**, gave 68 mg (68%) of needles from ethyl acetate–methanol, m.p. 177–178°; $[\alpha]_D$ +165°, $[\alpha]_{365}$ +629°.

Anal. Calc. for $C_{34}H_{50}O_{11}$: C, 64.33; H, 7.94; OCH_3, 4.98. Found: C, 64.31; H, 7.80; OCH_3, 4.94.

Methyl (17α-hydroxy-20-oxo-5β-pregnan-3α-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (**11**). — Compound **11** was prepared from 3α,17α-dihydroxy-5β-pregnan-20-one (17-hydroxypregnanolone), as in the synthesis of **1**. The product crystallized from ethyl acetate as needles, yield 47%, m.p. 213–213.5°; $[\alpha]_D$ −14°, $[\alpha]_{365}$ −11°.

Anal. Calc. for $C_{34}H_{50}O_{12}$: C, 62.75; H, 7.75; OCH_3, 4.77. Found: C, 62.72; H, 7.69; OCH_3, 5.01.

Methyl (17α-hydroxy-20-oxo-5β-pregnan-3α-yl 2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate (**11a**) *from* **11**. — Treatment of **11** (100 mg) with titanium tetrachloride, as in the preparation of **9a** from **9**, gave 83 mg (83%) of needles from ethyl acetate–methanol, m.p. 211–212°; $[\alpha]_D$ +104°, $[\alpha]_{365}$ +313°.

Anal. Calc. for $C_{34}H_{50}O_{12}$: C, 62.75; H, 7.75; OCH_3, 4.77. Found: C, 62.66; H, 7.50; OCH_3, 4.91.

Methyl (17-oxoandrost-5-en-3β-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (**12**). — Compound **12** was prepared from 3β-hydroxyandrost-5-en-17-one (dehydroisoandrosterone) as in the synthesis of **1**. Crystallization from methanol furnished needles (77%), m.p. 194–194.5°; $[\alpha]_D$ −11°, $[\alpha]_{365}$ −102°; lit.[20] m.p. 195–195.5°, $[\alpha]_D$ −11° (chloroform).

Methyl (5α,6β-dibromo-17-oxoandrostan-3β-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (**13**) *from* **12**. — Bromination of compound **12** was effected as in the preparation of **8** from **7**. Crystallization from dichloromethane–methanol afforded needles (74%), m.p. 153–154° (dec.); $[\alpha]_D$ −40°, $[\alpha]_{365}$ −19°.

Anal. Calc. for $C_{32}H_{44}Br_2O_{11}$: C, 50.27; H, 5.80; Br, 20.90. Found: C, 50.14; H, 5.84; Br, 21.06.

Methyl (5α,6β-dibromo-17-oxoandrostan-3β-yl 2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate (**13a**) *from* **13**. — Treatment of compound **13** with titanium tetrachloride was conducted as in the preparation of **8a** from **8**, except that a reaction time of

3 h was used. Crystallization from dichloromethane–methanol gave needles (90%), m.p. 180.5–181.5° (dec.); $[\alpha]_D$ +77°, $[\alpha]_{365}$ +323°.

Anal. Calc. for $C_{32}H_{44}Br_2O_{11}$: C, 50.27; H, 5.80; Br, 20.90. Found: C, 49.96; H, 5.72; Br, 21.85.

*Methyl (17-oxoandrost-5-en-3β-yl 2,3,4-tri-*O*-acetyl-α-*D*-glucopyranosid)uronate* (**12a**) *from* **13a**. — Debromination of compound **13a** was performed as in the preparation of **7a** from **8a**. The product crystallized from aqueous methanol as needles (88%), m.p. 156.5–157°; $[\alpha]_D$ +136°, $[\alpha]_{365}$ +539°.

Anal. Calc. for $C_{32}H_{44}O_{11}$: C, 63.56; H, 7.33; OCH_3, 5.13. Found: C, 63.67; H, 7.30; OCH_3, 5.04.

*Methyl (17-oxo-5α-androstan-3α-yl 2,3,4-tri-*O*-acetyl-β-*D*-glucopyranosid)uronate* (**14**). — Compound **14** was prepared from 3α-hydroxy-5α-androstan-17-one (androsterone) as in the synthesis of **1**. Crystallization of the product from ethyl acetate–hexane afforded needles in a yield of 42%, m.p. 181–182°; $[\alpha]_D$ +17°, $[\alpha]_{365}$ +196°; lit.[22] m.p. 175.5–176°, $[\alpha]_D$ +12° (chloroform).

*Methyl (17-oxo-5α-androstan-3α-yl 2,3,4-tri-*O*-acetyl-α-*D*-glucopyranosid)uronate* (**14a**) *from* **14**. — Treatment of compound **14** (100 mg) with titanium tetrachloride was conducted as in the preparation of **9a** from **9**. The product was crystallized (with difficulty) from aqueous methanol (yield, 70%); m.p. indefinite*; $[\alpha]_D$ +135°, $[\alpha]_{365}$ +541°.

Anal. Calc. for $C_{32}H_{46}O_{11}$: C, 63.35; H, 7.64; OCH_3, 5.11. Found: C, 63.20; H, 7.39; OCH_3, 5.09.

*Methyl (17-oxo-5β-androstan-3α-yl 2,3,4-tri-*O*-acetyl-β-*D*-glucopyranosid)uronate* (**15**). — Compound **15** was prepared from 3α-hydroxy-5β-androstan-17-one (etiocholanolone) as in the synthesis of **1**. The product crystallized from ethyl acetate–hexane as needles (63%), m.p. 181–182°; $[\alpha]_D$ +32°, $[\alpha]_{365}$ +235°; lit.[22] m.p. 176.5–178°, $[\alpha]_D$ +29° (chloroform).

*Methyl (17-oxo-5β-androstan-3α-yl 2,3,4-tri-*O*-acetyl-α-*D*-glucopyranosid)uronate* (**15a**) *from* **15**. — Anomerization of compound **15** was performed as in the preparation

*The melting points of **14a**, **15a**, **16a**, **20**, and **21** were indefinite, and are therefore not recorded. Extended drying over a suitable desiccant *in vacuo* at 60–100° was without effect. In order to determine whether these derivatives were contaminated with unreacted free steroid, samples of each were treated with acetic anhydride and pyridine, recovered in the usual way, and examined by t.l.c. This procedure would readily reveal contaminating free steroid, as the mobilities of the methyl ester triacetates and the acetates of the steroidal aglycons differ greatly. In all cases, this test gave negative results. (Contaminating free steroid can be detected by mass spectrometry, provided that the steroid is appreciably more volatile than the corresponding methyl ester triacetate; see ref. 2 in this regard).

Another possibility was that **14a**, **15a**, **16a**, and **20a** might contain some unreacted anomer; its presence or absence was difficult to establish with certainty by t.l.c., as the R_F differences between members of anomeric pairs are frequently too small to be useful. As an alternative test, fresh samples of each β-D precursor, namely **14**, **15**, **16**, and **20**, were treated with titanium tetrachloride for a period of time twice that originally employed. The crystallizing characteristics and melting-point behavior of the products were the same as those originally observed for **14a**, **15a**, **16a**, and **20a**.

These results, together with the satisfactory elemental analyses and the optical rotatory behavior, which was as expected, indicated that the compounds were substantially pure.

of **9a** from **9**. Crystallization from acetone–hexane gave needles (82%); $[\alpha]_D$ +148°, $[\alpha]_{365}$ +569°.

Anal. Calc. for $C_{32}H_{46}O_{11}$: C, 63.35; H, 7.64; OCH_3, 5.11. Found: C, 63.47; H, 7.43; OCH_3, 5.10.

Methyl (17-oxo-5α-androstan-3β-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (**16**). — Compound **16** was prepared from 3β-hydroxy-5α-androstan-17-one (iso-androsterone) as in the synthesis of **1**. The product crystallized from ethyl acetate–hexane as needles (63%), m.p. 147–148°; $[\alpha]_D$ +24°, $[\alpha]_{365}$ +217°; lit. m.p. 163–164°, $[\alpha]_D$ +19° (chloroform[23]; m.p. 168–170°, $[\alpha]_D$ +28° (chloroform)[20].

Methyl (17-oxo-5α-androstan-3β-yl 2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate (**16a**) *from* **16**. — Treatment of compound **16** with titanium tetrachloride, as in the preparation of **9a** from **9**, gave crystals from aqueous methanol (with difficulty) in a yield of 70%; $[\alpha]_D$ +164°, $[\alpha]_{365}$ +626°.

Anal. Calc. for $C_{32}H_{46}O_{11}$: C, 63.35; H, 7.64; OCH_3, 5.11. Found: C, 63.23; H, 7.44; OCH_3, 5.09.

Methyl ($25_{\alpha F}$-spirost-5-en-3β-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (**17**). — Compound **17** was prepared from $25_{\alpha F}$-spirost-5-en-3β-ol (diosgenin) as in the synthesis of **1**. The product crystallized from ethyl acetate–methanol as long needles (60%), m.p. 207–208°; $[\alpha]_D$ −86°, $[\alpha]_{365}$ −246°.

Anal. Calc. for $C_{40}H_{58}O_{12}$: C, 65.73; H, 8.00; OCH_3, 4.25. Found: C, 65.78; H, 7.96; OCH_3, 4.14.

Methyl (5α,6β-dibromo-$25_{\alpha F}$-spirostan-3β-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (**18**) *from* **17**. — Bromination of compound **17** was effected as in the preparation of **13** from **12**. Crystallization of the product from ethyl acetate–methanol (with avoidance of heat) gave needles (66%), m.p. 166–167° (dec.); $[\alpha]_D$ −99°, $[\alpha]_{365}$ −308°.

Anal. Calc. for $C_{40}H_{58}Br_2O_{12}$: C, 53.94; H, 6.56; Br, 17.94. Found: C, 53.69; H, 6.51; Br, 18.24.

Methyl (5α,6β-dibromo-$25_{\alpha F}$-spirostan-3β-yl 2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate (**18a**) *from* **18**. — Treatment of compound **18** with titanium tetrachloride was conducted as in the anomerization of **13**, except that the reaction time was extended to 4 h. Crystallization from dichloromethane–methanol afforded needles (68%), m.p. 217–218° (*dec.*); $[\alpha]_D$ +6°, $[\alpha]_{365}$ +6°.

Anal. Calc. for $C_{40}H_{58}Br_2O_{12}$: C, 53.94; H, 6.56; Br, 17.94. Found: C, 53.98; H, 6.72; Br, 17.34.

Methyl ($25_{\alpha F}$-spirost-5-en-3β-yl 2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate (**17a**) *from* **18a**. — Debromination of compound **18a** was performed as in the preparation of **12a** from **13a**. The product crystallized from dichloromethane–methanol as needles (82%), m.p. 218–219°; $[\alpha]_D$ +43°, $[\alpha]_{365}$ +131°.

Anal. Calc. for $C_{40}H_{58}O_{12}$: C, 65.73; H, 8.00; OCH_3, 4.25. Found: C, 65.70; H, 8.04; OCH_3, 4.25.

Methyl ($25_{\alpha F}$-5α-spirostan-3β-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (**19**). — Compound **19** was prepared from $25_{\alpha F}$-5α-spirostan-3β-ol (tigogenin) as in

the synthesis of **1**. Crystallization from methanol gave needles (43%), m.p. 234–235°; $[\alpha]_D$ −60°, $[\alpha]_{365}$ −171°.

Anal. Calc. for $C_{40}H_{60}O_{12}$: C, 65.55; H, 8.23; OCH_3, 4.23. Found: C, 65.68; H, 8.31; OCH_3, 4.33.

Methyl (25$_{\alpha F}$-5α-spirostan-3β-yl 2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate (**19a**) *from* **19**. — Anomerization of compound **19**, as in the preparation of **9a** from **9**, gave needles from ethyl acetate (70%), m.p. 246.5–247.5°; $[\alpha]_D$ +58°, $[\alpha]_{365}$ +172°.

Anal. Calc. for $C_{40}H_{60}O_{12}$: C, 65.55; H, 8.23; OCH_3, 4.23. Found: C, 65.57; H, 8.02; OCH_3, 4.16.

3α,17α-Dihydroxy-20β,21-(isopropylidenedioxy)-5β-pregnan-11-one. — To a concentrated solution of 3α,17α,20β,21-tetrahydroxy-5β-pregnan-11-one[24] (5 g) in warm methanol was added acetone (2 liters). The solution was cooled to room temperature, and *p*-toluenesulfonic acid (1.25 g) was added. After 20 min at room temperature, M sodium hydroxide (7.5 ml) was added, and the solution was concentrated almost to dryness *in vacuo*. The residue was dissolved in wet ethyl acetate, and the solution was washed with neutral brine, dried, and evaporated. Crystallization from acetone gave 5.05 g (90%) of prisms, m.p. 186–187°, $[\alpha]_D$ +40°.

Anal. Calc. for $C_{24}H_{38}O_5$: C, 70.90; H, 9.43. Found: C, 70.68; H, 9.15.

Methyl [17α-hydroxy-11-oxo-20β,21-(isopropylidenedioxy)-5β-pregnan-3α-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid]uronate (**21**). — Compound **21** was prepared from 3α,17α-dihydroxy-20β,21-(isopropylidenedioxy)-5β-pregnan-11-one as in the synthesis of **1**. The product crystallized from acetone–hexane as fine needles (69%); $[\alpha]_D$ +1°, $[\alpha]_{365}$ +48°; ν_{max}^{KBr} 3680–3550 (17-OH), 1705 (11-;carbonyl), 1160, 936, and 860 cm^{-1} (20,21-acetonide)[6].

Anal. Calc. for $C_{37}H_{54}O_{14}$: C, 61.48; H, 7.53; OCH_3, 4.29. Found: C, 61.19; H, 7.70; OCH_3, 4.40.

Methyl (17α,20β,21-trihydroxy-11-oxo-5β-pregnan-3α-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (**20**) *from* **21**. — A solution of compound **21** (1.5 g) in acetic acid (525 ml) and water (225 ml) was kept for 16 h at room temperature. After removal of the solvents *in vacuo*, the product was isolated in the usual way and gave, from acetone–hexane, 1.24 g (87%) of needles; $[\alpha]_D$ +4°, $[\alpha]_{365}$ +40°; ν_{max}^{KBr} 3700–3200 (OH) and 1701 cm^{-1} (11-carbonyl).

Anal. Calc. for $C_{34}H_{50}O_{14}$: C, 59.81; H, 7.38; OCH_3, 4.55. Found: C, 59.97; H, 7.60; OCH_3, 4.53.

Methyl (17α,20β,21-trihydroxy-11-oxo-5β-pregnan-3α-yl 2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate (**20a**) *from* **21***. — To a solution of compound **21** (200 mg) in benzene (10 ml) was added 0.1 ml of titanium tetrachloride. After 6 h at room temperature, the suspension was diluted with ethyl acetate, and the neutral product was isolated in the usual way. Crystallization from methanol gave 152 mg (80%)

*The acetonide **21** is the preferred starting material for the preparation of **20a**, as it is more soluble than **20** in benzene. The isopropylidene group is probably removed by hydrolysis during processing of the acidic reaction mixture.

of fine needles; $[\alpha]_D$ +116°, $[\alpha]_{365}$ +364°; ν_{max}^{KBr} 3700–3150 (OH) and 1701 cm^{-1} (11-carbonyl).

Anal. Calc. for $C_{34}H_{50}O_{14}$: C, 59.81; H, 7.38; OCH_3, 4.55. Found: C, 59.68; H, 7.16; OCH_3, 4.50.

Methyl [17α-hydroxy-11-oxo-20,21-(isopropylidenedioxy)-5β-pregnan-3α-yl 2,3,4-tri-O-acetyl-α-D-glucopyranosid]uronate (**21a**) *from* **20a**. — Acetonation of compound **20a**, as in the previous example, furnished needles from ethyl acetate–methanol (85%), m.p. 224.5–225.5°; $[\alpha]_D$ +116°, $[\alpha]_{365}$ +364°; ν_{max}^{KBr} 3680–3350 (17-OH), 1701 (11-carbonyl), 1160, 935, and 859 cm^{-1} (20,21-acetonide).

Anal. Calc. for $C_{37}H_{54}O_{14}$: C, 61.48; H, 7.53; OCH_3, 4.29. Found: C, 61.40; H, 7.65; OCH_3, 4.10.

Cholest-5-en-3β-yl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (**22**) *from* **1**. — To a solution of compound **1** (200 mg) in dry ether (20 ml), lithium aluminum hydride (100 mg) was added in one portion. After the suspension had been refluxed for 3 h, the excess of hydride was decomposed by the sequential addition of ethyl acetate and water. The mixture was diluted with ethyl acetate, and washed successively with 2M hydrochloric acid, dilute sodium hydroxide, water, and neutral brine, dried with anhydrous sodium sulfate, and evaporated *in vacuo*. The residue was treated with acetic anhydride and pyridine for 12 h at room temperature, and the resulting acetate was isolated in the usual way. Crystallization from ethyl acetate–methanol gave 130 mg of needles (64%), m.p. 161.5–162°; $[\alpha]_D$ −27°, $[\alpha]_{365}$ −90°; lit. m.p. 159–160°, $[\alpha]_D$ −24° (chloroform)[25]; m.p. 159–160°, $[\alpha]_D$ −23° (chloroform)[26]; m.p. 157–159°, $[\alpha]_D$ −25° (chloroform)[27].

Cholest-5-en-3β-yl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside (**22a**) *from* **1a**. — Reduction of compound **1a** (100 mg), as in the preparation of **22** from **1**, afforded 43 mg (42%) of needles from ethyl acetate–methanol, m.p. 195.5–196.5°; $[\alpha]_D$ +91°, $[\alpha]_{365}$ +257°; lit. m.p. 195°, $[\alpha]_D$ +88° (chloroform)[28]; m.p. 195–197°, $[\alpha]_D$ +88° (chloroform)[27].

5α-Cholestan-3β-yl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (**23**) *from* **3**. — Reduction of **3** (300 mg), as in the preparation of **22** from **1**, gave only 52 mg (17%)* of needles from ethanol, m.p. 174.5–175.5°; $[\alpha]_D$ +3°, $[\alpha]_{365}$ +5°; lit. m.p. 175°, $[\alpha]_D$ +5° (chloroform)[29]; m.p. 174–175° (ref. 30); m.p. 164–165°, $[\alpha]_D$ +6.3° (chloroform)[31].

5α-Cholestan-3β-yl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside (**23a**) *from* **23**. — Treatment of **23** (100 mg) in benzene (2 ml) with 0.05 ml of titanium tetrachloride

*When it was noted that the amount of mother liquor was very small, it became clear that much of the product, which has a very limited solubility in ether–ethyl acetate, had been lost during the processing. A modified procedure was therefore devised: after the addition of ethyl acetate and water, the remaining ether was evaporated with a stream of nitrogen. The residue was suspended in aqueous methanol, the pH adjusted to ~7, the solution re-evaporated, and the residue well dried (still in the original flask) *in vacuo* over an effective desiccant. The dry residue was treated with acetic anhydride and pyridine, and the acetate was recovered in the usual way. When a fresh sample of **3** was reduced as indicated, and subsequently processed by the modified technique, the yield of **23** was 85%. This method was also used for recovering the reduction products from **17**, **17a**, and **19**.

for 9 h at room temperature, followed by the usual processing, furnished 55 mg (55%) of needles from ethyl acetate–methanol, m.p. 186–187°; $[\alpha]_D$ +106°, $[\alpha]_{365}$ +303°; lit. m.p. 184°, $[\alpha]_D$ +114° (chloroform)[29]; m.p. 182°, $[\alpha]_D$ +108° (chloroform)[26].

5α-Cholestan-3α-yl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (**24**) *from* **4**. — Compound **24** was obtained by the reduction of **4**, as in the preparation of **22** from **1**. The product crystallized from acetone–hexane as needles (79%), m.p. 175–176°; $[\alpha]_D$ −11°, $[\alpha]_{365}$ −35°; lit. m.p. 174°, $[\alpha]_D$ −3° (chloroform)[29]; m.p. 170–172° (ref. 30).

5α-Cholestan-3α-yl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside (**24a**) *from* **24**. — Treatment of compound **24** with titanium tetrachloride was conducted as in the preparation of **23a** from **23**. Crystallization of the product from ethyl acetate–methanol gave needles (50%), m.p. 137–139°; $[\alpha]_D$ +97°, $[\alpha]_{365}$ +278°; lit. m.p. 130°, $[\alpha]_D$ +93° (chloroform)[29].

5β-Cholestan-3β-yl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (**25**) *from* **5**. — Compound **25** was obtained by the reduction of **5**, as in the preparation of **22** from **1**. Crystallization from ethanol–acetone gave needles (59%), m.p. 202.5–203.5°; $[\alpha]_D$ −3°, $[\alpha]_{365}$ −16°; lit.[30] m.p. 198–200°.

5β-Cholestan-3β-yl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside (**25a**) *from* **25**. — Anomerization of compound **25**, as in the preparation of **23a** from **23**, gave needles from ethyl acetate–methanol (62%), m.p. 120–121°; $[\alpha]_D$ +117°, $[\alpha]_{365}$ +332°.

Anal. Calc. for $C_{41}H_{66}O_{10}$: C, 68.49; H, 9.25. Found: C, 68.66; H, 9.20.

5β-Cholestan-3α-yl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (**26**) *from* **6**. — Reduction of compound **6**, as in the preparation of **22** from **1**, gave a filtrable gel from acetone–methanol (72%), m.p. 173–173.5°; $[\alpha]_D$ +11°, $[\alpha]_{365}$ +23°; lit.[30] m.p. 140–141° (?).

Anal. Calc. for $C_{41}H_{66}O_{10}$: C, 68.49; H, 9.25. Found: C, 68.45; H, 9.03.

5β-Cholestan-3α-yl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside (**26a**) *from* **26**. — Treatment of compound **26** with titanium tetrachloride, as in the preparation of **23a** from **23**, gave needles (71%) from ethyl acetate–methanol, m.p. 144.5–145.5°; $[\alpha]_D$ +110°, $[\alpha]_{365}$ +314°.

Anal. Calc. for $C_{41}H_{66}O_{10}$: C, 68.49; H, 9.25. Found: C, 68.37; H, 9.05.

$25_{\alpha F}$-Spirost-5-en-3β-yl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (**27**) *from* **17**. — Reduction of compound **17**, as in the preparation of **23** from **3**, gave needles from ethyl acetate–methanol (72%), m.p. 208–209°; $[\alpha]_D$ −77°, $[\alpha]_{365}$ −228°; lit. m.p. 200–202°, $[\alpha]_D$ −75° (chloroform)[32]; m.p. 204–205°, $[\alpha]_D$ −71° (*p*-dioxane)[33].

Anal. Calc. for $C_{41}H_{60}O_{12}$: C, 66.10; H, 8.12. Found: C, 66.24; H, 7.88.

$25_{\alpha F}$-Spirost-5-en-3β-yl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside (**27a**) *from* **17a**. — Reduction of compound **17a**, as in the preparation of **23** from **3**, gave a filtrable gel from ethyl acetate–methanol (62%), m.p. 161.5–162.5°; $[\alpha]_D$ +37°, $[\alpha]_{365}$ +107°.

Anal. Calc. for $C_{41}H_{60}O_{12}$: C, 66.10; H, 8.12. Found: C, 66.10, H, 7.98.

$25_{\alpha F}$-5α-Spirostan-3β-yl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (**28**) *from* **19**.

— Reduction of compound **19**, as in the preparation of **23** from **3**, gave plates from ethyl acetate–methanol (72%), m.p. 207–208°; $[\alpha]_D$ −41°, $[\alpha]_{365}$ −149°.

Anal. Calc. for $C_{41}H_{62}O_{12}$: C, 65.93; H, 8.37. Found: C, 66.06; H, 8.13.

$25_{\alpha F}$-5α-Spirostan-3β-yl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside (**28a**) *from* **28**. — Treatment of compound **28** with titanium tetrachloride, as in the preparation of **23a** from **23**, gave needles from ethyl acetate–methanol (71%), m.p. 210–211°; $[\alpha]_D$ +57°, $[\alpha]_{365}$ +164°.

Anal. Calc. for $C_{41}H_{62}O_{12}$: C, 65.93; H, 8.37. Found: C, 65.86; H, 8.22.

ACKNOWLEDGMENTS

This work was largely supported by a research grant, AM 01255, from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service. The author thanks Dr. David Fukushima for suggesting the use of titanium tetrachloride as an anomerizing agent, and for his good advice concerning Hudson's rules.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

VARIABLE CONTENT OF 2-ACETAMIDO-2-DEOXY-D-GLUCOSE AND NEUTRAL SUGARS IN MAMMALIAN HEPARINS*

J. A. CIFONELLI AND J. KING

Departments of Pediatrics and Biochemistry, The La Rabida-University of Chicago Institute, and The Joseph P. Kennedy, Jr. Mental Retardation Research Center, University of Chicago, Chicago, Illinois 60637 (U. S. A.)

(Received May 14th, 1969; in revised form, September 6th, 1969)

ABSTRACT

A wide range in the content of the linkage-region sugar units and of 2-acetamido-2-deoxy-D-glucose was found in heparin samples from different sources. All heparin preparations examined contained 2-acetamido-2-deoxy-D-glucose, and this constituent was not altered by bleaching reagents which significantly lowered the neutral sugar and L-serine content. This indicated that the variability of the 2-acetamido-2-deoxy-D-glucose content was not due to the methods of purification. Most of the purified heparin preparations showed single compact spots of varying mobilities on electrophoresis, although unbleached beef-lung fractions showed distinct double spots.

INTRODUCTION

The occurrence of 2-acetamido-2-deoxy-D-glucose in mammalian heparin preparations has been noted in several reports[1-3] and differing proportions of this component were found in samples obtained from different tissue sources. However, no clear evidence was presented to indicate the relationship between the *N*-acetylated hexosamine units and the heparin molecule. The nature of this relationship has been established recently by the action of nitrous acid on heparins obtained from tissues of animals[4,5], whales[6], and clams[7].

The present study provides data indicating widely differing proportions of 2-acetamido-2-deoxy-D-glucose in heparin preparations from different sources. Results have been obtained which indicate that such variations are not primarily due to conditions of preparation. A comparison is made of the concentration of this constituent with the concentrations of D-galactose, D-xylose, and L-serine in the heparin fractions. No correlation was found between the contents of these components and the anticoagulant activity.

*This work was supported by Grant No. AM-05996 from the National Institute for Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service.

EXPERIMENTAL

Materials. — The various heparin samples were obtained from the following sources. Purified beef-, hog-, and sheep-mucosa heparin fractions were supplied by Dr. H. H. R. Weber, The Wilson Laboratories, Chicago, Illinois. In addition, a crude, beef-lung preparation was obtained which had no further commercial purification after an initial ethanol precipitation. Purified, beef-lung heparin preparations were obtained from Dr. L. L. Coleman, Upjohn Co., Kalamazoo, Michigan. The beef-liver heparin was a gift from Dr. K. von Berlepsch, Hoffman-La Roche, Basel, Switzerland, and the whale-tissue heparin was obtained from Dr. Z. Yosizawa.

The hog-mucosa I fraction was prepared as a reference mucopolysaccharide standard with the financial support of The National Heart Institute. The bleached, beef-lung preparation has been described in ref. 8 as fraction A, preparation 2.

Methods. — Methods for the estimation of uronic acid, total hexosamine, total sulfate, and amino acids have been described previously[9]. Electrophoresis was accomplished, after application of 0.5% solutions to Sepraphore III strips, in pyridine–formic acid buffer, pH 3.0, as described by Mathews[10]. The strips were stained with a 1% Acridine Orange solution.

Purification of heparin preparations. — All commercial samples were purified by precipitation with cetylpyridinium chloride from 1.3 to 1.5M sodium chloride solutions, as described earlier[8].

The whale heparin sample was found to contain dermatan sulfate as a contaminant, and it was purified in the following way. The crude sample (300 mg) was dissolved in 20 ml of water, and the dermatan sulfate was precipitated as the copper complex[11]. After the supernatant solution was neutralized and dialyzed, cetylpyridinium chloride was added to precipitate the polysaccharide. The heparin was recovered in the usual way[8], and applied to a 2.2×30 cm column of Dowex-1 (X-2, Cl^-) to give fractions of 105, 120, and 20 mg, respectively. The 1.5M fraction was used for the present work.

The crude, beef-lung heparin preparation was fractionated as described for whale heparin, except that fractions eluted from Dowex-1 with 1.75 and 2.0M sodium chloride were used for isolation of the sample. The electrophoretic mobility of this sample (beef-lung II) is reported in Fig. 2 and in the Tables.

The hog-skin material was purified as reported previously[12], and the product was subjected to an additional precipitation with cetylpyridinium chloride in 1.4M sodium chloride solution.

Determination of 2-acetamido-2-deoxy-D-glucose in heparin. — This was done by determination of the hexosamine content remaining after treatment of heparin with nitrous acid, as described previously for the treatment of heparitin sulfate with nitrous acid[13]. Completion of the reaction was ascertained by noting the lack of hexosamine loss when the gel filtration products were treated with nitrous acid at room temperature, as described previously[13]. After gel filtration of the products, the individual peaks were analyzed by the Elson–Morgan procedure, and the hexos-

amine values obtained were compared to the total hexosamine present in the sample used in the reaction. This direct method was found more reliable than determinations based on differences between total hexosamine and the 2,5-anhydro-D-mannose formed after treatment of heparin with nitrous acid.

Estimation of the galactose and xylose contents of heparin. — The neutral sugar components present in the linkage region of heparin were determined with a Technicon Carbohydrate Autoanalyzer by using a borate column for chromatographic separations.

Hydrolyzates (2 ml) were prepared by heating 1.0% solutions of heparin in M hydrochloric acid for 3 h at 100°. The hydrolyzates were diluted with 5 vol. of water, and Dowex-3 (CO_3^{2-}) was added in portions with stirring until a pH of approximately 2.0 was reached. After filtration through a cotton plug, L-fucose (100 μg) was added as internal standard, and the filtrate was filtered through a 1.0×5 cm column of Dowex-50 (X-8, H^+, 200–400 mesh) to remove hexosamine-containing products. The solution was concentrated at 30°, *in vacuo* to near dryness, and the process was repeated several times after additions of methanol. The residue was dissolved in water (1.0 ml), and 0.2 ml was used for the sugar autoanalyzer.

Quantitative estimations of sugars were made from the peak areas by comparison with results obtained by known amounts of standard sugars. Corrections for technical losses were made from calculations based on internal standards.

Treatment of heparin with alkali and bleaching agents. — Hog-mucosa heparin, preparation III, was used for the following experiments: (*a*) a 1% solution of heparin was mixed with an equal volume of 0.2M sodium hydroxide and heated for 2 h at 100°. After neutralization with dilute acetic acid, ethanol (3 vol.) was added to precipitate the mucopolysaccharide. (*b*) To a 2% solution of heparin (5 ml) was added M acetate buffer (0.5 ml, pH 5.0) and hydrogen peroxide (0.5 ml, 30%). After the solution had been kept for 16 h at 24°, heparin was isolated by the addition of ethanol (3 volumes). (*c*) To a 10% solution of heparin was added an equal volume of 0.3M sodium hydroxide and 30% hydrogen peroxide (0.4 volume). After being kept for 2.5 h at 24°, the mixture was warmed and kept for 0.5 h at 70°, and the heparin was precipitated as previously described in (*a*). (*d*) Heparin (200 mg) was dissolved in 0.1 M sodium hydroxide (10 ml) and an equal volume of 5% sodium hypochlorite solution was added. After the solution had been kept for 3 h at 24° in the dark, one-half was acidified to pH 4.0 and heparin precipitated with ethanol (3 volumes). The remainder was precipitated in the same way after being kept for 24 h at 24°.

The beef-lung heparin II preparation was exposed to bleaching conditions as in (*d*) above, except that the reaction proceeded for 70 h before the heparin was precipitated.

RESULTS

Previous results have shown that commercial preparations of heparin, though of comparatively high purity, generally require further purification to remove small amounts of biologically inactive products[8]. Since the presence of contaminants

introduces uncertainties in estimating the minor components of heparin, all preparations used in this study were purified further before use.

Table I gives analytical data for purified heparin fractions from different sources. It is noteworthy that all preparations show a similarity in composition, although some variation in molar ratios of total sulfate to hexosamine occur. The molar ratios of 2-amino-2-deoxygalactose to 2-amino-2-deoxyglucose, as estimated from amino acid autoanalytical data, was found to be 0.01, or less, for all the samples used except for the heparins isolated from hog skin, and from sheep and beef mucosa which showed molar ratios of 0.02 to 0.03. The optical rotation of whale heparin is significantly higher than that shown by the other heparins, in agreement with the observation of Yosizawa[6]. The anticoagulant activities fell within the range generally reported, although the value found in the present work for whale heparin is lower than that found by others[2,3]. When compared on the basis of equivalent amounts of hexosamine, the anticoagulant activity of whale heparin and those of the purified, commercial preparations obtained from beef lung and hog mucosa were found to be similar.

Electrophoresis of heparin fractions. — Electrophoretic mobilities of various heparin samples are shown in Fig. 1. The relatively discrete spots shown by most of the purified preparations suggest reasonable homogeneity with respect to the ionic charge of individual fractions. The heparin samples fall into two categories, according to whether their mobilities are similar or slower than the mobility of beef-lung heparin. The mucosa and whale heparins moved more slowly than did the preparations obtained from hog skin, beef-liver, and beef-lung. Several of the hog-mucosa samples showed elongated (Fig. 1) rather than round spots, and beef-lung fractions, which had not been commercially purified, showed the presence of fast-moving double spots (Fig. 2). Fractionation of these preparations on Dowex-1 columns gave partial separation of the 2 components, although attempts to separate completely these fractions by the usual fractionation techniques were unsuccessful. In general, correlation of mobility with sulfate content is suggested by the results reported in Table I, although hog-skin and mucosa heparins appeared to be exceptions. However, the uncertainty of the sulfate estimation does not permit a conclusive interpretation of the results. Correlation of mobility with content of 2-acetamido-2-deoxyglucose of the heparins is also observed (Table II), the heparins having the larger concentrations moving the slower. However, the mobility of hog-mucosa III preparation, which had been treated with sodium hypochlorite, was increased to approximately that of beef-lung heparin (Fig. 2), although no change in 2-acetamido-2-deoxyglucose content was found. Similar, single, fast-moving spots were observed after treatment of unbleached, beef-lung heparin fractions which originally produced double spots.

Concentrations of the components forming the protein–carbohydrate linkage region in heparin. — Table II reports the molar ratios of D-galactose, D-xylose, L-serine, and 2-acetamido-2-deoxy-D-glucose to total hexosamine in different heparin fractions; large variations in the content of all minor components of heparins are observed.

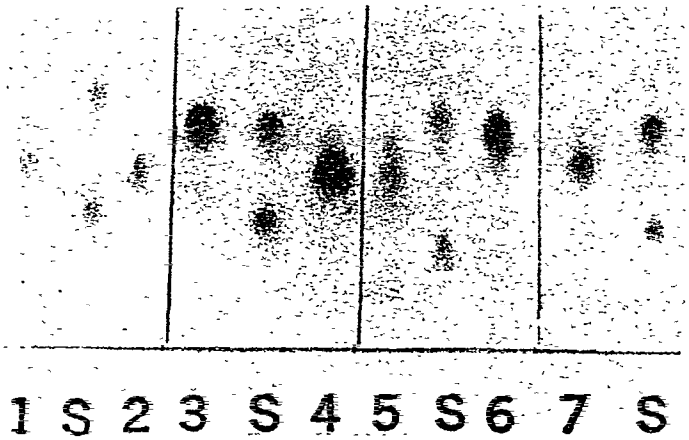

Fig. 1. Electrophoretic mobility of various heparin fractions. The heparin preparations were obtained from the following sources: whale tissues, 1.25M fraction (1); whale, 1.5M fraction (2); beef liver (3); beef mucosa (4); hog mucosa I (5); hog skin (6); sheep mucosa (7); and a standard mixture containing beef-lung heparin I (bleached) and heparitin sulfate (S). The conditions used are described in the Experimental Section.

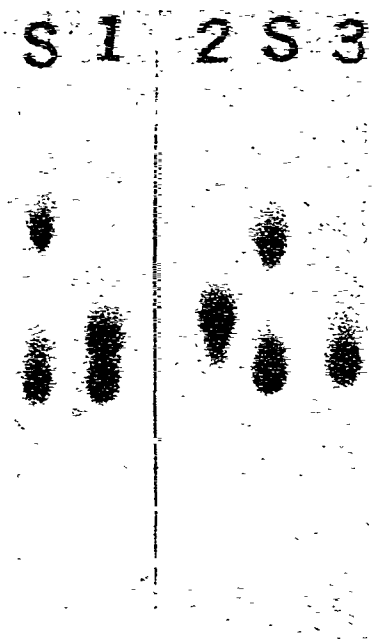

Fig. 2. Electrophoretic mobility of the following heparin preparations: beef-lung heparin II (unbleached) isolated from Dowex-1 by 1.75M sodium chloride (1); hog-mucosa fraction III (2); hog-mucosa fraction III after treatment with sodium hypochlorite for 24 h (3); and a standard mixture of heparitin sulfate and beef-lung heparin I (S).

The mucosal-tissue preparations had relatively high concentrations of neutral sugars and L-serine, indicating that the commercial isolation and purification procedures have had little effect on the residues in the linkage region. In contrast, the

TABLE I

COMPOSITION OF HEPARIN PREPARATIONS ISOLATED FROM DIFFERENT SOURCES[a]

Source	*Conditions of preparation*	*Uronic acid (%)*	*Hexosamine (%)*	*Sulfate/hexosamine (ratio)*	$[\alpha]_D^{24}$ *(degrees)*[b]	*Anticoagulant activity*	
						I.U./mg (ratio)	*I.U./hexosamine (μmole) (ratio)*
Hog mucosa I[c]	unbleached	38.7	23.8	2.33	+52	180	135
Hog skin	unbleached	35.4	24.8	2.15	+42		
Beef lung I[d]	bleached	39.0	23.2	2.46	+43	180	138
Beef mucosa	unbleached	49.2	24.2	2.40		152	113
Beef liver		48.5	24.8		+45	149	108
Sheep mucosa	unbleached	29.4	20.4	2.01			
Whale tissues		42.5	22.5	1.80	+67	176	139
Beef lung II	unbleached	47.6	25.1			161	115

[a]The results are not corrected for moisture content. [b]In water. [c]Prepared as a mucopolysaccharide reference standard. [d]Fraction A, preparation 2 as described in ref. 8.

TABLE II

CONTENTS OF MINOR COMPONENTS PRESENT IN HEPARIN PREPARATIONS[a]

Source	*Conditions of preparation*	D-*Galactose*[b]	D-*Xylose*[b]	L-*Serine*[c]	*2-Acetamido-2-deoxy-*D-*glucose*[d]
Hog mucosa I	Unbleached	0.029	0.022	0.026	0.11
Hog skin	Unbleached	0.003	0.003	traces	0.07
Beef lung I	Bleached	0.007	0.003	traces	0.02
Beef mucosa	Unbleached	0.050	0.035	0.044	0.14
Beef liver		traces	traces	traces	0.05
Sheep mucosa	Unbleached	0.040	0.020	0.016	0.14
Whale tissues		0.011	0.004	traces	0.31
Beef lung II	Unbleached	0.021	0.010	0.005	0.07

[a]Based on molar ratios to total hexosamine of the preparations. [b]Estimated from results obtained with the Technicon Carbohydrate Autoanalyzer. [c]Obtained from amino acid autoanalyses. [d]This value represents the hexosamine content remaining after treatment of heparin with nitrous acid.

remaining heparin fractions are deficient in linkage region components, although the procedure used for the hog-skin preparation did not affect these components in other heparin samples[14].

The higher content of L-serine relative to D-xylose in both the hog- and sheep-mucosa fractions could not be explained on the basis of extraneous peptide material, since only traces of amino acids other than L-serine were present. This discrepancy appears to be due to low values in the determination of D-xylose, since under the conditions of hydrolysis standard xylose was obtained in 70–80% yields. The values obtained for D-galactose lower than the theoretical content have previously been shown to be due to incomplete hydrolysis[14]. However, hydrolysis of the beef-liver preparations with N acid for 12 h, conditions designed to hydrolyze any (glucosyluronic)galactose residues present in the heparin fraction, failed to produce more than traces of free D-galactose, thus indicating that this sample contained practically no component involved in the protein–carbohydrate linkage.

Bleaching and other procedures used commercially for the final purification of heparin have been shown previously to destroy the L-serine residue almost completely[14]. The content of neutral sugars was decreased approximately by 50% in beef-lung heparin by commercial bleaching, as shown in Table III, and approximately 25% in hog-mucosa material. In contrast, the L-serine residues were destroyed almost completely.

In the present study, the effects on the linkage-region components produced by alkaline conditions alone and with hydrogen peroxide or sodium hypochlorite are shown in Table III. Neither hydrogen peroxide nor heating with 0.1M sodium hydroxide reduced the content of the linkage-region units. Treatment of hog-mucosa heparin with sodium hypochlorite, however, caused progressive loss of the neutral sugars and L-serine, although the content of the neutral sugar constituents after treatment for 24 h was approximately as high as that of unbleached beef-lung heparin

TABLE III

LINKAGE REGION COMPONENTS OF TREATED HEPARINS[a]

Source	*Conditions of treatment*	D-*Galactose*	D-*Xylose*	L-*Serine*	*2-Acetamido-2-deoxy*-D-*glucose*
Hog mucosa II	unbleached	0.034	0.025	0.028	0.14
	bleached	0.025	0.021	0.003	0.13
Beef lung I	unbleached	0.015	0.008	0.010	0.02
	bleached	0.007	0.003	traces	0.02
Hog mucosa III	unbleached	0.040	0.028	0.032	0.13
	0.1M NaOH, 100°	0.041	0.026	0.034	
	H_2O_2, pH 6	0.045	0.025		
	H_2O_2, 0.125M NaOH	0.037	0.017	0.023	
	NaOCl, pH 11, 3 h	0.022	0.008	traces	
	NaOCl, pH 11, 24 h	0.017	0.005		0.14
Beef lung II	unbleached	0.021	0.010	0.005	0.07
	NaOCl, pH 11, 70 h	0.005	0.001		0.05

[a]Given as molar ratios to total hexosamine.

preparations. Treatment of beef-lung heparin II with sodium hypochlorite for 70 h gave a product nearly free of D-xylose and L-serine and having little hydrolyzable D-galactose (Fig. 3).

N-Acetylhexosamine content of heparin preparation. — The determinations of 2-acetamido-2-deoxy-D-glucose were based on the amount of 2-amino-2-deoxy-D-

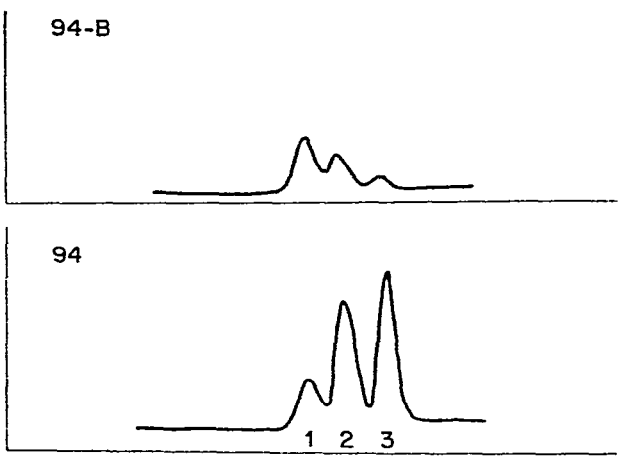

Fig. 3. Neutral sugar content of beef-lung heparin II before (94) and after bleaching with sodium hypochlorite (94-B). The quantities shown were obtained by analysis of the hydrolyzate of 2 samples of polysaccharide (4 mg), corrected to similar hexosamine content: L-fucose (10 μg), added as internal standard (1); D-galactose (2); and D-xylose (3). The patterns were obtained with a Technicon Carbohydrate Autoanalyzer using a Dowex-1-borate column.

glucose remaining after treatment of the heparin preparations with nitrous acid, as described elsewhere[13]. Table II illustrates the wide range of values obtained with heparin samples used in this study. It is evident that all fractions contain 2-acetamido-2-deoxy-D-glucose, although, in beef-lung heparin I, this sugar represents approximately only 2% of the total hexosamine content. In contrast, unbleached beef-lung heparin II was found to have 7%, and the mucosa heparins more than 10% of their hexosamine content as 2-acetamido-2-deoxy-D-glucose. The acetyl groups in the products resulting from the nitrous acid degradation were identified by g.l.c.[1] as well as by i.r. spectroscopy which showed absorption maxima at 1650 and 1560 cm^{-1}, characteristic of the acetamido group.

In addition to the samples listed in Table II, two other beef-lung preparations, both unbleached, were examined. One showed a content of approximately 2%, and the other 5% of 2-acetamido-2-deoxy-D-glucose. Also, six additional hog-mucosa samples, including bleached and unbleached fractions, had 10–15% hexosamine unreactive after treatment of the samples with nitrous acid. Other fractions showing intermediate concentrations of 2-acetamido-2-deoxy-D-glucose include beef-liver and hog-skin heparins. The latter material had previously been reported[12] to contain nearly twice the amount of unreactive hexosamine as the more highly purified preparation shown in Table II. The earlier result was obtained by calculating the difference between the total hexosamine value and the 2-deoxy-2-sulfoamino-D-glucose value, as determined by the nitrous acid reaction[16]; because this difference was small, the earlier result was naturally more uncertain than the estimation of hexosamine recovered after the degradation reaction. The whale-tissue heparin showed a content of 2-acetamido-2-deoxy-D-glucose double that of the mucosa-tissue substances, in agreement with the results of Yosizawa *et al*[2,6].

DISCUSSION

Numerous illustrations of microheterogeneity in acid glycosaminoglycans (mucopolysaccharides) have been made recently. In addition to the numerous reports concerning variability of sulfate content and distribution within the molecule, several examples extending heterogeneity to differences of component type have been noted. These include minor amounts of 2-amino-2-deoxy-D-galactose in cartilage keratan sulfate[17,18], and occurrence of D-glucuronic acid units in dermatan sulfate[19] and of L-iduronic acid units in heparin[20,21].

The occurrence of varying amounts of 2-acetamido-2-deoxy-D-glucose in heparin preparations[1–3,22] is another example of component type heterogeneity. Definitive evidence that such residues are integral constituents of heparin and are not contributed by related types of contaminating mucopolysaccharides appears not to have been reported. Therefore, the following data are presented in support of the presence of 2-acetamido-2-deoxy-D-glucose units in heparin: (*1*) no mammalian acid mucopolysaccharide other than heparin has been shown to precipitate with cetylpyridinium chloride in 1.5M sodium chloride; (*2*) products obtained by treatment of

heparin with nitrous acid give patterns of gel filtration with Sephadex G-50 which differ significantly from those obtained with the degradation products of heparitin (heparan) sulfate; (3) 2-amino-2-deoxy-D-galactose-containing mucopolysaccharides, as well as hyaluronic acid and keratan sulfate, do not react with nitrous acid, and are readily detected after gel filtration of the products resulting from the treatment of heparin, since only the unreactive substances emerge in the void volume.

The present study illustrates the wide range of concentrations of 2-acetamido-2-deoxy-D-glucose occurring in mammalian heparins, the ratio of this component to total hexosamine varying from approximately 2 to 31%. Quantitative determination of the D-galactose, D-xylose, and L-serine content of these substances revealed that, of the samples examined, only that isolated from beef-mucosa tissue has sufficient residues to form complete oligosaccharide linkage-chains, on the basis of the molecular-weight values generally given[23,24]. Analysis of the fractions obtained from hog- and sheep-mucosa tissues gave results indicating that most of the molecules contained complete linkage-chains.

The degradation of the linkage regions of the remaining preparations suggests that the relatively low 2-acetamido-2-deoxy-D-glucose content found in some of these preparations may be due to the conditions of preparation. That this is unlikely, however, is suggested by the following observations: heparin from whale tissues contained approximately twice as much *N*-acetylated hexosamine units as the mucosa-tissue preparations, although the content of linkage-region components was considerably reduced. Also, the hog-skin fraction, prepared under conditions shown not to affect the L-serine content[9], contained low proportions of neutral sugars and L-serine and only one-half or less as much 2-acetamido-2-deoxy-D-glucose as did the mucosa-tissue heparins. Furthermore, treatment of heparin with sodium hypochlorite for up to 70 h, as illustrated in Table III, did not decrease the 2-acetamido-2-deoxy-D-glucose content, although most of the D-xylose and D-galactose units were destroyed.

The treatment of purified commercial heparin preparations with 0.1N alkali resulted in insignificant losses of L-serine or neutral sugars. However, treatment with hydrogen peroxide, as used in the bleaching of heparin, caused minor losses of both L-serine and D-xylose, but little or no loss of D-galactose. In contrast, sodium hypochlorite destroyed much more of these components (Fig. 3), as might be expected from previous work[25]. Despite these effects from the bleaching steps, preparations from ox-lung or hog-mucosa tissues, which were unbleached, contained 2-acetamido-2-deoxy-D-glucose concentrations similar to those of the bleached materials, thus strengthening the suggestion that the conditions normally used for purification of heparin are unlikely to be responsible for the variations of the *N*-acetyl component.

Of particular interest has been an appraisal of the chemical relationship between heparin and heparitin sulfate. The presence of 2-acetamido-2-deoxy-D-glucose in variable and often relatively high concentrations in heparin fractions, as well as the variable proportions of this residue in heparitin sulfates, add support to previous suggestions of the possible derivation of one polysaccharide from the other. The finding by Silbert[26] that *N*-sulfation accompanies *N*-deacetylation of an

"*N*-acetylated glycosaminoglycan related to heparin" conforms with the presence of a variable content of 2-acetamido-2-deoxy-D-glucose in heparin preparations.

The results of this study further indicate a lack of correlation between anticoagulant activity of a heparin preparation and its content of the minor components. Similar biological activity has been found in preparations having a content of 2-acetamido-2-deoxy-D-glucose varying from approximately 2 to 31% of the total hexosamine, and in substances having a content of linkage-region constituents ranging from an undetectable amount to a theoretical content.

The highly variable anticoagulant activities of heparin preparations frequently reported in the literature and the general lack of a chemical basis for comparing these results indicate the desirability of relating the biological activity of heparin samples to a more reliable quantitative indicator than weight alone. Since hexosamine is readily determined in all mammalian-heparin preparations, it is suggested that this constituent be used for comparison of biological activities, as illustrated in Table I.

The separation of heparin preparations into fractions having different electrophoretic mobilities has been noted previously[15,27,28]. Most of the purified heparin samples used in this study showed single, relatively compact spots on electrophoresis, although double spots were observed for some of the beef-lung preparations. After treatment with bleaching agents, however, both the slower moving and double spotted fractions produced single spots having increased mobility, thus indicating that the mobility of heparin fractions may be influenced by the conditions of preparation.

ACKNOWLEDGMENTS

We wish to thank Dr. H. H. R. Weber, Wilson Laboratories and Dr. L. L. Coleman, The Upjohn Co. for supplying numerous hog-mucosa and cattle-lung heparin samples, Dr. K. von Berlepsch, Hoffman-La Roche for a gift of cattle-liver heparin, and Dr. A. Dorfman for making available the whale heparin. We are grateful to Dr. H. H. R. Weber for providing anticoagulant values and Mrs. L. Roth for technical assistance.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

APPLICATION OF THE WITTIG AND KOLBE REACTIONS TO URIDINE DERIVATIVES: ATTEMPTED SYNTHESIS OF THE NUCLEOTIDE ANALOGUE, 5'-CARBOXYMETHYL-5'-DEOXYURIDINE

P. HOWGATE, A. S. JONES, AND J. R. TITTENSOR
Chemistry Department, University of Birmingham, Birmingham 15 (Great Britain)

(Received August 6th, 1969; in revised form, September 11th, 1969)

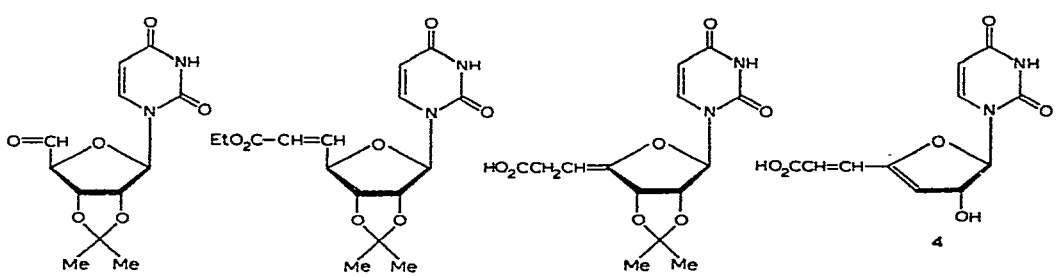
ABSTRACT

The Wittig reaction between (ethoxycarbonylmethylene)triphenylphosphorane and 1-(2,3-*O*-isopropylidene-β-D-*ribo*pentodialdo-1,4-furanosyl)uracil gave 1-(5,6-dideoxy-2,3-*O*-isopropylidene-β-D-*erythro*-hept-4-enofuranosyluronic acid)uracil and 1-(3,5,6-trideoxy-β-D-*glycero*-hept-3,5-dienofuranosyluronic acid)uracil. An anodic (Kolbe) coupling reaction between methyl hydrogen succinate and 1-(β-D-ribofuranosyluronic acid)uracil gave, in low yield, a mixture of 5'-carboxymethyl-5'-deoxyuridine [1-(5,6-dideoxy-β-D-*ribo*-heptofuranosyluronic acid)uracil] with its α-L-*lyxo* isomer, as their methyl esters. 5-Methoxycarbonylethyluracil was also formed.

INTRODUCTION AND DISCUSSION

Analogues of nucleotides in which the phosphate group has been replaced by a phosphorothioate[1] or by a phosphonate[2-4] group have been synthesised, and some have interesting biochemical properties. We thought it of interest, therefore, to synthesise a nucleotide analogue in which the phosphate group of the natural nucleotide is replaced by a carboxymethyl group, *e.g.*, 5'-carboxymethyl-5'-deoxyuridine (**7a**).

To achieve this, 1-(2,3-*O*-isopropylidene-β-D-*ribo*-pentodialdo-1,4-furanosyl)-uracil (**1**) was obtained by the oxidation of 2,3-*O*-isopropylideneuridine with the Pfitzner–Moffatt reagent. When compound **1** was treated with (ethoxycarbonyl-



1 2 3 4

methylene)triphenylphosphorane in an attempt to obtain the unsaturated ester **2**, no reaction occurred. However, when the phosphorane was generated *in situ* by the action of sodium ethoxide on (ethoxycarbonylmethyl)triphenylphosphonium bromide, a rapid reaction occurred, and five products were detected. Two of these (**3** and **4**) were acids, two were esters that gave **3** and **4**, respectively, on alkaline hydrolysis, and the fifth was uracil.

Compound **3** had a u.v. absorption spectrum typical of a uridine derivative, and mass-spectral analysis indicated a molecular ion of *m/e* 324. The n.m.r. spectrum (methyl sulphoxide-d_6) was in accord with the proposed structure as follows: (δ values) H-1′ 5.80; H-2′ 5.10–4.89 (d); H-3′ 5.33 (d) ($J_{2',3'}$ 6 Hz); H-5′ 5.1–4.9 (t); H-6′ 2.76 (d); H-6 7.57 (d); H-5 5.53 (d) ($J_{5,6}$ 8.5 Hz); isopropylidene methyl protons, 1.34 and 1.26. Irradiation at 2.76 brought about the collapse of the H-5′ triplet at δ 5.1–4.9 to a singlet, and irradiation at 4.9 caused the collapse of the H-6′ doublet at δ 2.76 to a singlet. These results show that the compound was 1-(5,6-dideoxy-2,3-*O*-isopropylidene-β-D-*erythro*-hept-4-enofuranosyluronic acid)uracil (**3**).

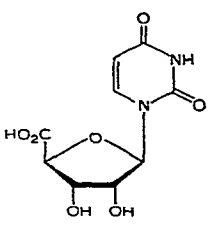
Compound **4** had a higher extinction coefficient and λ_{max} at longer wavelength than a simple uridine derivative, thus indicating the presence of an additional chromophore. The n.m.r. spectrum indicated that the compound was 1-(3,5,6-trideoxy-β-D-*glycero*-hept-3,5-dienofuranosyluronic acid)uracil (**4**); δ values: H-1′ 5.44 (d); H-2′ 4.82 (m); HO-2′ ~3.54; H-3′ 6.12 (d); H-5′ 6.72 (d); H-6′ 6.06 (d) ($J_{5,6}$ 16 Hz); H-6 7.15, H-5 5.59 ($J_{5,6}$ 8 Hz).

The compound did not contain a *cis*-diol function, as shown by the fact that the presence of borate did not increase its electrophoretic mobility at pH 9. Both **3** and **4** decomposed in a few days at room temperature in water to give uracil.

It appeared that **3** had been produced by rearrangement and then hydrolysis of the expected product **2**, under the basic conditions of the reaction. Compound **4** could have arisen by elimination of the isopropylidene group of **2** followed by hydrolysis. Alternatively, elimination of the isopropylidene group from the aldehyde **1** could have led to an αβ-unsaturated aldehyde which could react with the Wittig reagent to give **4**, after hydrolysis. In this connection, it was noted that treatment of **1** with sodium ethoxide under the conditions of the Wittig reaction rapidly gave uracil. Although no αβ-unsaturated aldehyde was detected, this result does indicate that **1** undergoes a ready elimination reaction.

As an alternative route to the desired compound, the anodic (Kolbe) coupling reaction between (β-D-ribofuranosyluronic acid)uracil (**5**, "uridine-5′-carboxylic acid") and methyl hydrogen succinate was investigated. When the electrolysis was carried out in the presence of sodium methoxide in methanol, uracil, the methyl ester of **5**, and two other products were formed. One of these was also formed when a mixture of uracil and methyl hydrogen succinate was subjected to the Kolbe reaction and was shown by u.v. and n.m.r. spectroscopy to be 5-methoxycarbonylethyluracil (**6**). The other product (11%) was considered to be a mixture of the required 5′-methoxycarbonyl-5′-deoxyuridine [1-(5,6-dideoxy-β-D-*ribo*-heptofuranosyluronic acid)uracil] with its α-L-*lyxo* isomer (as their methyl esters), on the basis of its ele-

mental analysis, behaviour on borate electrophoresis, u.v. absorption spectrum, and n.m.r. spectrum. The last of these showed a complex pattern which could be interpreted in accordance with this assignment.



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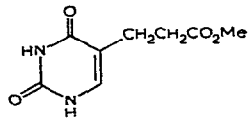

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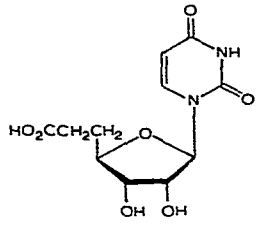
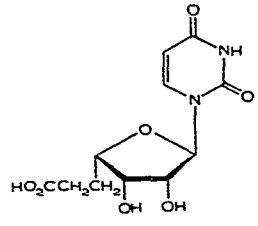


7a



7b

The C-6′ protons appeared as two partially overlapping triplets (δ 2.36, J ~6 Hz; 2.52, J 6.5 Hz), and H-4′ appeared as a triplet (δ 4.02, J 6 Hz) superimposed on a broad peak. Similar systems, namely, methyl-5,6-dideoxy-2,3-*O*-isopropylidene-β-D-allofuranoside and the corresponding β-D-*gulo* compound, have been studied by other workers[5,6]. In these cases, when H-4 was *trans* to H-3, $J_{3,4}$ was less than 0.5 Hz, and the signal appeared as a triplet (due to splitting by the protons at C-5). When H-4 was *cis* to H-3, $J_{3,4}$ was *ca.* 3 Hz, and so the triplet formed by coupling with the C-5 protons was further split by H-3 into a triplet of doublets. Also, in the β-D-allofuranoside, the protons on C-6 appeared at higher field than those of the β-D-gulofuranoside. The signals obtained for H-4′ and the protons on C-6′ of **7** indicated, therefore, that the major component of the mixture was **7a**. Furthermore, any long-range shielding by the ester carbonyl group affecting the H-5 and H-6 aromatic protons would be expected to be greater in the case of **7a**. In the spectrum of **7**, signals due to H-5 and H-6 were at δ 7.53 and 5.58, and at 7.65 and 5.69. The former pair were ascribed to **7a** and the latter to **7b**. It appeared that the two isomers were present in the ratio of 2:1. Although they could be separated by t.l.c., separation on a preparative scale was not achieved.

Thus, although the required compound had been formed, the poor yield and difficulty in separating it from its epimer rendered the synthesis unsatisfactory. The results did show, however, that the carboxyl group at the 5′ position of a nucleoside derivative will undergo a Kolbe reaction with a suitable carboxylic acid. It was further shown that 1-(β-D-ribofuranosyluronic acid)uracil did not undergo any self-coupling under the conditions of the Kolbe reaction.

EXPERIMENTAL

1-(2-3-O-Isopropylidene-β-D-ribo-pentodialdo-1,4-furanosyl)uracil ("*2′,3′-O-isopropylideneuridine-5′-aldehyde*")[7,8]. — 2′,3′-*O*-Isopropylideneuridine (1.1 g) was dissolved in a mixture of dry methyl sulphoxide (6 ml), benzene (3 ml), pyridine (0.32 ml), and trifluoroacetic acid (0.16 ml). Dicyclohexylcarbodiimide (2.5 g) in dry benzene (2.5 ml) was added. The mixture became warm, and *N,N′*-dicyclo-

hexylurea was deposited after 5 min. After 24 h at room temperature, water (20 ml) was added, the precipitate of *N,N'*-dicyclohexylurea was filtered off, washed with water, and then with ethanol, and the combined filtrate and washings were concentrated *in vacuo* to a syrup. Paper chromatography in butyl alcohol–ethanol–water (4:1:5) showed that all of the starting material (R_F 0.70) had reacted, and that one major product (R_F 0.86) had been formed. A small proportion of uracil (R_F 0.45) and an unidentified product (R_F 0.54) were also present. The major product gave a positive reaction with aldehyde-specific sprays.

As much methyl sulphoxide as possible was removed from the syrup by prolonged vacuum distillation (40–60°, 0.03–0.01 mmHg), and the residue was dissolved in acetone, silica gel (Fisons MFC; 5 g) was added, and the mixture was concentrated to dryness. The yellow powder so obtained was applied to the top of a column (34 × 2 cm) of the same grade of silica gel, and the column eluted with acetone–chloroform (1:9). The required product, free from uracil and the unidentified product, was eluted in 300 ml of eluant. This solution was evaporated to dryness, and the residue was extracted with ether in attempts to remove methyl sulphoxide which was still present. The residue was then dissolved in acetone and evaporated to dryness to give 1-(2,3-*O*-isopropylidene-β-D-*ribo*-pentodialdo-1,4-furanosyl)uracil (0.7 g) as a white powder. The n.m.r. spectrum showed this to be the correct compound, but that it still contained methyl sulphoxide; the elemental analysis agreed with this (Found: C, 48.7; H, 5.56; N, 8.3. $C_{12}H_{14}N_2O_6 \cdot \frac{1}{2}(Me_2SO)$ calc.: C, 48.6; H, 5.29; N, 8.7%).

Wittig reaction using (ethoxycarbonylmethyl)triphenylphosphonium bromide and 1-(2,3-O-isopropylidene-β-D-ribo-pentodialdo-1,4-furanosyl)uracil. — (Ethoxycarbonylmethyl)triphenylphosphonium bromide (7.2 g) was dissolved in dry ethanol (100 ml), a solution of sodium ethoxide (2.3 g) in dry ethanol (30 ml) was added, followed by a solution of 1-(2,3-*O*-isopropylidene-β-D-*ribo*pentodialdo-1,4-furanosyl)uracil (4 g) in dry ethanol (50 ml), and the mixture was boiled under reflux in an atmosphere of nitrogen. After 3 min, a precipitate had formed, and examination by t.l.c. (silica gel; butyl alcohol–ethanol–water, 4:1:5) showed that no starting material remained. The reaction mixture contained uracil, two acidic components (**3**, R_F 0.35; **4**, R_F 0.24), and two neutral components. The precipitate, which contained only the acidic components, was centrifuged off, and to the supernatant and washings (300 ml), conc. ammonia (sp. gr. 0.88, 100 ml) was added, and the mixture was allowed to stand for 5 h at room temperature and then for 24 h at 0°. This process converted the two neutral components into the acidic components **3** and **4**. The solution was evaporated to dryness, ethanol (60 ml) was added, and the resulting solid (which contained uracil, **3** and **4**) was combined with the precipitate from the reaction mixture and dissolved in water (20 ml). After standing at 0°, a precipitate of triphenylphosphine oxide separated; this was filtered off, and the mixture in the filtrate was fractionated on a column (90 × 2.8 cm) of Merck Kieselgel 7734 with butyl alcohol–ethanol–water (4:1:5). This separated uracil, **3**, and **4**.

1-(3,5,6-Trideoxy-β-D-*glycero*-hept-3,5-dienofuranosyluronic acid)uracil (**4**,

1.1 g) was obtained as the sodium salt dihydrate; λ_{max} 268.5 nm, ε_{max} 18.7×10^3 at pH 2; λ_{max} 266 nm at pH 11 (Found: C, 41.0; H, 4.54. $C_{11}H_9N_2NaO_6 \cdot 2H_2O$ calc.: C, 40.7; H, 4.32%).

Compound **3** was obtained as a white, hygroscopic powder (0.52 g) which still contained methyl sulphoxide that could not be removed by normal methods. It was removed, however, by reduction to methyl sulphide during an attempted hydrogenation of the compound. 1-(5,6-Dideoxy-2,3-*O*-isopropylidene-*β*-D-*erythro*-hept-4-enofuranosyluronic acid)uracil (**3**) was isolated as the sodium salt, m.p. 208° (dec.); λ_{max} 261 nm, ε_{max} 10.7×10^3 at pH 2; no change in λ_{max} occurred, but ε_{max} was diminished when the pH was raised to 11. (Found: C, 44.2; H, 5.0; N, 7.3. $C_{14}H_{15}N_2NaO_7 \cdot 2H_2O$ calc.: C, 44.0; H, 5.0; N, 7.3%).

Anodic coupling (Kolbe) reaction of 1-(β-D-ribofuranuronosyl)uracil with methyl hydrogen succinate. — The apparatus described by Weedon[9] was used. 1-(*β*-D-Ribofuranosyluronic acid)uracil[10] (200 mg, 0.8 mmole) and methyl hydrogen succinate (3 g, 22.7 mmoles) were dissolved in methanol (12.5 ml) containing sodium methoxide (25 mg). The cell was cooled to −10°, and a current of 0.9–0.95 amp (36 volts d.c. supply; electrodes, 15-mm square, 2–3 mm apart) was passed for 40 min with reversing as the anode became coated with carbon (the pH increased from 5.6 to 6.4). Eleven such reaction mixtures were combined and concentrated to a dark green syrup. Water (100 ml) was added, and methyl adipate was removed by extraction with ether (5 × 100 ml). The aqueous layer was concentrated to 15 ml, and the mixture fractionated by counter-current distribution in ethyl acetate–water in a 120-tube apparatus. After 300 transfers, the product [5-methoxycarbonylethyluracil (37 mg), m.p. 213°] with the highest R_F value, was isolated (Found: C, 47.9; H, 5.3; N, 14.3. $C_8H_{10}N_2O_4$ calc.: C, 48.5; H, 5.1; N, 14.2%). It had λ_{max} 265 nm, ε_{max} 8.3×10^3 at pH 2; 290 nm at pH 12. This compound was also obtained when the electrolysis of uracil was carried out in the presence of methyl hydrogen succinate.

After a further 1,000 transfers, component **7** (10 mg, as the methyl esters) was obtained in crystalline form, m.p. 155°; λ_{max} 262 nm, ε_{max} 10.0×10^3 at pH 2. (Found: C, 47.7; H, 5.3; N, 9.1. $C_{12}H_{16}N_2O_7$ calc.: C, 48.0; H, 5.3; N, 9.3%). The total yield was 11%, but only a small amount was obtained crystalline, because it very slowly decomposed to give uracil; this phenomenon was repeatedly observed.

Paper chromatography of the original reaction mixture showed that, in addition to the compounds isolated, there was present a component which had R_F values identical with those of an authentic sample of the methyl ester of the starting material.

ACKNOWLEDGMENTS

The authors thank Professor M. Stacey for his interest, Dr. J. M. Pryce, Dr. G. R. Bedford, and Mr. D. Greatbanks of I.C.I. Ltd., Pharmaceuticals Division, for recording and interpretation of n.m.r. spectra, and the British Empire Cancer Campaign for financial assistance.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

COMPLEX FORMATION BETWEEN AQUEOUS ZINC CHLORIDE AND CELLULOSE-RELATED D-GLUCOPYRANOSIDES*

NORMAN J. RICHARDS AND DALE G. WILLIAMS
The Institute of Paper Chemistry, Appleton, Wisconsin 54911 (U. S. A.)

(Received July 7, 1969; in revised form, September 15th, 1969)

ABSTRACT

Measurements of n.m.r. chemical shifts, and the method of continuous variations by using measurements of optical rotation, indicate that zinc chloride forms complexes with vicinal hydroxyl groups of several D-glucopyranosides. I.r. and u.v. spectra of aqueous mixtures of zinc chloride and methyl β-D-glucopyranoside illustrate that the concentration and stability of this complex increases as the concentration of zinc chloride is increased. The effect of concentration of zinc chloride on the complexing with methyl β-D-glucopyranoside parallels the swelling and loss of crystallinity of cellulose, indicating that the swelling mechanism for cellulose in aqueous zinc chloride solutions depends on the formation of a complex with the vicinal hydroxyl groups on C-2 and C-3 of the D-glucopyranoside repeating unit.

INTRODUCTION

Complexes between salts of sodium, lithium, barium, calcium, strontium, and iron, and molecules of carbohydrates, are known to exist[1,2]. Since zinc chloride is similar to several of the salts for which complexes with carbohydrates are known, it seemed likely that zinc chloride would form complexes with carbohydrates. Any formation of a complex would probably be concentration-dependent, because of the marked changes of ionic distribution[3,4] with concentration in the aqueous zinc chloride system. With these facts in mind, the present study sought to: (1), determine whether or not a complex exists between zinc chloride and carbohydrates in water; (2), determine the effect of concentration of zinc chloride on formation of a complex; (3), locate the points of complexing on the D-glucopyranoside ring; and (4), relate the findings of this study to the known effects of zinc chloride on cellulose.

RESULTS AND DISCUSSION

Formation of a complex between zinc chloride and methyl β-D-glucopyranoside. — Methyl β-D-glucopyranoside was chosen as representative of the carbohydrates that

*A portion of a thesis submitted by Norman J. Richards in partial fulfillment of the requirements of The Institute of Paper Chemistry for the Ph. D. degree from Lawrence University, Appleton, Wisc., U. S. A., Jan., 1969.

would be expected to form a complex with zinc chloride. The existence of such a complex was demonstrated polarimetrically by the method of continuous variations, by n.m.r. chemical-shift measurements, and by u.v. absorbance measurements. These methods all reflect a change in the electronic distribution of the reacting species, indicating formation of a complex between them.

The values for the optical rotation of methyl β-D-glucopyranoside in dilute zinc chloride solutions (<3M) show maximum deviations of 0.60 ±0.08 and 0.86 ±0.15 degrees from expected values at 546 and 435.8 nm, respectively, in an equimolar solution of the salt and the carbohydrate. This deviation is indicative of a change in the electronic distribution about the asymmetric carbon atoms of the sugar.

The n.m.r. spectrum of an equimolar solution (M in D_2O) of methyl β-D-glucopyranoside and zinc chloride, compared with the spectrum without zinc chloride, exhibits chemical shift changes ($\Delta\nu$) of 2.05 ±0.32 and 1.85 ±0.32 Hz for the anomeric proton (H-1) and the remaining ring protons (H-2,3,4,5), respectively. This observation also reflects a change in the electronic distribution about the ring protons.

With a 10-cm cell, methyl β-D-glucopyranoside exhibits a weak general absorption in the u.v. region (220–340 nm) with no distinguishable peaks; zinc chloride has an absorption maximum at 257 nm. The observed absorbance of an equimolar solution (M) of zinc chloride and methyl β-D-glucopyranoside at 257 nm is 0.06 absorbance unit less than the value calculated from the pure component solutions (1.562). This difference indicates an electronic charge-transfer between the reacting species.

Effect of concentration of zinc chloride on formation of a complex. — The law of mass action predicts that the concentration of the complex would increase as the concentration of zinc chloride is increased. This would be reflected by a change in absorbance in the absorption spectrum at a constant frequency. In addition, the stability of the complex would also be expected to increase, because of the closer proximity of the reacting species resulting from the decrease of the amount of water available to hydrate the ions in solution. This is primarily an environmental effect on the zinc ions, which would cause a shift in the frequencies of the absorption spectra of the reacting species. The term stability describes the bonding between reacting species; the more stable the complex, the stronger the bond between the reacting species. This is reflected by an increase in the equilibrium (stability) constant.

The method of attenuated total reflectance (a.t.r.) was used to obtain i.r. spectra of methyl β-D-glucopyranoside in aqueous solutions of zinc chloride (Table I).

The a.t.r. spectrum of methyl β-D-glucopyranoside in 3.0M zinc chloride is essentially the same as that of the control without zinc chloride. Above 3.0M there are increasing shifts of frequency in the absorption peaks in the range 1100–950 cm^{-1}, and at concentrations greater than 8.0M a weak, broad absorption maximum appears at 870 cm^{-1}. The new absorption maximum disappears with dilution below 8.0M zinc chloride indicating an easily reversible phenomenon. Also, above 7.0M zinc chloride, the absorption at 1440 and 1390 cm^{-1} increases relative to that of the control having no zinc chloride.

There are no observed changes in the optical rotations or the n.m.r. spectra

TABLE I

SPECTRA OF METHYL β-D-GLUCOPYRANOSIDE IN VARIOUS AQUEOUS ZINC CHLORIDE SOLUTIONS

Zinc chloride, molarity	*Frequencies of absorption maxima, cm⁻¹*					
0.0	1440	1390	1075	1031	993	[a]
3.0	1440	1390	1073	1030	990	[a]
5.0	1440	1390	1072	1020	987	[a]
6.0	1440	1390	1072	1021	990	[a]
7.0	1440	1390	1069	1015	990	[a]
8.0	1440	1390	1069	1015	985	870
9.0	1440	1390	1066	1013	986	870
9.5	1440	1390	1068	1010	987	870
10.0	1440	1390	1069	1010	985	870
11.5	1440	1390	1069	1010	985	870

[a]Not determined.

of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside and hexa-*O*-methyl-*myo*-inositol in zinc chloride solutions below 3.0M in the salt. However, at 9.5M salt the a.t.r. spectra of these two compounds exhibit frequency shifts in the absorption peaks representing the carbon–oxygen bonds, and new absorption maxima appear in the range of 910–835 cm^{-1}. This observation indicates that the methoxyl groups do not block formation of a complex in concentrated zinc chloride solutions. Complexing is probably achieved through zinc species that are insufficiently hydrated because of their high concentration, as discussed below for the cellulose case.

The i.r. region in which the new absorption maxima are observed is sensitive to stereochemical changes[5]. Therefore, the appearance of new absorption maxima is attributed to alterations, caused by complexing in the stereochemical properties of the sugar. An increase in the concentration of the complex may account for the new absorption maximum at 870 cm^{-1}, but the increasing frequency shifts in the 1100–950 cm^{-1} range, with little change in shape of the bands, indicate that the stability of the complex increases also as the zinc chloride concentration is raised. The fact that the methyl ether group does not block complexing in concentrated

TABLE II

ABSORBANCES FOR MIXTURES OF METHYL β-D-GLUCOPYRANOSIDE[a] AND ZINC CHLORIDE

Zinc chloride, molarity	*Absorbance at 257 nm (1-cm cell)*		
	Obs.	*Calc.*	*Deviation*
3.0	0.20	0.19	0.01
5.0	0.32	0.32	0.00
6.0	0.40	0.39	0.01
7.0	0.59	0.61	−0.02
9.0	1.01	0.89	0.12
10.0	1.21	1.00	0.21

[a]The concentration of methyl β-D-glucopyranoside was constant at 0.5M.

solutions of zinc chloride also indicates increasing stability of the complex, which is probably achieved because of insufficient hydration of the reacting zinc species.

The u.v. absorbance data for mixtures of zinc chloride and methyl β-D-glucopyranoside are presented in Table II.

The difference between the calculated and observed absorbances is negligible below 7.0M zinc chloride, but above this concentration there are increasing differences. These are probably due to an increase in the concentration of the complex as the concentration of zinc chloride is increased.

The foregoing spectral data show that maximum alteration of the physical properties of methyl β-D-glucopyranoside occurs from 8–10M zinc chloride, indicating maximal concentration and stability of the complex in this range.

Location of the points of complexing on the D-*glucopyranoside ring.* — Location of the points of complexing was determined by a comparison of the behavior of partially and fully methylated D-glucopyranosides with that of the parent D-glucopyranosides. This was done by the method of continuous variations with optical rotation, and also with n.m.r. spectra. The n.m.r. spectra were measured for solutions containing equimolar amounts of zinc chloride and the D-glucopyranosides.

It is known that the n.m.r. chemical shift is dependent on the polarity of the solution[6]. However, below 1.5M zinc chloride the change in the chemical shift due to a change in the dielectric constant is negligible. This conclusion is supported by the observation that there is no change in the chemical shift as the concentration of zinc chloride is varied at a constant molar ratio of salt to model compound.

The efficiency of the methyl ether group, when it is substituted for a hydroxyl group, in blocking formation of a complex can be ascertained from the behavior of three compounds: methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside, hexa-*O*-methyl-*myo*-inositol, and ethyl 3,4,6-tri-*O*-methyl-β-D-glucopyranoside. The data presented in Fig. 1 show that no complex is formed between methyl 2,3,4,6-tetra-*O*-

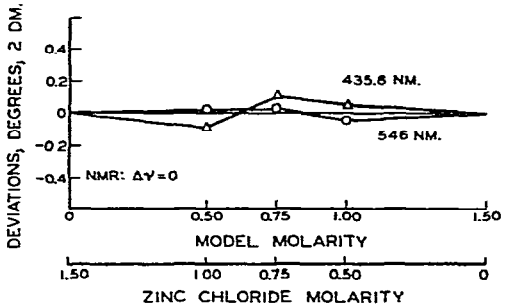


Fig. 1. Deviations of optical rotations: zinc chloride–methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside.

methyl-β-D-glucopyranoside and zinc chloride. In addition, no change was observed in the chemical shifts for any of these three compounds in the presence of zinc chloride, indicating that no complex is formed.

Lack of formation of a complex with ethyl 3,4,6-tri-*O*-methyl-β-D-glucopyranoside indicates that zinc chloride will not complex with a single hydroxyl group or with a hydroxyl–methoxyl group combination in a dilute aqueous solution of zinc chloride. In addition, methylation of all hydroxyl positions on the D-glucopyranoside ring blocks formation of a complex completely. Thus, two or more hydroxyl groups must be necessary for formation of a complex.

The polarimetric data-plots for continuous variations (Figs. 2 and 3) and the n.m.r. chemical-shift changes (Table III) for methyl β-D-glucopyranoside and methyl 4,6-di-*O*-methyl-β-D-glucopyranoside, show that the substituted D-glucoside forms complexes with zinc chloride as easily as does the unsubstituted one. Since monohydroxylic compounds do not form complexes, it appears that the vicinal hydroxyl groups on C-2 and C-3 must be the principal location at which complex formation takes place. Only one datum point was possible for Fig. 3 because of an insufficient supply of methyl 4,6-di-*O*-methyl-β-D-glucopyranoside.

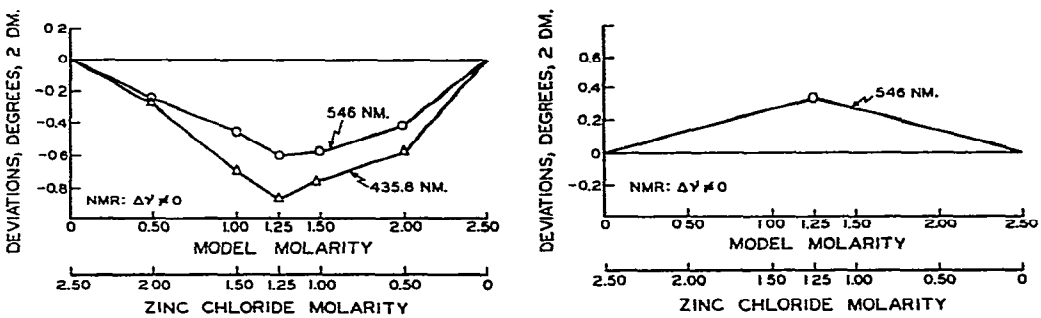
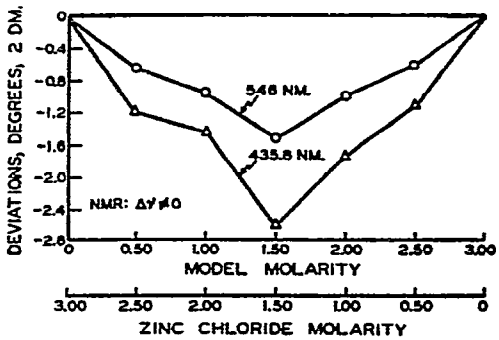


Fig. 2. Deviations of optical rotations: zinc chloride–methyl β-D-glucopyranoside.

Fig. 3. Deviations of optical rotations: zinc chloride–methyl 4,6-di-*O*-methyl-β-D-glucopyranoside.

When the data plots of continuous variations (Figs. 4 and 5) and the n.m.r. chemical-shift data (Table IV) for methyl α-D-glucopyranoside and methyl 3-*O*-methyl-α,β-D-glucopyranoside (~90% α-D anomer) are compared, a sharp decrease in the tendency of the substituted D-glucoside to complex with zinc chloride is evident. Thus, the absence of any vicinal hydroxyl groups significantly decreases complex formation. The residual complexing tendency of this compound is attributed to a slight ability of the zinc chloride to complex with pairs of hydroxyl groups that are not contiguous. This formation of a residual complex takes place either at C-2 and C-4 or at C-4 and C-6 with this particular compound. Only one datum point was determined for Fig. 5 because of an insufficient supply of methyl 3-*O*-methyl-α,β-D-glucopyranoside.

The behavior of the D-glucoside substituted at C-3 as confirming evidence for the observed indication of complex formation between zinc chloride and the vicinal hydroxyl groups on the sugar ring. The unsubstituted D-glucopyranosides (both α-D and β-D anomers) probably are capable of forming complexes involving hydroxyl groups at either C-2 and C-3 or at C-3 and C-4.



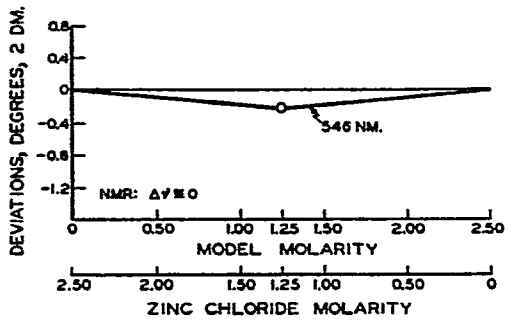


Fig. 4. Deviations of optical rotations: zinc chloride–methyl α-D-glucopyranoside.

Fig. 5. Deviations of optical rotations: zinc chloride–methyl 3-*O*-methyl-α,β-D-glucopyranoside (90% α-D anomer).

Although the location of the points of complexing was examined only for dilute solutions, the locations found would probably be the favored points for formation of a complex at higher concentrations of zinc chloride.

Possible mechanism for swelling of cellulose. — The effect of concentration of zinc chloride on the stability and concentration of the complex with methyl β-D-glucopyranoside (Tables I–II) closely parallels the effect on the swelling and loss of crystallinity in cellulose (Table V and Fig. 6). Of particular interest is the 8–10M range in which maximum swelling and loss of crystallinity are observed for cellulose. This is also the concentration range for which maximum stability and concentration of the complex occurs with methyl β-D-glucopyranoside. These parallels suggest that complex formation of a nature similar to that observed for the methyl D-glucopyranosides is

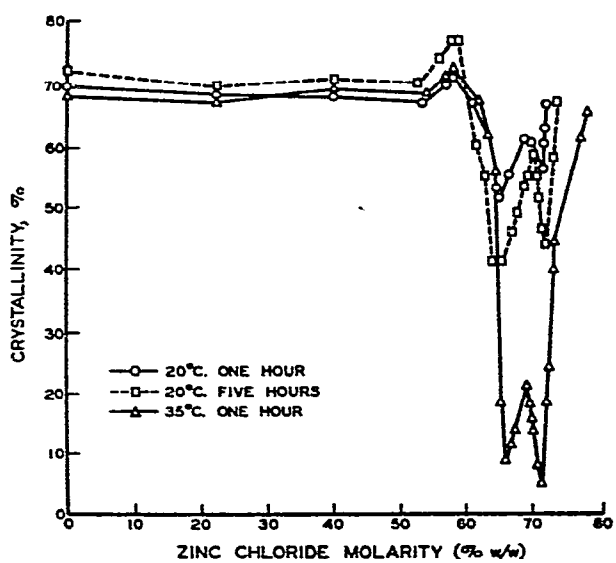


Fig. 6. Decrystallization of cotton by zinc chloride[8].

TABLE III

N.M.R. RESULTS FOR METHYL β-D-GLUCOPYRANOSIDE AND METHYL 4,6-DI-*O*-METHYL-β-D-GLUCOPYRANOSIDE

Compound and proton peak assignment	*Chemical shift changes (Δν), Hz*
M Methyl β-D-glucopyranoside + M $ZnCl_2$	
H-1	2.05
H-2,3,4,5	1.85
M Methyl 4,6-di-*O*-methyl-β-D-glucopyranoside + M $ZnCl_2$	
H-1	1.80
H-2,3,4,5	1.65

TABLE IV

N.M.R. RESULTS FOR METHYL α-D-GLUCOPYRANOSIDE AND METHYL 3-*O*-METHYL-α,β-D-GLUCOPYRANOSIDE (~90% α-D ANOMER)

Compound and proton peak assignment	*Chemical shift changes (Δν), Hz*
M Methyl α-D-glucopyranoside + M $ZnCl_2$	
H-1	1.30
H-2,3,4,5	1.40
0.5M Methyl 3-*O*-methyl-α,β-D-glucopyranoside + 0.5M $ZnCl_2$	
H-1 (α-D anomer)	0.10
H-1 (β-D anomer)	0.60
OCH_3 (3-*O*-methyl group)	0.00
OCH_3 (protons of β-D glycosidic methoxyl group)	0.10
OCH_3 (protons of α-D glycosidic methoxyl group)	−0.25
H-2,3,4,5	
A	0.20
B	0.15

TABLE V

EFFECT OF THE CONCENTRATION OF ZINC CHLORIDE ON THE SWELLING OF COTTON CELLULOSE[7]

$ZnCl_2$ concentration, weight percent	*Cellulose swelling, percent increase in weight relative to its dry weight*
10.53	108.3
20.51	140.0
20.97	166
40.81	223
54.8	535
55.6	872
57.2	1,090
58.7	1,764
60.6	2,430
61.6	5,955
62.8	7,340
63.6	9,035
64.7	10,000
67.4	13,000
68.3	12,500
68.9	13,000
71.0	12,600
73.0	13,000

probably responsible for the behavior of cellulose in zinc chloride solutions. Based on this interpretation, it is proposed that formation of the complex takes place by chelation, with zinc coordinating with the oxygen atoms of hydroxyl groups at C-2 and C-3 of the D-glucopyranoside repeating unit in the cellulose chain. This complexing would disrupt the hydrogen-bonded structure of the cellulose and cause swelling to occur.

The estimated hydration requirements of zinc chloride (listed in Table VI) illustrate how complexing is expected to increase as the zinc chloride concentration is increased. The values for the estimated water-to-zinc ratio required were calculated by using the reported concentrations[3] of $ZnCl^+$ (aq.), $ZnCl_2$ (aq.), and $ZnCl_4(H_2O)_2^{2-}$. Octahedral coordination was assumed in determining the hydration of the first two species, and $Zn(H_2O)_6^{2+}$ was determined by difference of the above species with the stoichiometric content of zinc. Since Cl^- (aq.) is also present, the calculated hydration represents a minimum value and therefore the trends indicated are valid. As the concentration of the water decreases, the concentration of the zinc chloride complex with the methyl D-glucopyranoside (or with cellulose) is increased. The increase in complexing is small until the actual water available is insufficient to hydrate the zinc ions. At this point ($\sim$8M) the lack of water creates a ligand deficiency, relative to the zinc ions in solution. This ligand deficiency is satisfied by a pair of vicinal hydroxyl groups on the D-glucopyranoside ring, and accounts for the marked increase in complexing with methyl β-D-glucopyranoside and the swelling and loss of crystallinity observed for cellulose in the 8–11M range.

As the water shortage becomes more severe, zinc chloride begins to self-associate to form a polymeric aggregate[3] at about 10M. Eventually, formation of this polymeric species, with the attendant increase in viscosity, becomes so extensive ($\sim$11.5M) that the swelling and crystallinity of cellulose approach values that are essentially the same as those observed in dilute solutions.

CONCLUSIONS

Zinc chloride complexes with vicinal hydroxyl groups at C-2 and C-3, and C-3 and C-4 of the D-glucopyranoside ring. Substitution by a methyl ether group blocks this complex formation in dilute zinc chloride solutions but not in concentrated ones. As the zinc chloride concentration is increased, the concentration and stability of the complex increase, reaching a maximum at 8–10M. Parallels between the formation of a complex with methyl β-D-glucopyranoside and the swelling and crystallinity behavior of cellulose indicate that formation of a complex between cellulose and zinc chloride is probably involved in the swelling mechanism.

EXPERIMENTAL

General methods. — Unless otherwise specified, all chemicals used were reagent grade. All solutions were prepared with triply distilled water.

TABLE VI

HYDRATION REQUIREMENTS OF THE AQUEOUS ZINC CHLORIDE SYSTEM

Stoichiometric concentration of zinc chloride		$ZnCl_4(H_2O)_2^{2-}$, *molarity*	$ZnCl^+$ *(aq.)*, *molarity*	$ZnCl_2$ *(aq.)*, *molarity*	$Zn(H_2O)_6^{2+}$, *molarity*	*Hydration requirements, mole ratio* (H_2O/Zn)	
Wt., %	*Molarity*					*Actual*	*Required*[a]
10	0.80	—	0.40	—	0.40	68.50	5.50
20	1.74	0.20	1.30	—	0.24	30.20	4.79
30	2.84	0.90	0.40	—	1.54	17.60	4.59
40	4.16	1.90	—	0.10	2.16	11.35	4.13
50	5.75	2.20	—	0.40	3.15	7.58	4.33
52	6.12	2.40	—	0.50	3.22	7.00	4.27
54	6.50	2.50	—	0.70	3.30	6.45	4.25
56	6.89	2.60	—	0.80	3.49	5.95	4.26
58	7.28	2.65	—	1.00	3.63	5.50	4.27
60	7.70	2.70	—	1.30	3.70	5.05	4.26
62	8.20	2.80	—	1.40	4.00	4.65	4.29
64	8.64	2.80	—	1.70	4.14	4.25	4.31
66	9.10	2.80	—	1.90	4.40	3.90	4.35
68	9.60	2.80	—	2.10	4.70	3.57	4.40
70	10.07	2.80	—	[b]	[b]	3.24	[b]
72	10.65	[b]	—	[b]	[b]	2.96	[b]
74	11.20	[b]	—	[b]	[b]	2.66	[b]
76	11.80	[b]	—	[b]	[b]	2.38	[b]
78	12.45	[b]	—	[b]	[b]	2.14	[b]
80	13.05	[b]	—	[b]	[b]	1.89	[b]

[a]Based on the analysis of Irish, McCarroll, and Young[3] for the concentration of $ZnCl_4(H_2O)_2^{2-}$, $ZnCl^+$ (aq.), and $ZnCl_2$ (aq.). Octahedral coordination is assumed for the latter two species and $Zn(H_2O)_6^{2+}$ is determined by difference with the stoichiometric zinc content. [b]Not determined.

Preparation and analysis of standard solutions of zinc chloride. — Standard solutions of zinc chloride were prepared by using the Fisher reagent-grade chemical without further purification. With the exception of the deuterated solutions used in n.m.r. work, solutions containing zinc chloride were prepared by dilution of these standard solutions. The concentration of the solutions was determined titrimetrically or by specific gravity. Both methods gave identical results and the simpler specific-gravity method was used routinely. The specific gravity was determined by weighing 25 ml of the solution to the nearest 0.1 mg in a sealed, 25-ml volumetric flask at room temperature. The concentration of the solution was obtained from a table of specific gravity *versus* concentration of zinc chloride as a function of temperature[9]. All determinations were made in duplicate. The titrimetric method followed the procedure of Shalfeev and Dobrovol'skaya[10].

Method of continuous variations. — The method of continuous variations employs the measurement of some property of a solution as a function of the molar ratio of the reacting species. At constant total molarity of the reacting species a maximum or minimum occurs at the molar ratio corresponding to the composition of the complex[11–14].

Preparation of the solutions for the method of continuous variations. — With the exception of methyl 4,6-di-*O*-methyl-β-D-glucopyranoside and methyl 3-*O*-methyl-α,β-D-glucopyranoside, the following method was used to prepare the solutions for the polarimetric measurements. All determinations were performed in duplicate at least, and all weighings were to the nearest 0.1 mg.

The amount of carbohydrate corresponding to the desired molarity was weighed in a 10-or 25-ml volumetric flask, and dissolved in a minimum of water. The amount of standard zinc chloride solution corresponding to the desired molarity was pipetted into the volumetric flask, and a single drop of 15% hydrochloric acid was added to disperse any floc of zinc oxychloride. The contents of the volumetric flask were then diluted to volume at equilibrium in a water bath at 20°. Pure component solutions were prepared by adding the calculated amount of carbohydrate or standard zinc chloride solution to a volumetric flask and diluting to volume. All solutions remained clear and transparent, even after storage for several months at room temperature (20–27°).

The optical rotation of each solution was measured to 0.01° with a Zeiss–Winkel polarimeter. An average of at least ten readings for solution and zero was obtained. Measurements of optical rotations were made at 546 and 435.8 nm with a filtered, mercury-light source. For methyl α- and β-D-glucopyranosides a constant temperature bath (20 ±0.02°) was used in conjunction with a 2-dm, jacketed polarimeter tube. All other compounds were measured with a 2-dm polarimeter tube at room temperature (24–28°).

The procedure for methyl 4,6-di-*O*-methyl-β-D-glucopyranoside and methyl 3-*O*-methyl-α,β-D-glucopyranoside was essentially the same as above, with the following modifications. A 1-ml volumetric flask was used. Instead of pipetting the zinc chloride into the volumetric flask, the calculated weight of zinc chloride, corrected

for impurities and water content, was weighed into the volumetric flask containing the model compound, and approximately 0.005 ml of 15% hydrochloric acid was added. The solution was then diluted to volume at equilibrium in a water bath at 20°. Measurements of optical rotation were made at 546 nm with a microbore, 2-dm polarimeter tube. An average of at least 25 readings for sample and zero was obtained.

N.m.r. spectra. — All n.m.r. spectra were obtained for deuterated solutions with a Varian Model A-60A analytical n.m.r. spectrometer. The chemical shifts of these spectra were measured for solutions in 99.8% deuterium oxide (Stohler Isotope Company) containing sodium 4,4-dimethyl-4-silapentanesulfonate (DSS) as an internal reference. All weighings were to the nearest 0.1 mg, and all chemical shifts reported were an average of five or more duplicate spectra for each deuterated solution. The deuterated solutions were prepared by weighing the amount of carbohydrate and zinc chloride corresponding to the desired molarity into the quartz-glass, coaxial sample tube used for n.m.r. measurements. Following this, a small amount of DSS in D_2O, and approximately 0.001 ml of concentrated hydrochloric acid was added, and the contents of the sample tube were diluted to a height of 5 cm in the tube; which corresponded to a volume of 0.434 ml.

I.r. spectra. — All i.r. data were obtained by the method of attenuated total reflectance[15,16] by using a silver chloride prism in a Perkin–Elmer Model 21 recording spectrometer. The aqueous solutions of model compound and zinc chloride were prepared by weighing the amount of model compound and standard zinc chloride solution, corresponding to the desired molarity, into a 25-ml volumetric flask, and diluting the contents to volume at equilibrium (water bath at 20°). For solutions less than 3M in zinc chloride, it was necessary to add a drop of 15% hydrochloric acid in order to disperse any zinc oxychloride floc. Above 3M no formation of floc occurred.

U.v. spectra. — The u.v. spectra were obtained by using a 1 or 10 cm silica-glass cell in a Cary Model 15 recording spectrophotometer. Solutions were prepared in the same manner as for the i.r. spectra.

Preparation of model compounds. — *Methyl α-D-glucopyranoside.* Methyl α-D-glucopyranoside (Pfanstiehl) was recrystallized three times from abs. ethanol; m.p. 167–168°, $[\alpha]_D^{20}$ +159.13° (lit.[17] m.p. 167° $[\alpha]_D^{20}$ +158–159°).

Methyl β-D-glucopyranoside. Methyl β-D-glucopyranoside (Pfanstiehl) was recrystallized twice from abs. ethanol; m.p. 108–109°, $[\alpha]_D^{20}$ −32.64° (lit.[17] m.p. 104° to 109–111°, $[\alpha]_D^{20}$ −32.5°).

Methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside. Prepared by the method of West and Holden[18], this product had m.p. 37–38°, $[\alpha]_D^{20}$ −17.3° (lit.[17] m.p. 40–41° and $[\alpha]_D^{20}$ −17.3°).

Methyl 4,6-di-O-methyl-β-D-glucopyranoside. The product prepared by Bayer[19] (crop number one) was used; m.p. of 77–78° (lit.[17] 77–79°).

Methyl 3-O-methyl-α,β-D-glucopyranoside. This product was prepared from 3-*O*-methyl-D-glucose by treatment with methanol in the presence of Amberlite IR-120 (H^+) resin following the method of Bayer[19]. The reducing sugars were removed from the product by treatment with hot alkali. The syrup that resulted

after removal of ions with a mixed-bed resin (Amberlite MB-3) was used without further purification. The n.m.r. spectrum indicated that the compound was about 90% α-D anomer.

Ethyl 3,4,6-tri-O-methyl-β-D-glucopyranoside. This product was prepared[20] by the *p*-toluenesulfonic acid-catalyzed ethanolysis of 3,4,6-tri-*O*-methyl-1,2-(ethyl orthoacetyl)-β-D-glucopyranose.

ACKNOWLEDGMENTS

For their assistance and many helpful suggestions the authors are grateful to Edgar Dickey and Donald Johnson. Thanks are also due to Lowell Sell for his help in the spectral work and to David Hultman for the sample of ethyl 3,4,6-tri-*O*-methyl-β-D-glucopyranoside.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

4,6-*O*-ALKYLIDENE DERIVATIVES OF D-GLUCOSE AND ITS METHYL PYRANOSIDES. AN IMPROVED ACETALATION PROCEDURE*

DAVID M. HALL AND OTTO A. STAMM†

Textile Engineering Department, Auburn University, Auburn, Alabama 36830 (U. S. A.) and Department of Industrial and Engineering Chemistry, Swiss Federal Institute of Technology, Zurich (Switzerland)

(Received April 11th, 1969; in revised form, September 26th, 1969)

ABSTRACT

In the preparation of 4,6-alkylidene acetals of D-glucose and its methyl pyranosides, addition of ethyl ether to the reaction mixture causes formation of the 4,6-*O*-alkylidene derivative as the major product. In this way, formation of 2,3-*O*-oxidodialkylidene derivatives of the methyl pyranosides may often be completely avoided, and an increased yield of the 4,6-monoacetal results. An improved method for the preparation of methyl β-D-glucopyranoside (3) by a modified Haworth methylation of D-glucose is also described.

INTRODUCTION

1,1-Dimethoxyethane has been reported[1,2] to give the 4,6-*O*-ethylidene derivative of D-glucopyranosides, uncontaminated by the 2,3-*O*-oxidodiethylidene derivative, but, with D-glucose, it failed to give an ethylidene derivative.

Mellies *et al.*[3] obtained small amounts of 4,6-*O*-ethylidene-α-D-glucose after considerable effort, and Helferich and Appel[4] experienced similar difficulties, but the yield was improved by later workers[5]. In the D-glucopyranoside series, Appel and Haworth[6] showed that a dilute solution of bromine in ethyl ether cleaves the 2,3-*O*-oxidodiethylidene group, possibly owing to a small proportion of hydrogen bromide produced by this reagent.

RESULTS AND DISCUSSION

It seemed possible that, were this explanation correct, presence of ethyl ether in the acetalating mixture might prevent formation, or cause hydrolysis, of any diacetal[3] or 2,3-oxidodialkylidene acetal[6] from either D-glucose or its methyl pyranosides. We have found that use of such a mixture does, indeed, increase the yields of the monocyclic acetal in both instances, while lessening or, in some cases, inhibiting formation of by-product.

*This work was begun, under a U. S. D. A. Fellowship, at the Swiss Federal Institute of Technology, and completed in the Textile Engineering Department of Auburn University under an Engineering Experiment Station Grant.

†Present Address: Geigy A. G., Basle, Switzerland.

The proportion of aldehyde employed appeared to be critical for the α-D-glucopyranosides. Large proportions of aldehyde, especially as the chain length was increased, gave more condensation at O-2 and O-3, presumably because of the raised solubility of the cyclic acetals of the α-D anomer in the aldehyde–ether mixture. The condensation at O-2 and O-3 could be eliminated by lessening the proportion of aldehyde; however, the proportion of unreacted D-glucopyranoside then increased. The monoacetals of the β-D-glucopyranosides studied are practically insoluble in ether, and their crystallization from the ether–aldehyde mixture shifts the equilibrium, and essentially precludes condensation at O-2 and O-3.

The procedure outlined in the Experimental section affords the highest yield of 4,6-acetal compatible with a minimal amount of unreacted material and minimal formation of 2,3-oxidodialkylidene acetal. Separation of the latter from the reaction mixture was achieved by virtue of its insolubility in water.

The 4,6-*O*-propylidene derivative (**16**) of methyl α-D-glucopyranoside is more readily crystallized, easier to purify, and obtained in higher yields than the corresponding ethylidene acetal (**14**). The latter has a low melting-point, and syrups are often obtained during attempts at crystallization. However, syrupy material can often be used directly; in this work, it was characterized as the crystalline 2,3-diacetate (**17**).

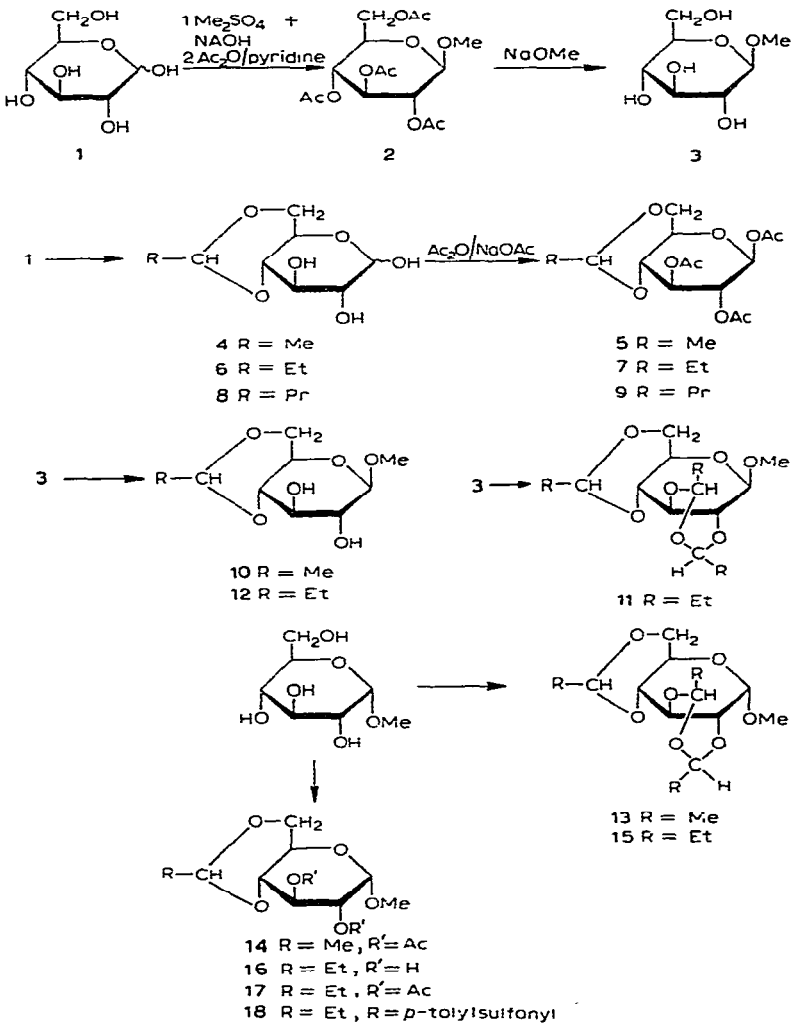
Good yields of the 4,6-acetals can be obtained from practical grades of D-glucose, but the presence of water (as in α-D-glucose monohydrate) lowers the yield. Products were obtained in high- or low-melting forms, depending on the solvent and procedure employed for recrystallization. Acetone was the preferred solvent for recrystallization, because D-glucose is almost insoluble in it. On using acetone, crystalline compounds different from those described by Mellies and co-workers[3] were obtained. They reported needles (m.p. 164–165°) for 4,6-*O*-butylidene-D-glucopyranose (**8**), but plates (m.p. 151–153°) could be obtained by rapidly cooling a solution thereof in acetone. A low-melting form of 4,6-*O*-ethylidene-D-glucose (**4**) (m.p. 144–148°) could be obtained by slowly cooling its solution in acetone; material of higher melting-point (166–170°) was obtained by rapid cooling. Similar results were obtained with the other 4,6-acetals.

In both the D-glucose and D-glucopyranoside series, the cleavage of the oxidodialkylidene group at O-2 and O-3 probably involves[7] the formation of the oxonium ion. As ethyl ether is a good solvent for the oxonium ion, it allows the ion to achieve maximum mobility; this may explain the lessening in efficiency of the reaction caused by water. The oxidodialkylidene group has an ether linkage connecting the two acetal groups. Possibly, the oxido oxygen atom may be involved in oxonium salt formation, thereby weakening this bond and promoting cleavage and ultimate removal of the group.

An improved synthesis of methyl β-D-glucopyranoside (**3**) was devised; this anomer is preferable for the foregoing synthesis, as it gives crystalline derivatives more readily than the α-D anomer[8]. The usual method of preparation of the β-D anomer involves treating a tetra-*O*-acetyl-α-D-glucopyranosyl halide[9] with methyl

alcohol and silver carbonate, or with magnesium methoxide[10], followed by deacetylation. As we needed relatively large amounts of this compound for our studies, a simpler procedure for its synthesis in the pure form was sought. A modification of Haworth's[11] aqueous-phase methylation[12] was found satisfactory. At low temperatures, the anomeric hydroxyl group of D-glucopyranose can be exclusively methylated by the action of methyl sulfate and sodium hydroxide, and essentially pure methyl β-D-glucopyranoside (3) is formed. Evidently, the reactivity of the anomeric hydroxyl group of the β-D form is many times that of the α-D form; as the β-hydroxyl group is methylated, the α–β equilibrium ratio is altered, and some of the α-D anomer changes to the β-D form.

It was found that the rate of addition of the methyl sulfate is the most critical variable; if the sulfate is added too slowly, the competing reaction with water becomes dominant, and little methyl β-D-glucopyranoside is formed. The methyl sulfate was



added as rapidly as possible, at 25–30°. The pH was kept at 9 ±0.5. The methyl β-D-glucopyranoside was purified by acetylation (to remove the sodium sulfate formed during methylation) followed by deacetylation.

EXPERIMENTAL

General. — Solutions were evaporated under diminished pressure unless otherwise indicated. Melting points were determined on either a Kofler hot-stage or a Nalge–Axelrod micro melting-point apparatus and are corrected. The analyses were performed either by the Micro-analytical Section of the Swiss Federal Institute or by Schwartzkopf Microanalytical Laboratory. For the pH measurements, a Corning, direct-reading, pH meter fitted with a combination type of electrode (E. H. Sargent) standardized against buffer at pH 9 was employed. The yields reported are those obtained after at least one recrystallization of the crude products.

Preparation of 4,6-O-alkylidene derivatives of D-*glucose.* — To 180 g (1 mole) of finely powdered, anhydrous D-glucose (dried over phosphorus pentaoxide for 24 h at 1 torr) was added, in three equal portions during 15 min, a solution of the aldehyde and concentrated sulfuric acid in diethyl ether; the mixture was shaken mechanically for 1 h, and kept for 24 h. Ethyl ether was then added until a filtrable slurry was obtained. Ethyl ether and solid potassium carbonate (5 g) were added to the precipitate, and the suspension was kept for several hours and filtered. Recrystallization was effected by extracting the acetal (50 g) with hot acetone (1 liter) containing ammonium hydroxide (1 ml), filtering the hot suspension, cooling the filtrate, and filtering off the acetal. The extractions were continued until only the potassium carbonate and a small amount of unreacted D-glucose remained. Depending on the method of recrystallization (rapid or slow cooling of the acetone solutions), a high- or low-melting product was obtained.

Preparation of 1,2,3-tri-O-acetyl derivatives of 4,6-monoacetals of D-*glucose.* — To acetic anhydride (100 ml) at 90° was added sodium acetate trihydrate (5 g), and the mixture was stirred until all of the acetate had dissolved. The 4,6-*O*-alkylidene derivative was added during 15 min, and, after a further 1 h at 90°, the solution was poured into water (500 ml). The solution was made neutral by addition of solid sodium carbonate, and the resulting crystals were filtered off and air-dried.

*Methyl 2,3,4,6-tetra-O-acetyl-β-*D-*glucopyranoside* (**2**). — In a five-necked flask, fitted with a stirrer, a thermometer, and a pH electrode (combination type), was placed a solution of α-D-glucose (**1**, 180 g, 1 mole) in water (250 ml). Methyl sulfate (140 g, 1.10 moles) was added dropwise during 8 h, the pH of the solution being kept at 9 ±0.5 (pH meter) by the addition of sodium hydroxide solution (35%), and the temperature being kept at 25–30°. The mixture was then stirred for 24 h. A small volume of concentrated, aqueous ammonia was added to decompose the excess of methyl sulfate, and the solution was evaporated under diminished pressure to a syrup which was dissolved in a solution of pyridine (50 ml) in acetic anhydride (1 liter), the temperature being kept at 50–60° by cooling in an ice bath. After 24 h at room temperature, the mixture was poured into cold water (3 liters), and the pH

was brought to 5 by addition of solid sodium carbonate. The mixture was filtered, giving crystals (*A*). A further amount of material was obtained by extracting the filtrate with chloroform. The chloroform layer was successively washed with 1% sulfuric acid, sodium hydrogen carbonate solution, and water, dried (calcium chloride), and evaporated. The resulting crystals were combined with precipitate *A*, and recrystallized from 7:3 (v/v) ethanol–water, to give 130–140 g (36%) of the tetraacetate **2**, m.p. 100–104°, $[\alpha]_D^{25}$ −21.1° (*c* 2.8, ethanol).

Methyl β-D-glucopyranoside (**3**). — The anhydrous tetraacetate **2** (110 g) was deacetylated by the Zemplén[13] method, with shaking for 2 h; ethyl ether (200 ml) was added, and the mixture was cooled overnight, and filtered. More product was recovered by concentration of the mother liquor. Recrystallization from methanol–ether gave 60 g (95%) of **3**; m.p. 110°, $[\alpha]_D^{22.5}$ −32.7° (*c* 3, water) [lit. m.p. 110° (ref. 14), 105° (ref. 15), 109–111° and $[\alpha]_D^{20}$ −32.5° (ref. 12)].

4,6-O-Ethylidene-D-glucopyranose (**4**). — Use of the general procedure for preparation of the 4,6-*O*-alkylidene derivatives, with D-glucose (180 g) and a solution of paraldehyde (100 ml) and concentrated sulfuric acid (3 ml) in ethyl ether (200 ml), gave, after one recrystallization, 185–195 g (90–95%) of fairly pure **4**, m.p. 166–170°; lit.[3,4] m.p. 179–182°.

1,2,3-Tri-O-acetyl-4,6-O-ethylidene-β-D-glucopyranose (**5**). — On acetylation, compound **4** (10 g) gave 14 g (88%) of **5** after two recrystallizations from 1:1 (v/v) ethanol–water; m.p. 124–125° (sublimes), $[\alpha]_D^{25}$ −40.1° (*c* 3.02, chloroform).

Anal. Calc. for $C_{14}H_{20}O_9$: C, 50.6; H, 6.1. Found: C, 50.9; H, 6.1.

4,6-O-Propylidene-D-glucopyranose (**6**). — An ice-cold solution of propionaldehyde (100 ml) and concentrated sulfuric acid (2.5 ml) in ethyl ether (200 ml) and D-glucose (180 g) gave 200 g (91%) of **6** after one recrystallization from acetone. The reaction is slightly exothermic, and it was sometimes necessary to cool the acetalating mixture intermittently to prevent loss of ether. If the reaction is terminated after 1 h, the yield is 70–75 g; with 130 ml of propionaldehyde, only 165 g of the acetal was obtained. Two recrystallizations from acetone gave platelets having m.p. 151–152°.

Anal. Calc. for $C_9H_{16}O_6$: C, 49.1; H, 7.3. Found: C, 49.0; H, 7.2.

1,2,3-Tri-O-acetyl-4,6-O-propylidene-β-D-glucopyranose (**7**). — Acetylation of compound **6** (5 g) gave, after one recrystallization from 1:1 (v/v) ethanol–water, 6.2 g (87%) of product (**7**). After a second recrystallization from the same solvent mixture, it was obtained as micro-needles, m.p. 112–113° (sublimes), $[\alpha]_D^{25}$ −25.8° (*c* 3.06, chloroform).

Anal. Calc. for $C_{15}H_{22}O_9$: C, 52.0; H, 6.4. Found: C, 51.9; H, 6.3.

4,6-O-Butylidene-D-glucopyranose (**8**). — A mixture of butyl aldehyde (150 ml), ethyl ether (190 ml), concentrated sulfuric acid (3 ml), and D-glucose (180 g) gave 205 g (89%) of **8** after one recrystallization. Two recrystallizations from acetone gave platelets, m.p. 152–154°; very slow cooling of an acetone solution gave needles having m.p. 149°; lit.[3] m.p. 164–165°.

1,2,3-Tri-O-acetyl-4,6-O-butylidene-β-D-glucopyranose (**9**). — Compound **8** (25 g) was acetylated similar to **1**, and, after three recrystallizations from 1:1 (v/v)

ethanol–water, gave platelets (35 g, 90%) having m.p. 162–164° (sublimes), $[\alpha]_D^{25}$ −36.6° (*c* 3.0, chloroform).

Anal. Calc. for $C_{16}H_{24}O_9$: C, 53.3; H, 6.7. Found: C, 53.3; H, 6.7.

Methyl 4,6-O-ethylidene-β-D-glucopyranoside (**10**). — *Method A.* To air-dried **3** (35 g) were added 1,1-dimethoxyethane (150 ml), and concentrated sulfuric acid (1 ml) dropwise, with stirring. After being shaken mechanically for 3 h, the suspension was filtered; the solid was shaken for 2 h with ether (100 ml) containing potassium carbonate (5 g), and the suspension was filtered. Recrystallization of the solid from methanol gave pure **10** (20 g), m.p. 188–190°; lit.[4] m.p. 189–190°. An additional 12 g (total 80%) was obtained by concentration of the mother liquors (after neutralization with potassium carbonate) to a small volume and addition of ether; m.p. 182–184°; this material could be converted into the higher-melting form by recrystallization from methanol or from 3:1 (v/v) methanol–ether.

Method B. To air-dried **3** (10 g) were added 20 small, glass beads and a solution of paraldehyde (10 ml) and concentrated sulfuric acid (0.25 ml) in ethyl ether (25 ml), and the mixture was shaken for 4 h. Ethyl ether (25 ml) was added, and the mixture was shaken for 20 h. After 24 h, the suspension was filtered, and the solid was washed with ether and then extracted with three 25-ml portions of chloroform. [The solid remaining was recrystallized from methanol–ether, to give unreacted starting material (0.5–0.75 g) having m.p. 110° alone or on admixture with methyl β-D-glucopyranoside.]

The washings and filtrate were combined, and made neutral (solid potassium carbonate), the suspension was filtered, and the filtrate was evaporated to dryness, to yield practically pure methyl 4,6-*O*-ethylidene-β-D-glucopyranoside. Water (100 ml) was added, and the suspension was filtered. The filtrate was evaporated to dryness, and the solid was recrystallized from methanol–ether–petroleum ether (b.p. 60–90°), to yield 9.2 g (81%) of **10**, as crystals having m.p. and mixed m.p. of 188–190° and $[\alpha]_D^{20}$ −78.9° (*c* 0.19, water); lit.[4] m.p. 189–190°, $[\alpha]_D^{22}$ −79.1° (water).

Methyl 2,3-O-(oxidodipropylidene)-4,6-O-propylidene-β-D-glucopyranoside (**11**). — To air-dried **3** (10 g) were added 20 small, glass beads and a solution of propionaldehyde (10 ml) and concentrated sulfuric acid (0.25 ml) in ethyl ether (25 ml). The mixture was treated as described for compound **10** (method *B*). [Unreacted starting material (1.0–1.5 g) was identified by m.p. and mixed m.p.]. Filtration of the aqueous suspension gave a solid (0.25 g) which was recrystallized from 9:1 (v/v) ethanol–water, giving needles of **11** having m.p. 118–120°; lit.[16] m.p. 117–118°.

Methyl 4,6-O-propylidene-β-D-glucopyranoside (**12**). — Evaporation of the aqueous filtrate from **11**, and recrystallization of the residue from methanol–ether–petroleum ether (b.p. 60–90°) gave 8.2 g (63%) of **12**, m.p. 153–155°, $[\alpha]_D^{25}$ +109.6° (*c* 2.92, chloroform).

Anal. Calc. for $C_{10}H_{18}O_6$: C, 51.2; H, 7.7. Found: C, 51.0; H, 7.7.

Methyl 4,6-O-ethylidene-2,3-O-(oxidodiethylidene)-α-D-glucopyranoside (**13**). — To 10 g of methyl α-D-glucopyranoside (dried over phosphorus pentaoxide at 1 torr) were added 20 small, glass beads and a solution of paraldehyde (10 ml) and con-

centrated sulfuric acid (0.25 ml) in ethyl ether (25 ml), and the mixture was treated as for **10** (method *B*). [The solid remaining after extraction with chloroform was recrystallized from methanol–ether to give 1.5–2 g of starting material (m.p., and mixed m.p. with starting material, 162–163°).]

The solution and washings were combined, and made neutral with solid potassium carbonate, the suspension was filtered, and the filtrate was evaporated to dryness. Distilled water (100 ml) was added, and the mixture was warmed to 50°, cooled to 20°, and filtered. The resulting solid (2.5 g) was recrystallized from methanol, to give needles of **13**, m.p. 183–184°, lit.[3] m.p. 183–183.5°.

*Methyl 2,3-di-*O*-acetyl-4,6-*O*-ethylidene-α-*D*-glucopyranoside* (**14**). — The aqueous filtrate from **13** was decolorized with activated carbon (Norit), and evaporated to dryness, and the resulting syrup was dried *in vacuo* over phosphorus pentaoxide. Acetic anhydride (50 ml) and pyridine (5 ml) were added, and the mixture was heated on a steam bath for 1 h, cooled, and kept at room temperature for 24 h. The mixture was poured into water, and brought to pH 5 by addition of sodium carbonate. The mixture was extracted twice with chloroform, and the extracts were combined, washed successively with sulfuric acid (1%) and sodium hydrogen carbonate solution (5%), dried (anhydrous calcium sulfate), and evaporated to dryness. Two recrystallizations from 1:1 (v/v) ethanol–water, and one from ether–petroleum ether gave 9.1 g (58%, based on starting materials) of **14** having m.p. 81–81.5°, $[\alpha]_D^{25}$ +118° (*c* 1.0, chloroform); lit.[17] m.p. 81°; $[\alpha]_D^{16}$ +117°.

*Methyl 2,3-*O*-(oxidodipropylidene)-4,6-*O*-propylidene-α-*D*-glucopyranoside* (**15**). — To anhydrous methyl α-D-glucopyranoside (10 g) were added 20 small, glass beads and a solution of propionaldehyde (10 ml) and concentrated sulfuric acid (0.25 ml) in ethyl ether (25 ml), and the mixture was treated as for compound **13**; unreacted α-D-glucoside (2 g) was recovered. Compound **15** (0.25 g) was precipitated by addition of water; it was recrystallized from 2:1 (v/v) ethanol–water or from ether–petroleum ether to a constant m.p. of 142–143°; lit.[3] m.p. 142.5–143.5°.

*Methyl 4,6-*O*-propylidene-α-*D*-glucopyranoside* (**16**). — Evaporation of the aqueous filtrate from **15** gave a solid that was dried *in vacuo* over phosphorus pentaoxide and then successively recrystallized from benzene–petroleum ether (b.p. 60–90°) and ether–petroleum ether to give 7.5 g (59%) of **16**, as needles having m.p. 103–104°, $[\alpha]_D^{25}$ +121.8° (*c* 1.85, chloroform); lit.[3] m.p. 102–103.5°, $[\alpha]_D^{25}$ +122.1° (chloroform).

When methyl α-D-glucopyranoside (10 g) was treated, as for the preparation of **15**, with 15 ml of propionaldehyde, instead of 10 ml, the products were unreacted α-D-glucoside (0.75 g), **15** (3 g), and **16** (6.2 g).

*Methyl 2,3-di-*O*-acetyl-4,6-*O*-propylidene-α-*D*-glucopyranoside* (**17**). — To **16** (2.5 g) were added 50 ml of acetic anhydride and 5 ml of pyridine, and the mixture was heated at 90° for 1 h, cooled to room temperature, and kept for 24 h. It was then poured into water, and the mixture was brought to pH 5 with solid sodium carbonate. The mixture was twice extracted with chloroform, and the extracts were combined, washed successively with sulfuric acid (1%) and sodium hydrogen carbonate solution (5%), dried (anhydrous calcium sulfate), and evaporated to a syrup which was

precipitated 3 times from ether by addition of petroleum ether. The syrup was dried *in vacuo* over phosphorus pentaoxide for several days at 25°, to give 2.5 g (73%) of a waxy solid, $[\alpha]_D^{25}$ +118.5° (*c* 2.13, chloroform).

Anal. Calc. for $C_{14}H_{22}O_8$: C, 52.8; H, 7.0. Found: C, 52.6; H, 7.0.

Methyl 4,6-O-propylidene-2,3-di-O-p-tolylsulfonyl-α-D-glucopyranoside (**18**). — To 1 g of **16** were added anhydrous pyridine (50 ml) and *p*-toluenesulfonyl chloride (5.0 g), and the mixture was heated for 8 h at 95–100°, and then kept for 3 days at room temperature. It was poured into ice water (1 liter), and the mixture was extracted with chloroform. The extract was successively washed with dilute sulfuric acid (2%), sodium hydrogen carbonate solution (2%), and water, dried (anhydrous calcium sulfate), and evaporated. The resultant material was recrystallized three times from ethyl ether, to give 1.2 g (50%) of very long, silky needles having m.p. 70–72°, $[\alpha]_D^{25}$ +47.7° (*c* 1.59, chloroform); lit.[16] m.p. 127.5–128°, $[\alpha]_D^{25}$ +46.1°. Evidently our product is a new crystalline form of this compound. On melting and slow cooling, the compound did not resolidify.

Anal. Calc. for $C_{24}H_{30}O_{10}S_2$: C, 53.1; H, 5.6. Found: C, 53.3; H, 5.7.

ACKNOWLEDGMENTS

One of us (D.M.H.) thanks Professor H. Zollinger and the Department of Industrial and Engineering Chemistry of the Swiss Federal Institute for the Fellowship under which this work was initiated. We thank the Auburn University Engineering Experiment Station for a grant that made the completion of this study possible, T. E. Lawler for determining the melting points and optical rotations, and Mrs. Penny Mullen and Mrs. Janice James for their assistance with the manuscript. We also acknowledge initial support given by the U. S. Department of Agriculture under Public Law 480 [Project No. UR-E 27-(20)-2].

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

ENZYMIC LIBERATION OF D-GALACTOSE FROM KERATAN SULFATE BY HUMAN TISSUES

P. A. ÖCKERMAN
Department of Clinical Chemistry, University Hospital, Lund (Sweden)
(Received May 14th, 1969; in revised form, July 26th, 1969)

ABSTRACT

Human and rat tissues were shown to contain an enzyme that liberated D-galactose from highly purified keratan sulfate. The enzyme had its maximum activity at pH 4.0, was preferentially localized in the lysosomes, and split off free D-galactose from the non-reducing end of the keratan sulfate molecule.

INTRODUCTION

Keratan sulfate is a polymer composed mainly of 2-acetamido-2-deoxy-D-glucose, D-galactose, and sulfate. The D-galactose and hexosamine residues are β-D-(1→4)-linked, and this disaccharide forms the repeating unit of the polysaccharide. In each polysaccharide chain, one molecule of 2-amino-2-deoxy-D-galactose is attached by a glycosidic linkage to serine or threonine in the peptide portion of the keratan sulfate–protein complex[1].

Although enzymes of bacterial origin are known to degrade keratan sulfate[2], no evidence has been presented to indicate the existence of keratan sulfate-degrading enzymes in mammalian tissues. We now describe the presence, in human and rat tissues, of an enzyme that catalyses the liberation of D-galactose from keratan sulfate.

MATERIALS AND METHODS

All keratan sulfate preparations used in this study were gifts from Dr. L. Å. Fransson, Department of Physiological Chemistry, University of Lund, and had been prepared[3] from nucleus pulposus and cornea by papain digestion. D-Galactose dehydrogenase was purchased from C. F. Boehringer and Sons, Mannheim, W. Germany.

Human tissues were obtained by liver biopsy at operation for cholelithiasis and 5–15 h post-mortem. All patients were adults.

The methods for homogenisation of tissues, centrifugation, gel filtration on Sephadex G-150, and fractionation of subcellular particles have been described[4,5]. Liberation of D-galactose from keratan sulfate was measured on whole homogenates, supernatant from homogenates, or eluates from gel filtration by incubating the

appropriate amount of keratan sulfate, buffer, and sample at 37° in 0.4-ml plastic tubes (Beckman–Spinco, Fullerton) in ordinary centrifuge tubes[6]. After incubation, the tubes were immediately cooled and centrifuged, and D-galactose was measured in the supernatant, immediately or after storage at −20°, by using a fluorimetric technique with D-galactose dehydrogenase. Of the incubation mixture, 50–150 μl was added to 1.35 ml of 40mM Tris-HCl buffer, pH 8.6, with 4mM neutral glutathione and 0.4mM NAD[7]. Fluorescence was read in an Aminco–Bowman spectrofluorimeter (American Instrument Company) at an excitation wavelength of 340 nm and an emission wavelength of 458 nm. After *ca.* 6–10 min, a stable reading was reached, and 5 μl (25 μg) of D-galactose dehydrogenase was added. After a new plateau had been reached, 1–5 μg of D-galactose was added in a volume of 10 μl, as internal standard.

Further experimental details are given in the Tables and Figures.

RESULTS

An aqueous, particle-free extract of human liver was fractionated[5] by gel filtration on Sephadex G-150. D-Galactose was liberated from keratan sulfate by two (I and II) of the three different fractions, which were active against a synthetic, unnatural β-D-galactoside (Fig. 1). The largest of these fractions, peak II, was used as enzyme source in all experiments, if not otherwise stated.

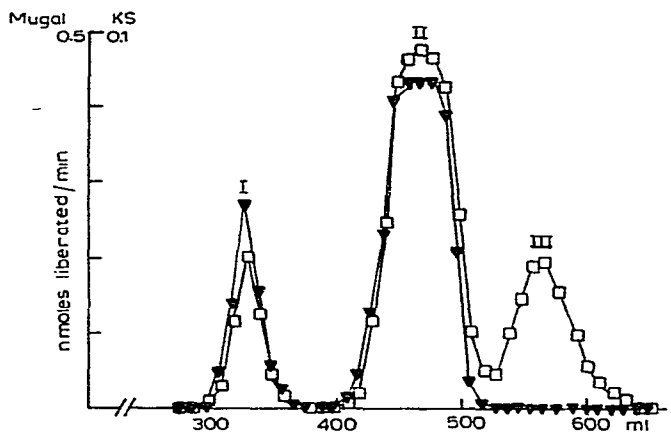


Fig. 1. Gel filtration of D-galactosidase activities in human liver. The supernatant from 10 g of homogenised human-liver was fractionated by gel filtration on Sephadex G-150, as described earlier[5]. β-Galactosidase was assayed on 100 μl of the fractions with 4-methylumbelliferyl β-D-galactopyranoside and keratan sulfate as substrates. MUGAL (□—□) = 4-methylumbelliferyl β-D-galactoside as substrate. KS (▼—▼) = keratan sulfate as substrate.

Peaks I and II, but not peak III, liberated D-galactose from keratan sulfate isolated from nucleus pulposus or from cornea (Table I).

To investigate the possibility that D-galactose was liberated from non-keratan sulfate impurities in the preparation, keratan sulfate from nucleus pulposus was

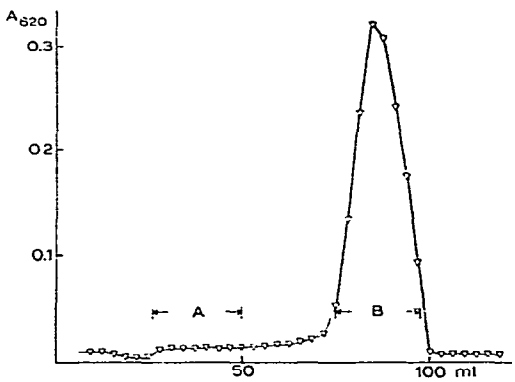
TABLE I

D-GALACTOSE LIBERATION[a] FROM KERATAN SULFATE PREPARED FROM CORNEA AND NUCLEUS PULPOSEUS

Enzyme source	*D-glactose liberated (μg) from keratan sulfate*	
	Cornea	*Nucleus pulposus*
Peak I	2.73	4.50
Peak II	4.20	8.85
Peak III	0	0

[a]Peaks I, II, and III from the experiment described in Fig. 1 were lyophilized, and dissolved in 6 ml of distilled and deionized water, and 100 μl was incubated for 2 h with 8 mg of keratan sulfate and 100 μl of acetate buffer (0.2M, pH 4.0).

fractionated by gel filtration on Sephadex G-75 (Fig. 2a) and by ion-exchange chromatography on DEAE-Sephadex (Fig. 2b). Practically all of the D-galactose liberated from the original keratan sulfate was recovered in the main fraction, both in the gel filtration and in the ion-exchange chromatography experiment (Table II). The keratan sulfate purified by gel filtration and chromatography was used in all of the following experiments, if not otherwise stated.



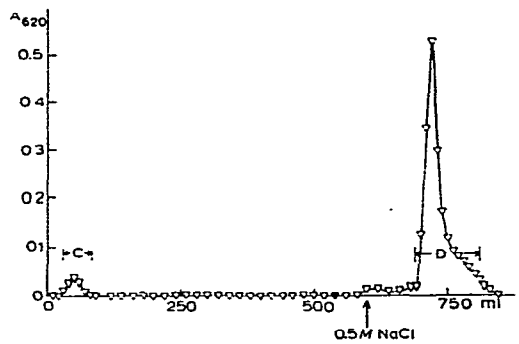


Fig. 2a. Gel filtration on Sephadex G-75 of keratan sulfate. Keratan sulfate (100 mg), isolated from nucleus pulposus, was dissolved in water (1 ml) and applied to a column (2 × 37 cm) of Sephadex G-75 in water and eluted with water. Fractions (1.25 ml) were analysed for hexose content by the anthrone method[8].

Fig. 2b. Ion-exchange chromatography on DEAE-Sephadex of keratan sulfate. Fraction *B* (85 mg) from the experiment described in Fig. 2a was dissolved in water (3 ml) and applied to a column, (2 × 31 cm) of DEAE-Sephadex A-25 equilibrated with 0.0025M phosphate buffer (pH 7.6) and eluted, first with 600 ml of a linear phosphate gradient from 0.0025M—0.05M and then with 0.5M NaCl. Fractions (5 ml) were assayed (0.5 ml) for hexose contents by the anthrone method[8].

The kinetics of D-galactose liberation were studied with peak II and purified keratan sulfate. The effects of substrate concentration, pH, time of incubation, and amount of enzyme are shown in Figs. 3a–d. The enzyme was found to have a prefer-

TABLE II

D-GALACTOSE LIBERATION[a] FROM FRACTIONS OF KERATAN SULFATE SUBJECTED TO GEL FILTRATION ON SEPHADEX G-75 AND ION-EXCHANGE CHROMATOGRAPHY ON DEAE-SEPHADEX

		Total weight of lyophilized fraction (*mg*)	*Amount of substrate in incubate* (*mg*)	D-*Galactose liberated* (*μg*)	D-*Galactose* (%) *liberated from original keratan sulfate*
Sephadex G-75	Fraction *A*	9	1	1.8	7.2
	Fraction *B*	87	1	2.4	92.8
DEAE-Sephadex	Fraction *C*	38[b]	2[b]	0.57	3.7
	Fraction *D*	3575[b]	35[b]	2.74	96.3

[a]Fractions *A*, *B*, *C*, and *D* (indicated by arrows) from the experiments described in Figs. 2a and 2b were lyophilized, and a weighed portion was used as substrate for peak II galactosidase activity. [b]Consisting largely of salt.

entially lysosomal localization in rat liver (Fig. 3e). The activity, when measured in human, whole-tissue homogenates, was greatest in kidney and liver. Smaller but still easily measurable activities were noted in spleen, lung, jejunum, and brain (Table III). No activity could be demonstrated in fresh or dialysed and lyophilized urine.

TABLE III

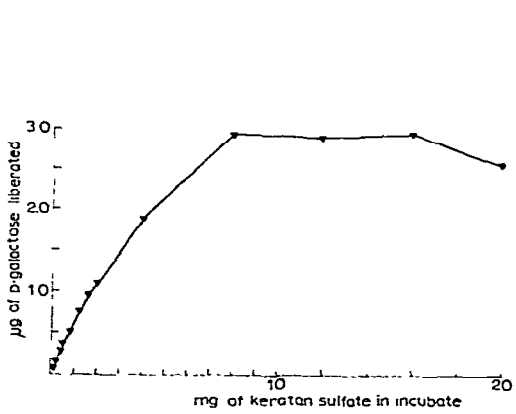
ACTIVITY IN VARIOUS HUMAN TISSUES[a] OF THE ENZYME LIBERATING D-GALACTOSE FROM KERATAN SULFATE

Tissue	*Number of samples*	*Mean mU/g (range)*
Kidney	4	85 (52–156)
Liver	13	60 (16–117)
Spleen	5	25 (16–40)
Lung	2	7.95–20.6
Jejunum	2	10.5–20.5
Brain	6	7.19 (4.29–10.6)
Urine	4	0

[a]Homogenized tissue (20 mg) was incubated with 8 mg of keratan sulfate at pH 4.0 in a volume of 200 *μl* for zero and 2 h; mU/g = international enzyme milliunits per g wet weight (nmoles of product formed, not nmoles of substrate split).

Identification of the product of enzyme activity was made by submitting the incubate to gel filtration on Sephadex G-150. The main fraction of keratan sulfate remained unchanged in its behaviour on gel filtration, but a low molecular peak appeared (Fig. 4). All of the hexose (anthrone reaction[8]) in this peak could be accounted for by D-galactose, as measured by D-galactose dehydrogenase and g.l.c. (Table IV). The amount of D-galactose liberated in this experiment, using a long incubation time and a large excess of enzyme, was *ca.* 4.5% of the total D-galactose in the purified keratan sulfate. When purified keratan sulfate was incubated extensively with a

large excess of rat-liver lysosomes, somewhat more D-galactose could be liberated than by peak II (Table V).



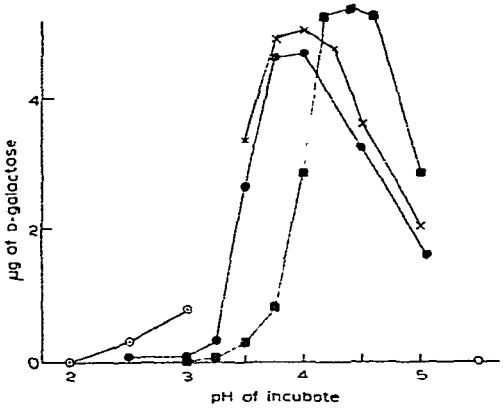
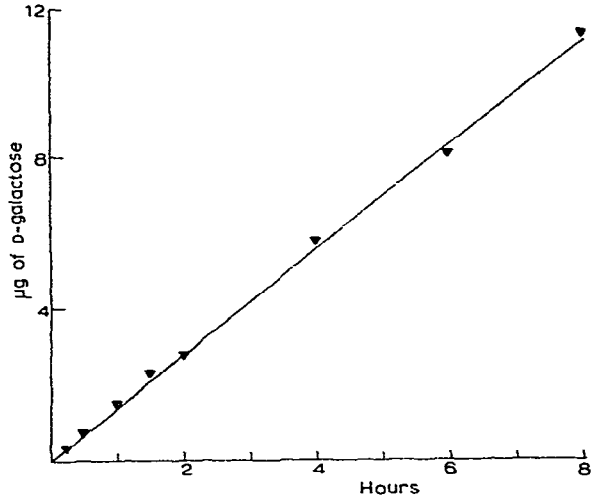


Fig. 3a. Effect of concentration of keratan sulfate on D-galactose liberation. Keratan sulfate (0.1–20 mg) was incubated for 2 h at pH 4.0, with 200 μl of a solution of 100 μl of lyophilized peak II dissolved in 6 ml of water.

Fig. 3b. pH-Dependency of D-galactose liberation from keratan sulfate. Incubation conditions as in Table I. Peak II used. Buffers: ×—× acetate, 0.25M; ⊙—⊙ glycine–HCl, 0.25M; ●—● formate, 0.25M; ■—■ citrate, 0.25M.



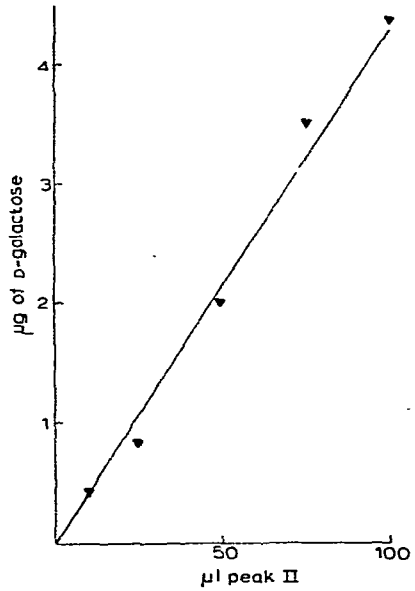


Fig. 3c. D-Galactose liberation as a function of time of incubation. Keratan sulfate (8 mg) and 100 μl of lyophilized peak II were incubated at pH 4.0 in a volume of 200 μl for the times indicated.

Fig. 3d. D-Galactose liberation as a function of amount of enzyme in the assay. Keratan sulfate (8 mg) was incubated for 2 h at pH 4.0 with various amounts of lyophilized peak II, dissolved in 6 ml of water; 100 μl of peak II contained material from 167 mg of liver.

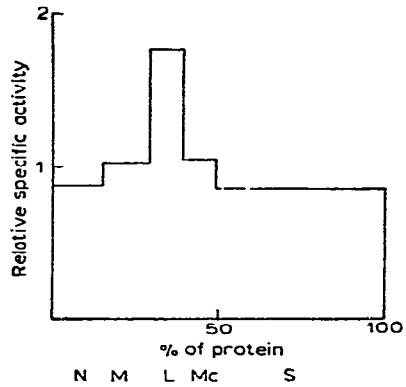


Fig. 3e. Subcellular localization of enzyme activity catalyzing the liberation of D-galactose from keratan sulfate. Rat liver was homogenized in 0.25M sucrose, followed by centrifugal fractionation of subcellular particles[4]. Relative specific activity = D-galactose liberation in fraction in per cent of total, divided by protein in fraction in per cent of total. N = nuclear fraction, M = mitochondrial fraction, L = lysosomal fraction, Mc = microsomal fraction, S = supernatant.

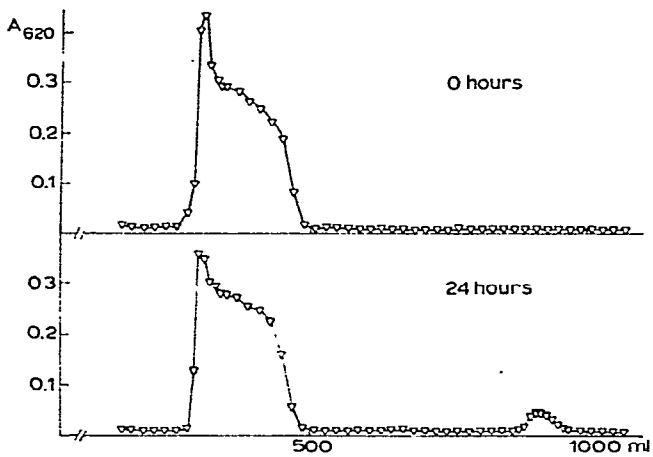


Fig. 4. Gel filtration of products of enzyme action. Keratan sulfate (20 mg) and 1 ml of lyophilized peak II from human liver were incubated for 24 h at pH 4.0 in a volume of 1.5 ml. 1.2 ml of supernatant, after centrifugation of the incubate, was applied to a column (3 × 145 cm) of Sephadex G-50 fine in water and eluted with water in 5-ml fractions. Hexose was analysed by the anthrone method[8] on 2-ml portions of each fraction. 0 h = zero minutes' blank, not incubated, containing enzyme, substrate, and buffer; 24 h = incubated sample.

To exclude further the possibility that D-galactose was liberated from a non-keratan sulfate impurity, D-galactose liberation was studied from three different preparations of keratan sulfate having different chain-length. These preparations had been extensively purified[3] from glycopeptide and other components by precipitation with cetylpyridinium chloride–sodium chloride. Table VI shows that D-galactose was liberated in inverse proportion to the chain length.

TABLE IV

ANALYSES[a] ON LOW-MOLECULAR FRACTION FROM THE EXPERIMENT DESCRIBED IN FIG. 4

D-Galactose	*Anthrone*	*D-Galactose dehydrogenase*	*G.l.c.*
Amount liberated (μg)	287	299	Galactose (other sugars < 5%)
Percentage of total D-galactose in keratan sulfate	4.3	4.6	—

[a]The anthrone-reacting, low-molecular peak in the experiment described in Fig. 4 was concentrated by lyophilization and assayed for D-galactose contents by the D-galactose dehydrogenase method and for sugar contents by g.l.c. of alditol acetates[10].

TABLE V

D-GALACTOSE LIBERATION FROM KERATAN SULFATE BY RAT-LIVER LYSOSOMES[a]

Keratan sulfate incubated (mg)	*D-Galactose liberated*	
	μg	*% of total D-galactose in the keratan sulfate added*
1.0	37	13.7

[a]Lysosomes (isolated as earlier described[4] from 10 g of rat liver) were incubated with 1 mg of keratan sulfate for 48 h at pH 4.0, and free D-galactose was measured with D-galactose dehydrogenase.

TABLE VI

D-GALACTOSE LIBERATION FROM KERATAN SULFATES OF DIFFERING CHAIN LENGTH[a]

Preparation of keratan sulfate[b]	*2-Amino-2-deoxy-D-galactose (% of total hexosamine in the molecule*[b]*)*	*Approximae molecular weight*	*Total D-galactose in keratan sulfate in assay (μg)*	*D-Galactose liberated (μg)*	*% liberated of total D-galactose*
II	21	4–5,000	62	2.7	4.4
III	11	10,000	68	1.6	2.4
IV	2	20,000–	61	0.6	1.0

[a]Keratan sulfates of differing chain length were incubated for 24 h at pH 4.0 with a large excess of enzyme (200 μl of lyophilized peak II from human liver). [b]From Antonopoulos *et al.*[3].

DISCUSSION

The fact that the substrate for the D-galactose-liberation followed keratan sulfate in all purification steps indicates that D-galactose was split off from keratan sulfate itself and not from an accompanying impurity, such as a different glycosaminoglycan or a glycopeptide. The amount of D-galactose that could be split off by extensive enzyme digestion is only a small part of the total D-galactose contents

(24, 4–27%; ref. 3) of the keratan sulfate preparations. D-Galactose in keratan sulfate is located either in the non-reducing end of the molecule or inside the molecule. The chain length of the molecule can be estimated by assay of 2-amino-2-deoxy-D-galactose, since there is one such residue in each molecule. Knowing the chain length, the maximal amount of D-galactose located at the non-reducing end, *viz.* 1.8% of the total D-galactose if the molecular weight is 20,000 (preparation IV in Table VI), 3.6% if the molecular weight is 10,000 (preparation III), and 7.2% if the molecular weight is 5,000 (preparation II). The liberation of D-galactose catalysed by peak II from these preparations was smaller and proportional to these figures. This affords further strong evidence that D-galactose was split off from keratan sulfate itself and not from an associated impurity. It also indicates that the liberated D-galactose was terminal D-galactose in the non-reducing end of the molecule.

The nature of the enzyme activity studied can be compared to that of other galactosidases. Studies with the synthetic substrate 4-methylumbelliferyl β-D-galactoside have shown[9] that human tissues contain two different β-D-galactosidases (*A* and *B*). As regards the properties shown in gel filtration, subcellular localization, and tissue distribution experiments, the activity studied in this investigation is very similar to the *A* enzyme. It is, therefore, possible that enzyme *A* is active not only against the synthetic substrate mentioned but also against keratan sulfate.

The fact that more D-galactose was liberated by rat-liver lysosomes than by more-purified enzyme preparations might indicate that keratan sulfate-degrading enzyme(s) of low activity, different from β-D-galactosidase *A*, may exist in mammalian tissues.

ACKNOWLEDGMENTS

This investigation was supported by The Swedish Medical Research Council (grant No. B69-13X-2307-02A), The University of Lund, Svenska Livförsäkringsbolags Nämnd för Medicinsk Forskning and Förstamajblommans Riksförbund. Skilful technical assistance was given by Mrs. I. Brandt and Mrs. B. Rosberg.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

ON THE STRUCTURE OF THE CARBOHYDRATE CHAINS OF THE BLOOD-GROUP SUBSTANCE (A+H)

N. K. KOCHETKOV, V. A. DEREVITSKAYA, L. M. LICHOSHERSTOV,
M. D. MARTYNOVA, AND S. N. SENCHENKOVA
Institute of Organic Chemistry, U. S. S. R. Academy of Sciences, Moscow (U. S. S. R.)
(Received August 4th, 1969)

ABSTRACT

The structure of the carbohydrate chains of blood-group substance (A+H) (BGS) in the region adjacent to the peptide backbone has been investigated. Two approaches were used: (1) a study of the degradation of BGS by a combination of chemical and enzymic (preparation from *Clostridium perfringens*) methods, and (2) a study of the alkaline degradation of BGS by measurement of the accumulated products of degradation of *N*-acetylhexosamines (3-acetamido-5-dihydroxyethylfuran) and D-galactose (metasaccharinic acid and 5-hydroxymethyl-2-furaldehyde). It has been shown that the carbohydrate–peptide linkage-unit contains a 2-acetamido-2-deoxy-D-galactose residue. Directly adjacent to this region is a chain of several *N*-acetylhexosamine residues bound by (1→3) linkages and partially branched at C-6.

INTRODUCTION

Recent structural studies of the blood-group substance (BGS) have shown the presence of a peptide backbone with carbohydrate chains attached by glycosidic bonds to serine and threonine residues[1–3]. The elucidation of the structure of some oligosaccharides released on degradation of BGS yielded information on the structure of the terminal part of the carbohydrate chains[3–6]. The structure of the carbohydrate chains in the region adjacent to the peptide backbone is unknown. We have developed two new routes for approaching this problem. Firstly, we have studied the degradation of BGS (A+H), isolated from the lining of pig stomachs, by an enzyme preparation from *Clostridium perfringens*, together with some chemical methods. Secondly, we have investigated the structure of the carbohydrate chains by estimation of the alkaline-degradation products of *N*-acetyl hexosamines and D-galactose. The latter approach, which has not been applied hitherto in studies of glycoprotein structure, may be of general value.

RESULTS AND DISCUSSION

Degradation of BGS by an enzyme preparation from Clostridium perfringens.

After treatment of BGS with the enzyme preparation, *ca.* 45% of the substance was split off as a fraction of low molecular weight; fucose (7.5%), and galactose

and *N*-acetylhexosamines (~35%) were released[7]. The peptide part of the polymer was largely unaffected, and the fragment (**1**) of high molecular weight was stable to repeated treatment with the enzyme preparation[8].

Since **1** still contains ~65% of carbohydrate, a more extensive degradation of BGS was sought by a combination of chemical and enzymic methods. BGS was treated in sequence with periodate and borohydride, and the resulting polyol (BGS I) was subjected to acid hydrolysis (pH 1.5, 3 h, 100°). The overall breakdown of BGS in this procedure is *ca.* 50%, whereas that of amino acids is less than 0.5%. The fragment (**2**) of high molecular weight subsequently isolated by gel filtration was then treated with the enzyme preparation, which resulted in cleavage of *ca.* 45% of the carbohydrate content. Further gel filtration gave a new fragment (**3**) of high molecular weight, which retained the amino acids characteristic of BGS, but in increased percentage (58%, *cf.* 16% for BGS) (see Table I). Fraction **3** contains *ca.* 40% of carbohydrate, no fucose, and ≯ 6% of galactose. The remainder of the carbohydrate content is *N*-acetylhexosamine.

TABLE I

CONTENT OF AMINO ACIDS AND HEXOSAMINES IN BGS AND ITS FRAGMENTS[a]

Preparation	*Amino acids % (a)*	*N-Acetyl-hexosamines % (b)*	*a/b*	*2-Acetamido-2-deoxy-D-galactose (μmoles)[b] (c)*	*2-Acetamido-2-deoxy-D-glucose (μmoles)[b] (d)*	*c/d*
BGS (A+H)	16.0	41.0	0.39	1.13	1.45	0.8
3	58.2	28.9	2.0	2.29	0.30	7.6
3 treated with Na_2CO_3	35.8	15.8	2.26	0.82	0.16	5.1

[a]Determined by using an amino acid analyser after hydrolysis with 4N HCl for 24 h at 100° *in vacuo*.
[b]Referred to 1 mg of substance.

The ratio of 2-acetamido-2-deoxy-D-galactose to 2-acetamido-2-deoxy-D-glucose (GluNac) in **3** is 7.6, whereas in the original BGS it is only 0.8. The preponderance of 2-acetamido-2-deoxy-D-galactose in **3** suggests that this hexosamine could constitute the sugar residues at the reducing end of the oligosaccharide chains and thereby be involved in the carbohydrate–peptide linkage, as well as forming part of the carbohydrate chains adjacent to the peptide backbone. The D-galactose and GluNac are mainly in the periphery of the molecule, since they are more readily split off during the enzymic and chemical degradation of BGS. This view was supported by the data obtained from the alkaline treatment (0.05M Na_2CO_3, 100°, 15 min) of **3**. The monosaccharides which are glycosidically linked to serine and threonine are the first to be destroyed. Analysis of the mixture of alkaline-degradation products of **3** showed the decrease of 2-acetamido-2-deoxy-D-galactose (1.47 μmoles, Table I) to be 10.5 times that of 2-acetamido-2-deoxy-D-glucose (0.14 μmole).

The degradation of BGS with alkali

β-Elimination of the carbohydrate chains from the serine and threonine units of the peptide chain is known to proceed upon treatment of BGS with alkali. The carbohydrate chains liberated are then eroded from the reducing end to form a series of oligosaccharides that are characteristic of the terminal sequence of the monosaccharides (*cf.* refs. 4 and 6).

The rapid degradation of the carbohydrate chains at the reducing end, which is observed on treating BGS with alkali, is characteristic of polysaccharides having (1→3) linkages[9]. The degradation of such chains should result in the formation of 3-acetamido-5-dihydroxyethylfuran and, probably, 2-acetamido-2,3-dideoxy-D-hex-2-enofuranose from *N*-acetylhexosamine residues. These compounds give the direct Ehrlich reaction, and the presence of chromogens in the mixture of alkaline-degradation products of BGS was reported earlier[10].

Metasaccharinic acid should be formed from the residues of galactose substituted at C-3, and isosaccharinic acid[11] from galactose residues substituted at C-4.

A preliminary study of the degradation of BGS in 0.05M Na_2CO_3 has shown that the accurate determination of the amount of metasaccharinic and isosaccharinic acids by periodate oxidation is complicated by the sensitivity of BGS to periodate. Therefore, both BGS and the product (BGS I) obtained after periodate oxidation and borohydride reduction were subjected to alkaline degradation[12].

The alkaline conditions chosen were based on model experiments with 2-acetamido-2-deoxy-D-glucose which was heated in 0.05M Na_2CO_3 at 50, 70, and 100° (Fig. 1); 70° proved to be optimal, and, at this temperature, chromogen forms rapidly and is stable upon prolonged heating.

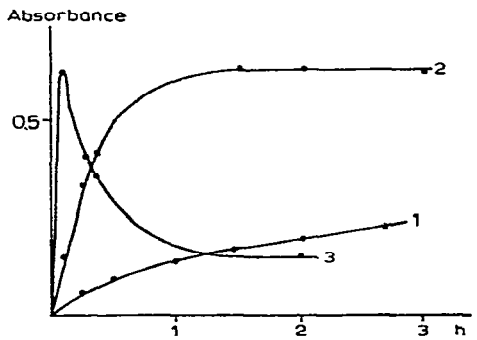


Fig. 1. The rate of formation of chromogen from 2-acetamido-2-deoxy-D-glucose on treatment with 0.05M Na_2CO_3 as determined by the Ehrlich reaction: 1, 50°; 2, 70°; 3, 100°.

Chromogen and metasaccharinic acid are formed upon treating BGS and BGS I in 0.05M Na_2CO_3 at 70°. Isosaccharinic acid was not detected[11], even after acid hydrolysis (0.5N HCl, 100°, 12 h) of the alkaline-degradation products of BGS. This indicates the absence of 4- and 4,6-substituted galactose residues in the eroded parts of the carbohydrate chains.

Upon treatment of BGS and BGS I with aqueous Na_2CO_3 (Fig. 2, Table II), chromogen was liberated rapidly; its release was markedly decreased in 4 h. Metasaccharinic acid formation began 7 min after treatment of BGS I with alkali, and the amount increased very slowly, corresponding, after 4 h, to ~0.5% of galactose (of BGS) destruction. During this time, 10.5% of the *N*-acetylhexosamines were cleaved (Table II).

The rates of *β*-elimination reactions of 2-acetamido-2-deoxy-D-glucosyl and D-galactosyl derivatives of the methylamide of *N*-benzyloxycarbonyl-L-serine were

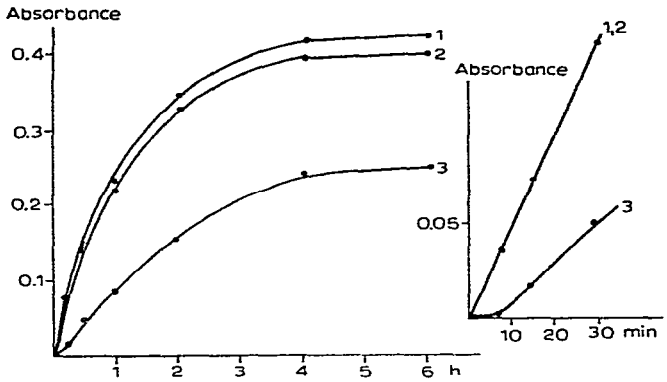


Fig. 2. The rate of formation of chromogen and metasaccharinic acid from BGS and BGS I on treatment with 0.05M Na_2CO_3 at 70°. 1, BGS; 2, BGS I (by Ehrlich reaction); 3, BGS I (by reaction with thiobarbituric acid).

TABLE II

THE NUMBER OF DEGRADED UNITS (% OF THE WEIGHT OF BGS AND BGS I) OF *N*-ACETYLHEXOSAMINES [CALCULATED FROM THE 3-ACETAMIDO-5-DIHYDROXYETHYLFURAN (I) FORMED AND FROM AMINO ACID ANALYSIS (AAA) DATA] AND OF GALACTOSE [CALCULATED FROM THE METASACCHARINIC ACID (II) AND 5-HYDROXYMETHYL-2-FURALDEHYDE (III) FORMED] UPON TREATMENT OF BGS AND BGS I WITH 0.05M Na_2CO_3 AT 70°.

Time of the treatment with Na_2CO_3 (min)	*BGS*[a]		*BGS I*[b]			
	N-Acetylhexosamines from I	*D-Galactose from III*	*N-acetylhexosamines*		*D-Galactose*	
			From I	*From aaa*	*From II*	*From III*
7	0.52	0.02	0.52	—	0.00	0.01
14	1.14	—	1.15	—	0.03	—
30	2.1	0.14	—	—	0.10	0.16
60	3.3	0.31	3.3	—	0.17	0.29
120	5.2	0.50	4.9	—	0.31	0.49
240	6.5	0.72	6.1	10.4	0.48	0.61
360	6.6	0.71	6.2	10.6	—	0.62
480	—	0.71	—	—	—	0.62

[a]The percentage of *N*-acetylhexosamines, galactose, and fucose in the original BGS is 43, 22, and 10%, respectively. [b]The percentage of *N*-acetylhexosamines, galactose, and fucose in original BGS I is 26, 10, and 0%, respectively.

approximately equal (Table III). Therefore, the difference in the rates of release of metasaccharinic acid and chromogen cannot be accounted for on this basis. Nor can the absence of metasaccharinic acid at the beginning of the degradation be accounted for by the differences in the rate of formation of metasaccharinic acid and chromogen. The degradation[13] of laminarin, having a known number of reducing ends (kindly provided by Professor D. G. Manners), upon treatment with 0.05M Na_2CO_3 at 70° results in rapid formation of metasaccharinic acid (Fig. 3). Thus, 3 moles of metasaccharinic acid are released in 7 min from each reducing end of laminarin, under conditions where no metasaccharinic acid was formed from BGS. The decrease in the glucose content (anthrone method) in laminarin after alkaline degradation corresponds precisely to the amount of metasaccharinic acid liberated.

TABLE III

DEGRADATION OF THE GLYCOSIDIC LINKAGE OF THE *O*-β-D-GALACTOSYL (5) AND *O*-2-ACETAMIDO-2-DEOXY-β-D-GLUCOSYL (6) DERIVATIVES OF *N*-BENZYLOXYCARBONYL-L-SERINE METHYLAMIDE UPON TREATMENT WITH 0.05M Na_2CO_3 AT 70°.

Treatment time (min)	*Cleavage of* 5 *(% of sample)*	*Cleavage of* 6 *(% of sample)*
15	77	72
30	96	97
60	100	100

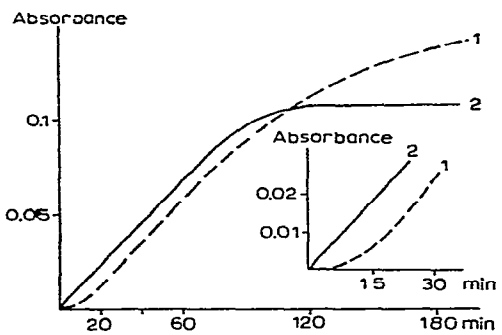


Fig. 3. The rate of formation of metasaccharinic acid from BGS I and laminarin on treatment with 0.05M Na_2CO_3 at 70°. 1, BGS I; 2, laminarin (by reaction with thiobarbituric acid).

Thus, the data obtained from the treatment of BGS and BGS I with 0.05M Na_2CO_3 at 70° indicate that (1→3)-linked galactose residues (as well as those[11] branched at C-6) do not contribute to the carbohydrate–peptide linkage and are essentially absent from the parts of the carbohydrate chains immediately adjacent to the peptide backbone. Apparently, these portions are made up of *N*-acetylhexosamine residues. The possible presence of a small number of (1→3)-linked galactose residues branched at C-4 or C-2 in this part of the BGS molecule is not excluded by this evidence. (1→3)-Linked galactose residues that are branched at C-4 are known to

afford 4-substituted metasaccharinic acid in alkaline medium, which cannot be directly determined[11]. However, after acid hydrolysis (0.5N HCl, 100°, 12 h) of the alkaline-degradation products of BGS, there was no increase in the yield of metasaccharinic acid. Hence, the degraded chains do not contain any 3,4-disubstituted galactose residues.

By analogy with glucose derivatives, 2,3-disubstituted galactose residues should be readily converted by alkali into 2-substituted 3-deoxyhex-2-enose derivatives which, under mild, acidic conditions, should yield 5-hydroxymethyl-2-furaldehyde[14]. Actually, 5-hydroxymethyl-2-furaldehyde is formed on heating 2,3-di-*O*-methyl-D-galactose in 0.05M Na_2CO_3 for 7 min or 2 h at 70°, followed by acidic treatment, to extents corresponding to 9 or 58% degradation. Similar treatment of D-galactose gave no 5-hydroxymethyl-2-furaldehyde. To detect 2,3-disubstituted galactose residues in the cleaved parts of the carbohydrate chains, BGS and BGS I were treated with aqueous Na_2CO_3 at 70°, and then with acid at 50°. The absorption measured at 284 nm then corresponds to 5-hydroxymethyl-2-furaldehyde[14]. The amount of 2-substituted 3-deoxyhex-2-enose formed and determined from 5-hydroxymethyl-2-furaldehyde increased slowly and, after 4 h, corresponded to 0.6–0.7% of galactose (of BGS). This, however, cannot radically change the conclusions that have been previously drawn, since the possible presence of 2,3-disubstituted galactose in the carbohydrate–peptide linkage unit has been ruled out. The presence of small proportions of 3- and 2,3-substituted galactoses (*ca.* 1.0% from BGS) in the reducing end of the carbohydrate chain has not been explained so far, but it might indicate the inhomogeneity of the BGS carbohydrate chains.

All of these data support the conclusion that there are 2-acetamido-2-deoxy-D-galactose residues in the region of the glycosylpeptide linkage of BGS.

After 4 h of alkaline degradation of BGS, chromogen formation ceases (Fig. 2), indicating completion of the process. The alkaline-degradation products obtained after 4 h of treatment of the BGS I with Na_2CO_3 were separated on Biogel P-6 into two fractions (Fig. 4). Almost all of the amino acids were in the fraction (**4**) of high molecular weight. The amino acid content of the low molecular fraction was 0.5% of BGS I (Table IV). The yield of **4** is *ca.* 50%, and, hence, the alkaline treatment results in cleavage of *ca.* 60% of the carbohydrate residues of BGS. The carbohydrate remaining bound to the peptide skeleton of the glycoprotein is not split off under the alkaline conditions chosen.

The analysis of the chromogens formed provides further information concerning the carbohydrate portion of BGS which is degraded by alkali. Chromogens are formed only from 3- and 6-monosubstituted and 3,6-disubstituted *N*-acetylhexosamines[15]. An unsubstituted chromogen should form from the residues of (1→3)-linked *N*-acetylhexosamines which arose from alkaline degradation of BGS carbohydrate chains; (1→6)-linked *N*-acetylhexosamines or (1→3)-linked units branched at C-6 should yield a substituted chromogen. Since (1→4)-linked residues are stable to alkali, the *N*-acetylhexosamine residues at the reducing end of BGS I carbohydrate chains should be bound by (1→3) links, with or without branches at C-6. Gel-filtra-

TABLE IV

AMINO ACID[a] CONTENT OF BGS I, AND FRACTIONS 4, AND LMF 4

Amino acids	*BGS I* (%)	*4* (%)	*LMF 4* (%)
Asparaginic acid	0.31	0.57	0.06
Threonine	5.09	5.48	0.13
Serine	2.20	1.43	0.13
Glutamic acid	0.76	1.38	0.16
Proline	3.10	6.78	0.13
Glycine	0.37	0.56	0.10
Alanine	0.91	1.75	0.11
Valine	0.92	1.47	0.11
Isoleucine	0.15	0.23	0.04
Leucine	0.64	1.12	0.07
Σ Amino acids	14.45	20.77	1.04

[a]Excluding the basic amino acids.

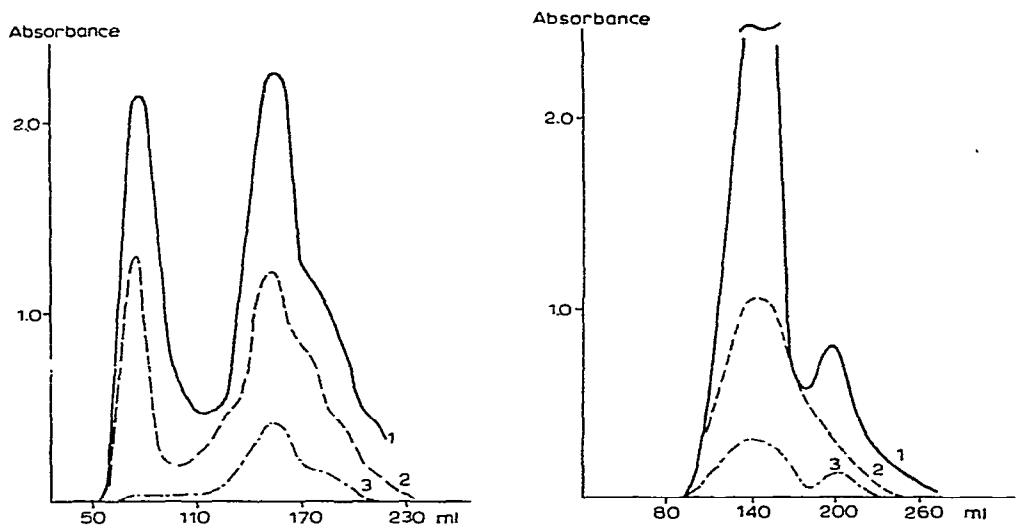


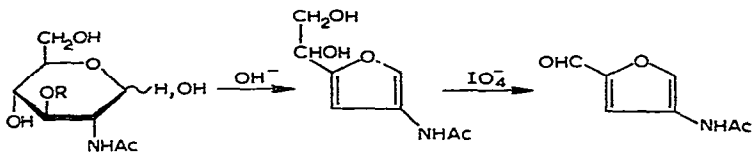
Fig. 4. Gel-filtration of products from alkaline hydrolysis of BGS I (0.05M Na_2CO_3, 70°, 4 h) on a column (1.5 × 60 cm) of Biogel P-6 in 0.1N CH_3COOH. Fractions were assayed by 1, absorbance at 232 nm; 2, by the phenol–sulphuric acid method; and 3, by the Ehrlich reaction.

Fig. 5. Gel-filtration of LMF 4 (0.05M Na_2CO_3, 70°, 4 h) on a column (1.5 × 60 cm) of Biogel P-2 in 0.1N CH_3COOH. Fractions were assayed by: 1, absorbance at 232 nm; 2, by the phenol–sulphuric acid method; and 3, by the Ehrlich reaction.

tion on Biogel P-6 (Fig. 4) shows that the chromogen formed from BGS I accumulated solely in the fraction of low molecular weight (LMF 4). The fractionation of LMF 4 on Biogel P-2 (Fig. 5) gave two chromogen peaks (I and II) which probably correspond to a substituted and an unsubstituted chromogen.

Further evidence for the presence of two types of chromogen in LMF 4 was

based on periodate oxidation of the unsubstituted chromogen to form 3-acetamido-2-furaldehyde, according to the scheme:



2-Acetamido-2-deoxy-D-glucose and its 3,6-dimethyl ether were used as model compounds. After heating in 0.05M Na_2CO_3 at 70°, with subsequent periodate oxidation, the chromogens formed were determined by the direct Ehrlich reaction. 3-Acetamido-2-furaldehyde reacted rapidly, but the resulting colour was unstable and disappeared in 15 min. 3-Acetamido-5-(1-hydroxy-2-methoxyethyl)furan was not oxidized with periodate, and colour formation in the Ehrlich reaction reached a maximum in 15–20 min. This distinctive behaviour of the chromogens allowed their separate determination. Thus, analysis of fractions I and II (Fig. 5) obtained from LMF 4 by this method confirmed that it is mainly the substituted chromogen which is formed from the fraction of higher molecular weight, whereas the fraction of low molecular weight affords the unsubstituted chromogen. The absorbances of chromogen reaction-products formed from equimolecular amounts of 3-substituted and 3,6-disubstituted 2-acetamido-2-deoxy-D-glucose in the Ehrlich reaction are nearly equal[15]. The ratio of the absorbances of the reaction products from fractions I and II (Fig. 5) is *ca.* 3:1, and, hence, most of the hexosamines constituting the reducing end of carbohydrate chains of BGS I are bound by (1→3) links and are branched at position 6. This conclusion is supported by the fact that experiments (A. V. Sharova) on the acid hydrolysis of BGS give oligosaccharides containing only hexosamine residues.

EXPERIMENTAL

BGS (A+H) from pig-stomach linings (at pH 3) and BGS I[12] were obtained by literature procedures. The enzyme preparation was isolated[7] from culture liquid of *Clostridium perfringens* (type A). Neutral monosaccharides were determined by the phenol–sulphuric acid[17] and anthrone methods[18], fucose by the Dische method[19], and hexosamines by the Elson–Morgan method after preliminary hydrolysis with 2N HCl for 2 h at 100°. Amino acids and hexosamines were determined on an amino acid analyser after hydrolysis with 4N HCl for 24 h at 100° *in vacuo*. Paper chromatography was carried out with 6:4:3 butyl alcohol–pyridine–water and detection with alkaline silver nitrate.

Acid hydrolysis of BGS I. — A solution of BGS I (1.6 g) in 96 ml of diluted HCl (pH 1.5) was heated for 3 h at 100°, neutralized with Dowex-2 (CH_3COO^-), and loaded onto a column (69 × 3.9 cm) of Sephadex G-50. Fractions (40 ml) were collected, and their absorbance at 232 nm was measured. The sugars were determined by the phenol–sulphuric acid method. The fractions corresponding to the high

molecular weight substance were collected, evaporated, and freeze-dried to yield 720 mg of HMF 2.

Enzymic hydrolysis of **2**. — To a solution of 715 mg of **2** in 71.5 ml of 0.01M phosphate buffer (pH 6.9), a 0.1% aqueous solution (36 ml) of the enzyme preparation was added, and the mixture was incubated for 18 h at 37°. Low molecular weight products were separated by gel-filtration on Sephadex G-50, and the high molecular weight substance was freed from enzyme by gel-filtration on Sephadex G-200. Yield: 400 mg of **3**.

The enzymic hydrolysis of BGS (A + H) was performed in a manner analogous to that described above.

Alkaline degradation of BGS (A + H) and BGS I. — BGS or BGS I (50 mg) was dissolved in 10 ml of 0.05M Na_2CO_3 and heated at 70°. Aliquots were taken for determination of chromogen (0.5 ml), metasaccharinic acid (2 × 0.2 ml), galactose (0.1 ml), and 5-hydroxymethyl-2-furaldehyde (0.5 ml), after 7, 15, 30, 60, 120, 240, 360, and 480 min.

The quantity of chromogen formed from the *N*-acetylhexosamines was determined by using Ehrlich's reagent[14]. Water (0.5 ml) and 1 ml of *p*-dimethylaminobenzaldehyde solution (10 mg/ml of 50% CH_3COOH) were added to the aliquot with shaking, and then 0.2 ml of conc. HCl was added. Absorbance was measured at 540 nm after 10–15 min.

Free and 6-substituted metasaccharinic acids and isosaccharinic acid were determined by the method of Barker *et al.*[11], and 4- and 4,6-substituted metasaccharinic acids and 5-substituted isosaccharinic acid were determined[11] after preliminary hydrolysis of the alkaline-degradation products with 0.5N HCl for 12 h at 100°.

5-Hydroxymethyl-2-furaldehyde (**7**) was determined by the following procedure: 0.1N HCl (0.54 ml) was added to the aliquot (0.5 ml) which was then diluted with water to 4 ml. The absorbance of the solution was measured at 284 nm, the solution was then heated at 50° for 12, 15, 18, and 24 h, and the absorbance was remeasured. The quantity of **7** was calculated by using the molar extinction coefficients[14] (Table II). The reaction mixture was extracted twice with butyl alcohol, and the u. v. spectrum of the extract was identical to that of **7** (ref. 14). No **7** was found when D-galactose was treated with 0.05M Na_2CO_3 at 70°.

*Alkaline degradation of 2-acetamido-2-deoxy-*D*-glucose.* — A solution of 5 mg of the sugar in 5 ml of 0.05M Na_2CO_3 was heated at 50, 70, and 100°. Aliquots (0.5 ml) were taken after 5, 15, 30, 60, 120, and 180 min, and the quantity of chromogen formed was determined by the aforementioned method (Fig. 3).

Alkaline degradation of laminarin. — This was accomplished under the conditions used for BGS degradation, with laminarin concentrations of 0.1 and 0.05 mg/ml. Aliquots (0.2 ml) were taken from the reaction mixture after 3, 7, 10, 20, 40, 60, 120, and 180 min, and the metasaccharinic acid was determined[11] (Fig. 2). Glucose was determined by the anthrone method in aliquots taken from the original reaction mixture and after heating for 120 min.

*Alkaline degradation of 2,3-di-*O*-methyl-*D*-galactose.* — The sugar (2 mg)

was dissolved in 40 ml of 0.05M Na_2CO_3 and heated at 70°. Aliquots (1 ml) were taken after 7, 15, 30, 60, 90, and 120 min, 0.1N HCl (1.03 ml) was added, the solution was diluted with water to 3 ml (final HCl concentration, 0.01N), and the u. v. spectrum of the mixture was recorded after heating for 24 h at 50°.

Alkaline degradation of O-β-D-*galactosyl and* O-(*2-acetamido-2-deoxy-β-*D-*glucosyl*) *derivatives of* N-*benzyloxycarbonyl*-L-*serine methylamide.* — The reaction was performed under the same conditions as for the degradation of BGS, using a 0.5 mg/ml solution of glycosides. Aliquots (0.2 ml) were taken after 15, 30, 60, and 120 min. The determination of the degree of destruction (see Table III) was based on the estimation of formaldehyde[20] after periodate oxidation. The amount of formaldehyde is equivalent to that of free monosaccharides formed from glycosides upon β-elimination.

The separation of products of alkaline degradation of BGS. — BGS I (200 mg) was dissolved in 40 ml of 0.05M Na_2CO_3 and heated for 4 h at 70°. The solution was neutralized with Amberlite IR-120 (H^+) batchwise, concentrated under diminished pressure, loaded onto a column (60 × 1.5 cm) of Biogel P-6, and eluted with 0.1N CH_3COOH. Fractions (6 ml) were collected, and the absorbance was measured at 232 nm. Fractions were assayed for sugar content by the phenol–sulphuric acid method, and chromogen was determined by the method mentioned above. Fig. 4 shows the gel-filtration pattern. The fractions corresponding to the fragment (**4**) of high molecular weight (fraction 1) and that (LF **4**) of low molecular weight (fraction 2) were concentrated and freeze-dried to give **4** (92 mg) and LF **4** (76 mg). LF **4** (76 mg) was dissolved in 2 ml of 0.1M CH_3COOH, loaded onto a column (60 × 1.5 cm) of Biogel P-2, and eluted with 0.1N CH_3COOH. Fractions (6 ml) were collected, their absorbance at 232 nm was measured, and the content of sugars and chromogen was determined in aliquots. Fig. 5 shows the gel-filtration pattern. The fractions corresponding to fragments of high molecular weight (fraction I) and low molecular weight (fraction II) were concentrated and freeze-dried to give fraction I (42 mg) and fraction II (20 mg).

Determination of the substituted chromogen in the presence of unsubstituted one. — $NaIO_4$ solution (0.2 ml; 0.0618M $NaIO_4$ in 3M H_3PO_4) was added to 0.5 ml of the solution under investigation, and the mixture was shaken and left at room temperature for 20 min. A portion (1 ml) of a Na_3AsO_3 solution (10% of Na_3AsO_3 in 0.5M Na_2SO_4) was added, and the solution was shaken until the brown colour disappeared (2–3 min) and then diluted with water to 2 ml. Then 2 ml of a solution containing 10 mg of *p*-dimethylaminobenzaldehyde per ml of 50% CH_3COOH and 0.4 ml of conc. HCl were added, and the absorbance at 540 nm was measured after 15–20 min.

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Note

An enzymic method for the determination of chain lengths of polysaccharides

D. W. NOBLE AND R. J. STURGEON
Department of Brewing and Biochemistry, Heriot-Watt University, Edinburgh (Great Britain)
(Received July 9th, 1969; in revised form, August 11th, 1969)

INTRODUCTION

The average chain-length of (1→3)- and (1→4)-linked polysaccharides is obtained by a measure of the release of one molecular proportion of formic acid from each non-reducing end-group on periodate oxidation of the polymer. Titrimetric[1,2] and potentiometric[3] methods have been described for the determination of formic acid, together with manometric[4], enzymic[5,6], and spectrophotometric[7] procedures. In the controlled degradation of polysaccharides by periodate oxidation, reduction, and partial hydrolysis with acid (Smith degradation), glycerol is released from non-reducing hexose units. Thus, a (1→4)-linked glucan gives rise to glycerol from the non-reducing, terminal residues, and erythritol from the (1→4)-linked residues. Identification and quantitative analysis of these alditols by further oxidation has also been used to give information on the chain length of polysaccharides[8]. In addition, alditols may also be analysed by means of gas–liquid chromatographic techniques[9,10].

We now describe an enzymic method for the determination of glycerol which is released during the final step of the Smith degradation of a (1→4)-linked glucan.

EXPERIMENTAL

Glycerol kinase (GK) was purchased from C. F. Boehringer, α-glycerophosphate dehydrogenase (GDH), nicotinamide adenine dinucleotide (NAD), and adenosine triphosphate (ATP) were purchased from Sigma Chemical Company.

Spectrophotometric measurements were made at 340 nm at 25° in a Unicam SP 800 spectrophotometer fitted with an external recorder. Formic acid was determined potentiometrically[1] at pH 6.0 with sodium hydroxide (0.01N). Total hexose was measured by the orcinol–sulphuric acid method[11].

Standardised procedure for the periodate oxidation and Smith degradation of samples. — Oligosaccharides (1 mg) and polysaccharides (5 mg) were dissolved in water (1 ml), and sodium metaperiodate was added to a final concentration of 0.015M. The oxidation was carried out in the dark at room temperature. Portions (0.40 ml)

were removed at intervals, and the excess of periodate was destroyed by the addition of ethylene glycol (0.20 ml). An aqueous solution of sodium borohydride (10 mg/ml, 0.30 ml) was added, and the samples were allowed to stand for 18 h at room temperature. Sulphuric acid (N, 1 ml) was added, the samples were heated for 1 h at 100°, cooled, and neutralised with sodium hydroxide to pH 7.0, and the volumes were adjusted to 5 ml.

Determination of glycerol. — To portions (0.1 ml) of the oxidised, reduced, and hydrolysed samples, the following additions were made: buffer [M hydrazine hydrate, 0.2M glycine, Mg^{2+} (2×10^{-3}M): pH 9.8, 2.7 ml], ATP (0.05M, 0.10 ml), NAD (0.02M, 0.05 ml), GDH (100 units/ml, 0.02 ml).

After determination of the initial absorbance, the reaction was started by the addition of GK (85 units/ml, 0.02 ml). Absorbances were measured continuously until no further change was observed (10–15 min).

RESULTS

The reactions used in the assay for glycerol are based on two coupled enzyme-reactions[1,2]:

$$\text{glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{L-glycerol 1-phosphate} + \text{ADP}$$

$$\text{L-glycerol 1-phosphate} + \text{NAD} \xrightarrow{\text{GDH}} \text{dihydroxyacetone (1,3-dihydroxy-2-propanone) phosphate} + \text{NADH} + \text{H}^+.$$

Portions of glycerol solutions containing 0.5–5.0 μg of glycerol were analysed by the standard procedure. The changes in absorbance were determined at 340 nm by using 10-mm cuvettes (capacity 3.0 ml) for solutions containing 2.5–5.0 μg of glycerol, and 10-mm cuvettes (capacity 0.75 ml) for solutions containing 0.5–2.5 μg of glycerol. The extinction coefficient of NADH at 340 nm is taken as 6.22×10^6 cm^2/mol. The resulting calibration was linear up to 5.0 μg of glycerol.

The effect on the enzymic assay of various inorganic ions and of fragments from the Smith degradation of polysaccharides was investigated. Borate, iodate, and sulphate ions in the concentration used in the oxidation, reduction, and hydrolysis stages had no effect on the calibration of the assay. No change in absorbance was observed when glycerol was replaced by D-glucose, D-galactose, D-mannose, D-mannitol, L-threitol, erythritol, ethylene glycol, glycolaldehyde, D-glyceraldehyde, or *O*-β-D-glucopyranosyl-(1→1)-D-glycerol. In the presence of glycerol (2.0 μg) and 200 μg of each of the individual compounds, no inhibition of the enzymic reaction was observed.

Determination of the glycerol released on hydrolysis of periodate-oxidised and borohydride-reduced methyl α-D-glucopyranoside. — A solution of methyl α-D-glucopyranoside (2.5 mg/ml) was treated with sodium metaperiodate by using the standard conditions. Portions were removed at various time intervals, and the glycerol content was determined by the standard procedure (see Fig. 1).

Determination of the glycerol released on hydrolysis of periodate-oxidised and borohydride-reduced oligo- and poly-saccharides. — The quantity of glycerol produced

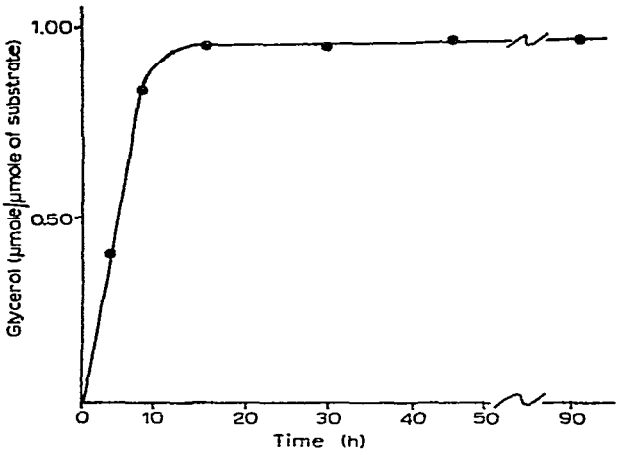

Fig. 1. Release of glycerol from methyl α-D-glucopyranoside.

from the oxidation of a series of oligosaccharides by the standard procedure is given in Table I. The periodate oxidation of a number of polysaccharides by the standard procedure was followed by the production of formic acid and glycerol to provide an estimate of the average chain-length (CL) of the polymers by the two procedures (see Table II).

TABLE I

RELEASE OF GLYCEROL FROM PERIODATE-OXIDISED AND BOROHYDRIDE-REDUCED OLIGOSACCHARIDES

Substrate	*Oxidation time (h)*	*Glycerol (% of theory)*
Cellobiose	90	97.1
	190	99.7
Laminaribiose	90	95.0
	190	98.6
Maltose	180	99.1
O-β-D-Glucopyranosyl-(1→1)-D-glycerol[a]	—	98.5

[a]Periodate-oxidation and borohydride-reduction step was omitted, and the material was hydrolysed with acid. The ratio of D-glucose:glycerol was 1:0.96.

DISCUSSION

Many of the existing methods for the estimation of the chain lengths of polysaccharides depend on determination of the formic acid liberated during oxidation with sodium metaperiodate. This may involve the use of relatively large amounts of polymer (50–500 mg). In titrimetric methods, care has to be exercised in the inter-

TABLE II

RELEASE OF GLYCEROL FROM PERIODATE-OXIDISED AND BOROHYDRIDE-REDUCED POLYSACCHARIDES

Substrate	*Oxidation time (h)*	*CL (formic acid)*	*CL (μmoles of "anhydroglucose"/ μmole of glycerol)*
Amylopectin (potato, Red Star)	208	24	23
Amylopectin (malted barley)	224	20	18
Glycogen (rabbit liver)	240	15	14
Glycogen (rat liver)	230	13	13
Glycogen (*Ascaris lumbricoides*)	230	12	12
Laminarin	410	28	25

pretation of results when overoxidation due to hydrolysis of formic esters or titration of other acids may occur[14]. Similarly, in the estimation of alditols derived from the oxopolysaccharides, interference due to inorganic ions has to be overcome[8,10]. The enzymic assay for glycerol does not suffer from these disadvantages. L-Glycerol 1-phosphate is reported to be the only substrate for glycerophosphate dehydrogenase[13]. Glycerokinase will phosphorylate[13] dihydroxyacetone to dihydroxyacetone phosphate in the presence of ATP, but, since this does not involve the production of NADH, there will be no interference in the assay. The hydrolysis of formic esters has been shown[15] to occur with high concentrations of periodate ion and at pH values of 1.0 and 7.0, respectively, with the formation of substituted hydroxymalonaldehyde structures. These would then release glycerol on subsequent reduction with borohydride and hydrolysis. The release of glycerol corresponded to the degree of polymerisation of a number of oligosaccharides, and the glycerol derived from polysaccharides gave values for the average chain-lengths of these polymers in agreement with values obtained from the release of formic acid, suggesting that minimal hydrolysis of formic esters had taken place.

The method can only be applied to measurements of the average chain-lengths of polysaccharides containing (1→3)- and (1→4)-linkages, since, in those polymers containing either (1→2)- or (1→6)-linkages, glycerol can be expected to be produced from these residues, as well as from the non-reducing end-group, after borohydride reduction and hydrolysis of the oxidised polysaccharides.

ACKNOWLEDGMENTS

We thank Professor D. J. Manners for his interest in this work, Dr. J. C. P. Schwarz for a sample of L-threitol, Dr. D. E. Brundish for a sample of *O*-β-D-glucopyranosyl-(1→1)-D-glycerol hexaacetate, and the Brewers' Society for a grant (D. W. N.).

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Note

P.m.r. spectra and conformation of cellulose oligosaccharides in solution in methyl sulphoxide

A. J. MICHELL
CSIRO, Division of Forest Products, P.O. Box 310, South Melbourne 3205 (Australia)

(Received June 3rd, 1969; in revised form, August 15th, 1969)

Casu and co-workers have used the p.m.r. technique to study the hydrogen bonding and conformations of some glucoses, disaccharides (including cellobiose), and polyglucoses of the α-D-(1→4) series[1] and of some of their *O*-methylated derivatives[2] in solution in methyl sulphoxide. We now present the results of similar studies on other cellulose oligosaccharides, which are of interest because of their structural relationship to the celluloses[3].

The 100-MHz p.m.r. spectra of β-D-glucose and of the oligosaccharides are shown in Fig. 1 and of methyl β-D-glucoside and methyl β-D-cellobioside in Fig. 2. The chemical shifts and splittings are given in Table I.

Bands in the spectra of β-D-glucose and methyl β-D-glucoside have been assigned[1] to H-1 and a number of the hydroxyl-proton resonances, on the basis of their chemical shifts and splittings and their response to the addition of deuterium oxide. This work has been repeated and confirmed, and, in addition, spin-decoupling experiments have been carried out. After the addition of deuterium oxide to a solution of β-D-glucose in methyl sulphoxide, no new signals attributable to α-D anomers were found, indicating that the exchange took place without mutarotation. When the decoupling field was applied at the position of the axial H-1 signal, changes near τ 7.0 were observed in the spectra of both deuterated materials, showing that there was a proton at this field spin-coupled to the anomeric proton. This result is consistent with that of Lemieux and Stevens[4] who found that in a mixture of glucoses at tautomeric equilibrium the H-2 resonance of β-D-glucose occurred at τ 6.68 (tetramethylsilane as external reference).

In the spectra of cellobiose and cellotriose, in addition to the signals at τ 3.38 arising from the HO-1 resonance of the β-D anomer, there are signals near τ 3.70 arising from the HO-1 resonance of α-D anomers[1]. For cellobiose, the ratio of the band integrals was *ca.* 4:1, and for cellotriose *ca.* 2:5. However, in the spectra of cellotetraose and cellopentaose, only very weak, unresolved signals near τ 3.38 were observed for the HO-1 proton.

In the spectra of deuterated cellobiose and cellotriose, there are doublets at τ 5.12 (3.0 Hz) and apparent triplets (probably pairs of doublets)[1] at τ 5.72 (7.0 Hz) arising from the equatorial and axial H-1 resonances, respectively, and having

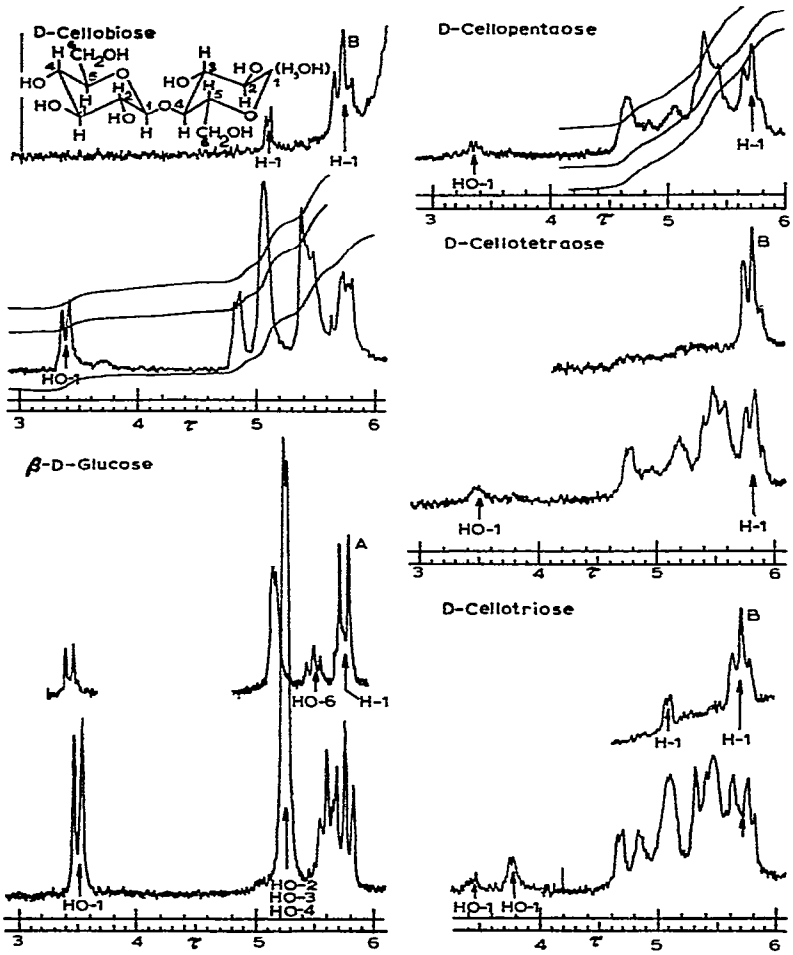

Fig. 1. Partial 100-MHz p.m.r. spectra of β-D-glucose and of some cellulose oligosaccharides. A: After partial exchange with D_2O. B: After almost complete exchange with D_2O.

integral ratios of 1:7 for cellobiose and 1:4 for cellotriose. The spectra of deuterated cellotetraose, however, showed only the triplet-shaped signal at τ 5.74 for H-1. These results indicate that α-D anomers composed 20% of the sample of cellobiose and 65% of the sample of cellotriose, but only very low percentages of the samples of cellotetraose and cellopentaose. The i.r. spectra (KBr) indicated similar compositions; the spectrum of cellobiose showing strong type 2b absorption near 890 cm^{-1} characteristic of β-D anomers, but only weak type 2a absorption near 835 cm^{-1} characteristic of α-D anomers[5], that of cellotriose type 2a and 2b bands of comparable intensity, and those of cellotetraose and cellopentaose type 2b bands only. The spectra of cellotetraose and cellopentaose were almost identical, providing further evidence of the structural similarities of the higher oligosaccharides[6].

The splittings (7.0 Hz) of the H-1 signals of the oligosaccharides are similar

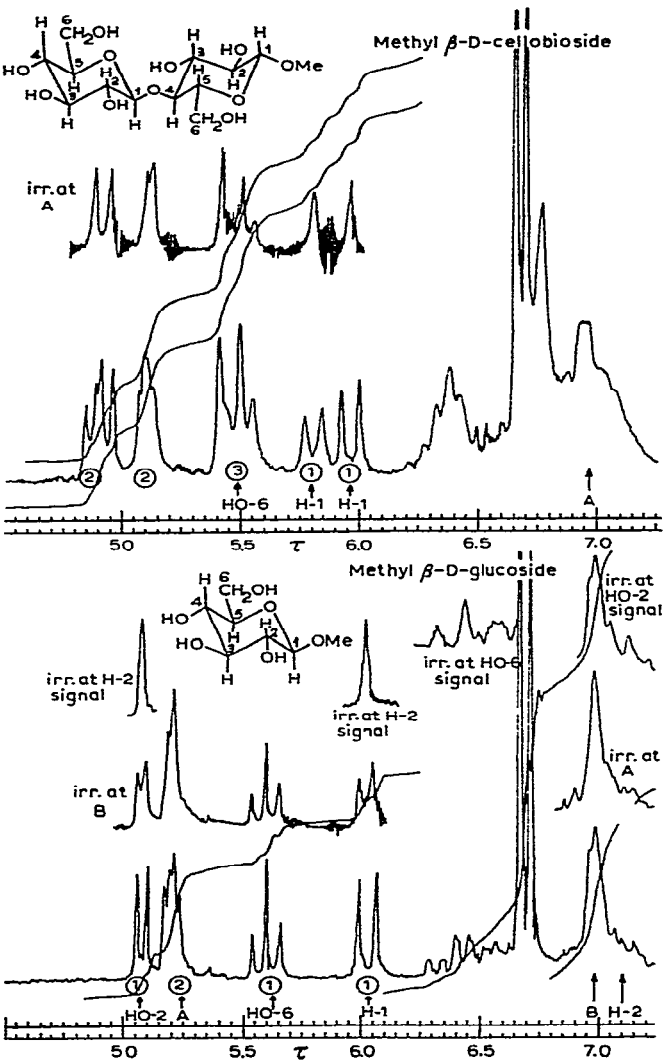

Fig. 2. Partial 100-MHz p.m.r. and double-resonance spectra of methyl β-D-glucoside and methy β-D-cellobioside. The intensities are shown thus: O.

to that (6.5 Hz) found[1] for β-D-glucose and indicative[1] of the *C1* (D) conformation. Therefore, the pyranose units in the oligosaccharides also exist substantially in this conformation, which has been found in some of the crystalline materials by X-ray diffraction studies[3,7]. Decoupling experiments showed that the splittings in each case arose from coupling of H-1 to a proton absorbing near τ 7.0.

In the spectrum of the methyl glycosides (Fig. 2), the H-1 resonance appears at higher field than in the spectra of the oligo-glucoses, so that two doublets arising from H-1 resonances were observed in the spectrum of methyl β-D-cellobioside. These doublets collapsed when the decoupling field was applied near τ 7.0.

Carbohyd. Res., 12 (1970) 453–458

TABLE I

CHEMICAL SHIFTS (τ, FROM TETRAMETHYSILANE AS INTERNAL REFERENCE) AND SPLITTINGS (Hz, IN PARENTHESES) OF SOME CELLULOSE OLIGOSACCHARIDES AND RELATED COMPOUNDS IN Me_2SO-d_6

Compound	*HO-1 doublet*		*Non-anomeric OH signals under τ 5*	*HO-6 triplet eq*	*Other OH groups*	*H-1*		*H-2*
	eq	*ax*				*eq*[a]	*ax*	*ax*[b]
β-D-Glucose	3.50 (6.5)		[c]	5.55 (5.5)[d]	5.25 (3.5)[e]	[c]	5.75 (6.5)[f]	7.1
Methyl β-D-glucoside			[c]	5.55 (6.0)	5.01 (4.5)[e], 5.16[g]		5.95 (7.2)[e]	7.0
Cellobiose	3.38 (6.5)	3.72[g]	4.83 (3.5)[e]	[h]	5.06[g], 5.38[g]	5.12 (3.0)	5.72 (7.0)[f]	7.0
Methyl β-D-cellobioside			4.83 (4.8)[e], 4.89 (4.3)[e]	5.44	5.05[g], 5.35		5.75 (7.0)[f], 5.90 (7.5)[e]	6.9
Cellotriose	3.38[g]	3.70 (4.5)	4.62 (4.0)[e], 4.78[g]	[h]	5.04[g], 5.26,[g] 5.42[g]	5.12 (3.0)	5.74 (7.0)[f]	7.0
Cellotetraose	3.38[g]	[c]	4.67[g], 4.84[g]	[h]	5.08[g], 5.35[g]	[c]	5.74 (7.0)[f]	6.9
Cellopentaose	3.38[g]	[c]	4.67[g], 4.84[g]	[h]	5.08[g], 5.35[g]	[c]	5.74 (7.0)[f]	6.9

[a]Doublet observed after D_2O exchange. [b]Chemical shift inferred from spin-decoupling measurements. [c]Peak not observed. [d]Splittings from spectra of partly deuterated sample. [e]Apparent doublet. [f]Triplet observed after D_2O exchange (see text). [g]Unresolved peak. [h]Peak not assigned.

In each of the spectra (Figs. 1 and 2), with the exception of those of the monosaccharides, there is at least one band below τ 5, arising from non-anomeric hydroxyl groups. For cellobiose, there is a 1-proton doublet, but for cellotriose and the higher oligosaccharides, two bands appear below τ 5. In the case of cellotriose, the bands are of approximately equal intensity (one proton each), but, in the cases of cellotetraose and cellopentaose, the lower field band is the more intense, with their joint intensities approximating to three and four protons, respectively. These results, although derived from only very approximate data of intensity for the higher oligosaccharides, appear to support Casu's contention[1] that the non-anomeric hydroxyl absorption below τ 5 in the cellobiose spectrum arises from the "internal" hydroxyl group HO-3. However, the group of bands below τ 5 in the spectrum of methyl β-D-cellobioside contains two hydroxyl resonances at τ 4.83 and 4.89, which are coupled to the same protons as the anomeric protons, namely protons absorbing near τ 7.0. If it was only H-2 that absorbed at this field, these results would suggest that the two bands arose from the two HO-2 resonances. However, the band at τ 5.05 was also sharpened by the decoupling field, suggesting that other CH signals might lie close to that of H-2. If these are H-3 signals, as seems likely, the "internal" HO-3 signal might contribute to the hydroxyl absorption below τ 5, but, since the intensity of the absorption requires two protons, it cannot be the sole contributor. In the spectrum of methyl β-D-glucoside, the hydroxyl resonance at lowest field is again coupled to the same resonance as the anomeric proton, but, as it arises from only a single hydroxyl group, is here assigned to the HO-2 resonance.

The non-anomeric hydroxyl resonances do not shift regularly with the number of units, as was found for the α-D-(1→4) series[1].

Casu and co-workers[1] suggested that the "internal" HO-3 signal in the cellobiose spectrum would be at lower field than the HO-3 signal in β-D-glucose, because of its participation in a hydrogen bond to the ring oxygen-atom of the contiguous unit. Such a bond is present in crystalline cellobiose[7] and is favoured for crystalline cellotetraose[3]. Its strength is unknown, but an analogous bond in the *cis* isomer of 2-(2-tetrahydropyranyloxy)cyclohexanol[8] causes a shift of *ca.* 200 cm^{-1} in the O–H stretching frequency in carbon tetrachloride. This shift is twice as large as that produced by a hydrogen bond between *syn* diaxial groups in 2,3,6-tri-*O*-methyl-α-D-glucose[9]. This bond is disrupted by methyl sulphoxide[2].

The possibility of a hydrogen bond existing between contiguous units in cellobiose dissolved in methyl sulphoxide must therefore remain open, but the intensities of the bands in the methyl β-D-cellobioside spectrum show that oligosaccharide hydroxyl groups, other than those involved in such a bond, can absorb below τ 5. This result throws some doubt on Casu's interpretation[1] of such bands in terms of inter-residue hydrogen-bonding.

EXPERIMENTAL

The sample of β-D-glucose, m.p. 150°, $[\alpha]_D^{20}$ +18.6 → +52.2° (*c* 2, water), was

obtained from the Nutritional Biochemicals Corporation; and methyl β-D-glucoside, m.p. 113°, $[\alpha]_D^{20}$ −32.4° (*c* 4, water), was provided by Dr. A. Meller. Cellobiose, m.p. 226°, $[\alpha]_D^{20}$ +35.3° (*c* 2, water), was obtained from Kerfoot and Co. The sample of methyl β-D-cellobioside-^{14}C was provided by Dr. J. W. Green[10]. The preparation of cellotriose, m.p. 203–206°, $[\alpha]_D^{20}$ +22.4° (*c* 2, water); cellotetraose, m.p. 248–252°, $[\alpha]_D^{20}$ +16.5° (*c* 2, water); and cellopentaose, m.p. 264–268°, $[\alpha]_D^{20}$ +12.4° (*c* 2, water), has been described previously[11]. The purities of the oligosaccharide samples were checked by paper chromatography and by g.l.c. of their trimethylsilyl ethers.

The deuterated methyl sulphoxide (99.5%, Stohler Isotopic Chemicals) was used without further purification. Deuteration of the dissolved sugars was accomplished by adding a few drops of D_2O to the filtered, saturated solution in a conventional n.m.r. tube.

The p.m.r. spectra were recorded on a Varian HA-100 spectrometer in the frequency-swept mode, with a probe temperature of 30°, by using sodium trimethylsilyl-1-propanesulphonate and tetramethylsilane (τ 10.0) as internal references for the sugar spectra and hexamethyldisiloxane (τ 9.94) for the glycoside spectra. A Hewlett Packard Audio Oscillator, Model 201 C, was employed in the decoupling experiments, and tetramethylsilane was used as an external reference for the lock system of the spectrometer.

The infrared spectra were measured on a Perkin–Elmer 457 spectrophotometer by using samples dispersed in potassium bromide discs.

ACKNOWLEDGMENTS

The author is grateful to Mr. I. Willing of CSIRO Division of Applied Chemistry for obtaining the p.m.r. spectra, to Dr. A. Meller and Dr. J. W. Green for providing samples, and to Mr. L. Harewood and Mrs. J. Hamilton for assistance in the preparation of the oligosaccharides.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

Note

Stannic chloride-catalysed synthesis of glycosides*

T. R. INGLE AND J. L. BOSE
National Chemical Laboratory, Poona 8 (India)

(Received December 30th, 1968; in revised form, August 27th, 1969)

In 1953, Lemieux and Shyluk[1] reported that the reaction between phenol and penta-*O*-acetyl-β-D-glucopyranose in benzene solution for short periods at reflux temperature, in the presence of anhydrous stannic chloride as a catalyst, gave phenyl tetra-*O*-acetyl-β-D-glucopyranoside. The carbonium ion generated by the elimination of the acetoxyl anion at C-1 of the pentaacetate and stabilised by the anchimeric assistance[2] of the acetoxyl group at C-2, was ascribed[1] a prominent role in the formation of the phenyl β-D-glucoside.

Since no other aryl β-D-glycoside has been prepared so far by the application of the above method[1], we have investigated its possible general applicability for the synthesis of aryl β-D-glycosides and thioglycosides from sugar acetates having 1,2-*trans*-related groups. Several of these glycosides are required for studies in genetics.

The reaction of the pentaacetates of β-D-glucopyranose and β-D-galactopyranose and the tetraacetate of β-D-xylopyranose with phenol was studied, and also the reaction of the former compounds with *o*-nitrothiophenol. Monitoring of the reactions by thin-layer chromatography revealed the formation of both of the anomeric glycosides, the β-D anomer predominating in all cases except in the reaction between phenol and penta-*O*-acetyl-β-D-galactopyranose. In each case, the α- and β-D anomers were isolated in the crystalline state.

Lemieux and Shyluk[1] did not observe the formation of the α-D anomer during their preparation of phenyl tetra-*O*-acetyl-β-D-glucopyranoside. The results reported herein indicate that pathways alternative to that suggested by Lemieux and Shyluk are available. A preliminary account of these observations has been already reported[3].

EXPERIMENTAL

Reagent-grade phenol and anhydrous stannic chloride were freshly distilled, *o*-nitrothiophenol was prepared by the literature procedure[4], and benzene was thiophene free and dried over sodium.

The purity of the acetylated aryl glycosides was checked by thin-layer chromatography (t.l.c.) on silica gel with acetone–light petroleum [(*a*) 1:3, and (*b*) 2:3];

*Communication No. 1309, National Chemical Laboratory.

(*c*) benzene–ethyl acetate (7:3) containing methyl sulphoxide (2%); (*d*) toluene–ether (1:2). Detection was effected with 10% methanolic sulphuric acid at 110°.

The R_F values were in the following order: acetylated aryl α-D-glycoside > acetylated aryl β-D-glycoside > acetylated sugar > decomposition products.

Melting points were taken on a Kofler block and are uncorrected.

Reaction between phenol and penta-O-acetyl-β-D-glucopyranose. — Penta-*O*-acetyl-β-D-glucopyranose (3.9 g, 10 mmoles) was added to a stirred solution of phenol (1.88 g, 20 mmoles) in benzene (100 ml), followed by stannic chloride (1 ml, 8.55 mmoles), under anhydrous conditions. The mixture was refluxed with stirring for 10 min at 95–100°, and then poured into ice and water. The benzene layer was washed successively with water (2 × 100 ml), saturated aqueous sodium hydrogen carbonate (2 × 50 ml), and water (100 ml), dried (Na_2SO_4), and concentrated. The syrupy residue (3.6 g) contained both the anomeric phenyl tetra-*O*-acetyl-D-glucopyranosides, with the β anomer predominating (t.l.c.). Some decomposition products and traces of unreacted penta-*O*-acetyl-β-D-glucopyranose were also present. Crystallisation from methanol (5 ml) gave almost pure β-D anomer (1.5 g, 35.4%). Further recrystallisation from ethanol gave phenyl tetra-*O*-acetyl-β-D-glucopyranoside (1.4 g), m.p. and mixed m.p. 124–125°, $[\alpha]_D^{30}$ −21.8° (*c* 0.9, chloroform); lit.[1] m.p. 125–126°, $[\alpha]_D$ −22.5° (*c* 2, chloroform).

Preparative thin-layer chromatography (p.l.c.) (solvent *a*) of the product from the mother liquor, followed by crystallisation from ethanol, gave phenyl tetra-*O*-acetyl-α-D-glucopyranoside (0.9 g), m.p. and mixed m.p. 113°, $[\alpha]_D^{30}$ +162.2° (*c* 1, chloroform); lit.[5] m.p. 114°, $[\alpha]_D$ +168° (chloroform). An additional amount of the β-D anomer was also obtained. The total yield of the β-D anomer was 2.2 g (51.9%) and that of the α-D anomer was 0.9 g (21.3%).

Reaction between phenol and penta-O-acetyl-β-D-galactopyranose. — Phenol (1.88 g, 20 mmoles), penta-*O*-acetyl-β-D-galactopyranose (3.9 g, 10 mmoles), stannic chloride (1 ml, 8.55 mmoles), and benzene (100 ml) were treated as described above. Crystallisation of the crude product mixture (3.1 g) from methanol (5 ml) gave almost pure α-D anomer (1.3 g) which, on recrystallisation from methanol, gave phenyl tetra-*O*-acetyl-α-D-galactopyranoside (1.1 g), m.p. and mixed m.p. 131°, $[\alpha]_D^{28}$ +170° (*c* 2, benzene); lit.[6] m.p. 131–132°, $[\alpha]_D^{28}$ +173.3° (benzene).

P.l.c. of the product (1.6 g) obtained from the mother liquor gave more of the pure α-D anomer (0.4 g), m.p. 131°; and phenyl tetra-*O*-acetyl-β-D-galactopyranoside (0.5 g), m.p. (from carbon tetrachloride) and mixed m.p. 123°, $[\alpha]_D^{28}$ −26.1° (*c* 1, benzene); lit.[6] m.p. 123–124°, $[\alpha]_D^{17}$ −26.4° (benzene).

The total yield of the α-D anomer was 1.7 g (40.1%), and that of β-D anomer 0.5 g (11.8%).

Reaction between phenol and tetra-O-acetyl-β-D-xylopyranose. — Phenol (1.88 g, 20 mmoles), tetra-*O*-acetyl-β-D-xylopyranose (3.18 g, 10 mmoles), stannic chloride (1 ml, 8.55 mmoles), and benzene (100 ml) were treated as above. More-extensive degradation occurred in this reaction. Crystallisation of the crude reaction product (1.8 g) from ethanol (3 ml) gave the β-D anomer (0.8 g) which, on recrystallisation

from ethanol, gave phenyl tri-*O*-acetyl-β-D-xylopyranoside, m.p. and mixed m.p. 146–147°, $[\alpha]_D^{28}$ −50.1° (*c* 1, chloroform); lit.[7] m.p. 148°, $[\alpha]_D$ −50.5° (chloroform).

P.l.c. of the syrup obtained from the mother liquor gave more β-D anomer (0.2 g) and phenyl tri-*O*-acetyl-α-D-xylopyranoside (0.6 g), m.p. (from carbon tetrachloride–isopentane) and mixed m.p. 62–63°, $[\alpha]_D^{28}$ +132° (*c* 2, chloroform); lit.[7] m.p. 64–65°, $[\alpha]_D$ +135° (chloroform).

The total yield of β-D anomer was 1.0 g (28.4%), and that of the α-D anomer, 0.6 g (17.0%).

Reaction between o-*nitrothiophenol and penta*-O-*acetyl*-β-D-*glucopyranose.* — Stannic chloride (1 ml, 8.55 mmoles) was added to a solution of *o*-nitrothiophenol (3.1 g, 20 mmoles) in benzene (100 ml). Penta-*O*-acetyl-β-D-glucopyranose (3.9 g, 10 mmoles) was then added in small amounts with stirring. The reaction was then carried out, and the products were worked up, as described above. Crystallisation of the crude product mixture (2.6 g) from ethanol, with recrystallisation from methanol, gave *o*-nitrophenyl tetra-*O*-acetyl-1-thio-β-D-glucopyranoside (1.2 g), m.p. 140°, $[\alpha]_D^{28}$ −67.0° (*c* 1.9, benzene) (Found: C, 49.60; H, 4.82; S, 6.81. $C_{20}H_{23}NO_{11}S$ calc.: C, 49.48; H, 4.74; S, 6.61%

P.l.c. [solvent (*d*)] of the syrup obtained from the mother liquor gave more (0.4 g) of the β-D anomer and *o*-nitrophenyl tetra-*O*-acetyl-1-thio-α-D-glucopyranoside (0.7 g), m.p. 143–144° (from methanol), $[\alpha]_D^{28}$ +284.5° (*c* 1, chloroform) (Found: C, 49.35; H, 4.91; S, 6.74. $C_{20}H_{23}NO_{11}S$ calc.: C, 49.48; H, 4.74; S, 6.61%).

The total yield of the β-D anomer was 1.6 g (32.9%), and that of the α-D anomer, 0.7 g (14.4%).

Reaction between o-*nitrothiophenol and penta*-O-*acetyl*-β-D-*galactopyranose.* — This reaction was carried out, as described above, using penta-*O*-acetyl-β-D-galactopyranose (3.9 g, 10 mmoles). The crude product mixture was crystallised and recrystallised from methanol to give *o*-nitrophenyl tetra-*O*-acetyl-1-thio-β-D-galactopyranoside (1.4 g) as pale-yellow plates, m.p. and mixed m.p. 128°, $[\alpha]_D^{28}$ −54.1° (*c* 1, chloroform); lit.[8] m.p. 128°, $[\alpha]_D^{28}$ −54.19° (*c* 0.77, chloroform).

P.l.c. [solvent (*c*)] of the syrup obtained from the mother liquor gave more (0.5 g) β-D anomer, and *o*-nitrophenyl tetra-*O*-acetyl-1-thio-α-D-galactopyranoside (0.8 g), as pale-yellow needles, m.p. 153–154° (from ethanol), $[\alpha]_D^{28}$ +298.3° (*c* 0.5, chloroform) (Found: C, 49.51; H, 4.70; S, 6.72. $C_{20}H_{30}NO_{11}S$ calc.: C, 49.48; H, 4.74; S, 6.61%).

The total yield of the β-D anomer was 1.9 g (39.2%), and that of the α-D anomer, 0.8 g (16.15%).

Deacetylation of the acetylated glycosides. — The acetylated glycosides were deacetylated with catalytic amounts of sodium methoxide in methanol[9]. The new compounds prepared in this way are as follows: *o*-nitrophenyl 1-thio-β-D-glucopyranoside, m.p. 149–150° (from acetone), $[\alpha]_D^{28}$ −132.7° (*c* 1, methanol) (Found: C, 45.59; H, 5.09; S, 10.32. $C_{12}H_{15}NO_7S$ calc.: C, 45.41; H, 4.77; S, 10.10%); *o*-nitrophenyl 1-thio-α-D-glucopyranoside, m.p. 94–95° (from light petroleum), $[\alpha]_D^{28}$ +217.8° (*c* 1, methanol) (Found: C, 45.21; H, 4.86; S, 10.27%); *o*-nitrophenyl

1-thio-α-D-galactopyranoside, m.p. 161–162° (from acetone–light petroleum), $[\alpha]_D^{28}$ +463° (*c* 1, methanol) (Found: C, 45.34; H, 4.91; S, 9.91%).

ACKNOWLEDGMENT

The authors are grateful to Professor A. B. Foster of Chester Beatty Research Institute, London, for helpful suggestions.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

Note

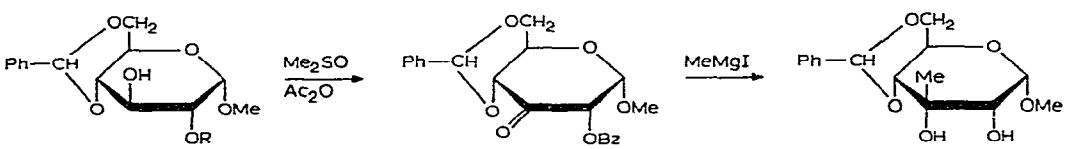
Efficient syntheses of methyl 2-*O*-benzoyl-4,6-*O*-benzylidene-α-D-glucopyranoside and methyl 2-*O*-benzoyl-4,6-*O*-benzylidene-α-D-*ribo*-hexopyranosid-3-ulose

FRANCIS A. CAREY AND KEITH O. HODGSON
Department of Chemistry, University of Virginia, Charlottesville, Virginia 22901 (U. S. A.)

(Received July 28th, 1969)

In connection with studies directed toward the synthesis of branched-chain sugars *via* keto sugars, the usefulness of *N*-benzoylimidazole[1] as a selective acylating agent was examined using methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (**1**) as a model compound.

In contrast to other benzoylation procedures[2] a high degree of discrimination between the hydroxyl groups at C-2 and C-3 was observed leading to the exclusive formation of methyl 2-*O*-benzoyl-4,6-*O*-benzylidene-α-D-glucopyranoside (**2**) in 78% yield. The physical properties coincided with those reported for **2**, and the n.m.r. spectrum was in accord with that of **2**. Additional support for the proposed structure was obtained from subsequent transformations.



1 R=H
2 R=Bz

3

4

Oxidation of **2** with methyl sulfoxide–acetic anhydride[3] proceeded smoothly to give crystalline methyl 2-*O*-benzoyl-4,6-*O*-benzylidene-α-D-*ribo*-hexopyranosid-3-ulose (**3**) in a 72% yield. The n.m.r. spectrum of **3**, which firmly supports the proposed structure, shows the protons at C-1 and C-2 as an AB quartet between δ 5.3 and 5.7 on which the benzylidene proton is superimposed. The simplicity of this spectrum results from the absence of a proton at C-3, and corresponds to no other reasonable formulation. Further confirmation of the structure of **3** (and therefore of that of **2**) was obtained by treating **4** with excess methylmagnesium iodide to give **4** having n.m.r. and m.p. identical with those reported in the literature[4].

Since conversion of keto sugars into a variety of interesting amino[5], branched-chain[6], and unsaturated sugars[7] can be easily accomplished, the mild, selective, blocking and oxidation sequence described in this note may be of value in other systems.

EXPERIMENTAL

Melting points were determined in glass capillaries with a Thomas–Hoover apparatus and are corrected. N.m.r. spectra were recorded on a Hitachi Perkin–Elmer R-20 spectrometer in deuteriochloroform, and chemical shifts are reported in p.p.m. (δ) from the internal tetramethylsilane adsorption peak.

Benzoylation of methyl 4,6-O-benzylidene-α-D-glucopyranoside. — Imidazole (13.6 g, 0.2 mole) was dissolved in purified chloroform (150 ml), and benzoyl chloride (14.1 g, 0.1 mole) was added slowly with cooling. The suspension was filtered to remove imidazole hydrochloride and the filtrate was added to methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (**1**, 28.7 g, 0.1 mole) in chloroform (200 ml) and heated for 10 h at reflux. After being extracted with sodium hydrogen carbonate solution (50 ml) and two 50-ml portions of conc. sodium chloride solution, the solution was dried with sodium sulfate and evaporated to give crude **2** which was recrystallized from acetone–water (30.1 g, 78%), m.p. 166.5–168.5°; (lit.[2]: m.p. 169–170°).

Oxidation of methyl 2-O-benzoyl-4,6-O-benzylidene-α-D-glucopyranoside (**2**). — A solution of **2** (5 g, 12.9 mmoles) in acetic anhydride (50 ml) and methyl sulfoxide (80 ml) was kept for 10 h and the pH was adjusted to 8 with a 2M sodium carbonate solution. The mixture was extracted with chloroform (250 ml). The extracts were washed with three 35-ml portions of water, dried with sodium sulfate, and evaporated to give **3** as a syrup which soon crystallized. The crude product was washed with hot hexane (25 ml) to give 3.56 g (72%), m.p. 210–212°. The analytical sample melted at 211–213°.

Anal. Calc. for $C_{21}H_{20}O_7$: C, 65.62; H, 5.24; Mol. weight 384. Found: C, 65.81; H, 5.13; Mol. weight 372 (Rast).

Reaction of methyl 2-O-benzoyl-4,6-O-benzylidene-α-D-ribo-hexopyranosid-3-ulose with methylmagnesium iodide. — The Grignard reagent was prepared from magnesium (5.0 g, 0.21 mole) and methyl iodide (17 ml, 0.27 mole) in dry ether (250 ml). To the Grignard reagent was added **3** (3.56 g, 9.3 mmoles) in dry benzene (200 ml). After 24 h at 25°, the reaction mixture was added to a saturated ammonium chloride solution (500 ml), and the organic layer separated. The aqueous layer was extracted with four 150-ml portions of chloroform, and the combined organic extracts were dried with magnesium sulfate and evaporated to yield 4.2 g crude product which crystallized. Washing with ether gave methyl 4,6-*O*-benzylidene-3-*C*-methyl-α-D-allopyranoside (1.1 g, 40%) which melted at 201–203° (lit.[4] 210–212°) after recrystallization from hexane–chloroform. The n.m.r. spectrum was identical with that reported[4]: δ 7.3–7.7 (5-proton multiplet, Ph), 5.55 (1-proton singlet, benzylidene H), 5.25 (1-proton doublet, J 4 Hz, H-1), 3.46 (3-proton singlet –OMe), and 1.40 (3-proton singlet, C-3 Me).

Anal. Calc. for $C_{15}H_{20}O_6$: C, 60.80; H, 6.80. Found: C, 60.74; H, 6.75.

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Note

Preparation of D-apiose phenylosotriazole*

PAUL K. KINDEL
Department of Biochemistry, Michigan State University, East Lansing, Michigan 48823 (U. S. A.)
(Received July 28th, 1969; in revised form, September 17th, 1969)

During studies on the cell-free biosynthesis of D-apiose [3-*C*-(hydroxymethyl)-*aldehydo*-D-*glycero*-tetrose][1], it was necessary to prove the identity of radioactive material having the same R_F as D-apiose. One criterion used was to mix the radioactive material with D-apiose, prepare a crystalline derivative, and determine if the specific activity of the derivative remained constant over several recrystallizations. Several crystalline derivatives of D-apiose have been described[2-4], but because most are colored, their usefulness in scintillation counting is limited. The colorless diisopropylidene acetal[4] has the disadvantage of being volatile[5]. D-Apiose phenylosotriazole [3-*C*-(hydroxymethyl)-*aldehydo*-D-*glycero*-tetrose phenylosotriazole], a new derivative described herein, can be prepared from D-apiose in an overall yield of at least 65% and is ideally suited for this type of experiment since it is easily prepared, is colorless, has desirable crystallization properties, and is readily soluble in solvents normally used in scintillation counting.

EXPERIMENTAL

Analytical. — Melting points were determined with a Kofler micro hot-stage (A. H. Thomas Company, Philadelphia, Pa., U. S. A.) and are uncorrected.

D-*Apiose phenylosazone* [3-C-(*hydroxymethyl*)-aldehydo-D-glycero-*tetrose phenylosazone*] (**1**). — A solution of D-apiose[2,6,7] (0.2575 g, 1.715 mmole) in 20 ml of 5M acetate buffer (pH 4.8) was heated to 80° and phenylhydrazine hydrochloride (2.48 g, 17.15 mmole) in 14 ml of the same buffer at 80° was added all at once. After heating this solution for 1 h at 90–95°, 34 ml of distilled water was added slowly with swirling. The osazone separated initially as a mixture of yellow solid and oil. The oil solidified on cooling. After 2 h at 4°, the precipitate was collected by suction filtration, washed with 3 ml of a 1:1 dilution of the above buffer with water and then with 3 ml of water, both at 4°. The well-drained precipitate was immediately dissolved completely in 6 ml of hot abs. ethanol. Distilled water was added to the hot ethanol solution slowly with stirring, until the concentration of ethanol was 15% by volume. After 3–4 h at 4°, the amorphous precipitate was filtered off, washed with

*Michigan Agricultural Experiment Station Journal Article 4811.

1 ml of cold 10% aqueous ethanol, and dried to constant weight *in vacuo* over phosphorus pentaoxide to give 0.4844 g (86%), m.p. 153–157°; lit.[2] for crystalline **1**, m.p. 156–157°.

D-*Apiose phenylosotriazole* (**2**). — A suspension of powdered **1** (0.4844 g, 1.475 mmole) in 37 ml of water was heated to boiling and a solution of cupric sulfate pentahydrate (0.4052 g, 1.623 mmole) in 25 ml of water at 80–90° was added all at once. The suspension was refluxed for 1 h. After cooling and filtering, the solution was extracted continuously with diethyl ether for 10 h. The ether solution was evaporated to about 45 ml, heated with 0.1–0.2 g of Darco G-60 decolorizing carbon for 5 min, and filtered. After further evaporation of the ether solution to about 15 ml, compound **2** crystallized readily, as cream-colored plates, by adding boiling petroleum ether (b.p. 30–60°) to incipient cloudiness and then adding several seed crystals. Seed crystals were obtained by evaporating a small portion of the diethyl ether solution to dryness and scratching the syrup. After 2 h at 4° the crystals were filtered off; yield 0.2664 g (77%), m.p. 94–96°. Compound **2** was redissolved in diethyl ether, treated again with Darco G-60, and crystallized as described above, to give white crystals, m.p. 95–96°. Compound **2** could also be crystallized from diethyl ether alone.

Anal. Calc. for $C_{11}H_{13}N_3O_3$: C, 56.16; H, 5.57; N, 17.86. Found: C, 56.28; H, 5.33; N, 17.96.

Oxidation of **2** *with sodium metaperiodate.* — Periodate oxidation of **2** gave the known 2-phenyl-1,2,3-triazole-4-carboxylic acid[8] and formaldehyde. Compound **2** (0.2352 g, 1.0 mmole) was dissolved in 14 ml of water with agitation. To this solution was added 10 ml of 0.3M sodium metaperiodate (3.0 mmole). The suspension obtained was kept for 1 h at 25° and 2 h at 4°, and then the precipitate was collected on a weighed sintered glass disk by suction filtration and washed with two 5-ml portions of ice-cold water. The filtrate and washings were adjusted to 100 ml and immediately analyzed as described below. After drying to constant weight, the precipitate weighed 0.1669 g (0.88 mole per mole of **2**) and melted at 193.5–194.5°. Recrystallization from 10:1 hot water–abs. ethanol did not alter the m.p.; lit.[8] for 2-phenyl-1,2,3-triazole-4-carboxylic acid, m.p. 191–192°.

Anal. Calc. for $C_9H_7N_3O_2$: C, 57.14; H, 3.73; N, 22.21. Found: C, 57.03; H, 3.67; N, 22.28.

An 0.5-ml aliquot of the filtrate was appropriately diluted and analyzed for formaldehyde by using chromotropic acid[9]. The analysis showed that 1.98 moles of formaldehyde was produced per mole of **2**. This value did not increase over 24 h. A second aliquot (20 ml) was acidified with sulfuric acid and then treated with sodium arsenite. The colorless solution was adjusted to pH 6–7 with sodium hydroxide and then 0.561 g of 5,5-dimethyl-1,3-cyclohexanedione in 80 ml of warm water was added. After 10 min at 90°, 6 ml of glacial acetic acid was added and the solution was kept for 12 h at 4°. The amount of crystalline formal dimethone (m.p. 189–190°) obtained, corresponded to the formation of 1.94 moles of formaldehyde per mole of **2**. Analysis of a third aliquot (3 ml) for unreacted periodate by the iodine–arsenite

procedure[10] showed that 1.98 moles of periodate had been consumed per mole of **2** oxidized.

These data are consistent with the structure of **2**.

ACKNOWLEDGMENTS

This investigation was supported by research grant AM-08608 from the National Institutes of Health and by the Michigan Agricultural Experiment Station.

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Carbohydrate Research
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Preliminary communication

Circular-dichroism spectra of cuprammonium complexes of furanosides

(MISS) S. T. K. BUKHARI and R. D. GUTHRIE*
School of Molecular Sciences, The University of Sussex, Brighton BN1 9QJ (Great Britain)
(Received December 2nd, 1969)

Studies of the cuprammonium complexes formed by vicinal hydroxyl groups in pyranosides yielded valuable information about their conformational equilibria; no such studies on furanosides have been reported. We have recently modified Reeves' original technique[1], by measuring[2,3] the circular-dichroism spectra of cuprammonium complexes, rather than by taking measurements at only one wavelength.

The most readily available furanoside derivatives are nucleosides, and twenty such compounds have been studied, as well as some simple alkyl furanosides. In these types of compounds, the only possible position for the vicinal diol group is at C-2 and C-3. It would be expected that compounds with the *ribo* or *lyxo* configurations would form copper chelates, whereas those with the *arabino* or *xylo* arrangement would not. A complication in the case of nucleosides is that the heterocyclic moiety is known to form copper complexes (see, for example, ref.4), and this was demonstrated to occur in cuprammonium solution, since (2-deoxy-D-*erythro*-pentofuranosyl)-pyrimidines and -purines, and 2',3'-*O*-isopropylidenenucleosides all caused a change in specific resistance when dissolved in the reagent (*cf.* ref.1.). However, these two classes of compounds did not give any observable Cotton effects, whereas ribofuranosylnucleosides, and simple ribofuranosides and lyxofuranosides, each having a *cis* 2,3-diol group, showed Cotton effects at about 300 and 600 nm of opposite sign; similar behaviour has been observed with pyranosides[2]. It was concluded, therefore, that the observation of Cotton effects demonstrates the presence of a diol:copper chelate.

All of the nucleosides studied showed a negative Cotton effect at *ca.* 600 nm (see Fig.1), showing[2] the presence of a λ chelate ring[5]. Such a chelate ring would be formed from a furanosyl ring in a T_3^2, C-3'*exo* or C-2'*endo* conformation**. From n.m.r. studies[7], it has been suggested that adenosine has a T_3^2 conformation in solution. The authors[7,8] further commented that methyl ribofuranosides existed in the 'opposite' conformation to their nucleoside counterparts, namely a T_2^3 form. Methyl β-D-ribofuranoside gave a positive band at about 600 nm (Fig.1), showing[2] the presence of a δ chelate ring, consistent with the furanose ring being in a T_2^3, C-3'*endo* or C-2'*exo* conformation.

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*To whom enquiries should be addressed.

**Only conformations of the furanosyl ring that involve displacement of C-2 or C-3, or both, out of the mean plane of the ring have been considered (*cf.* ref.6).

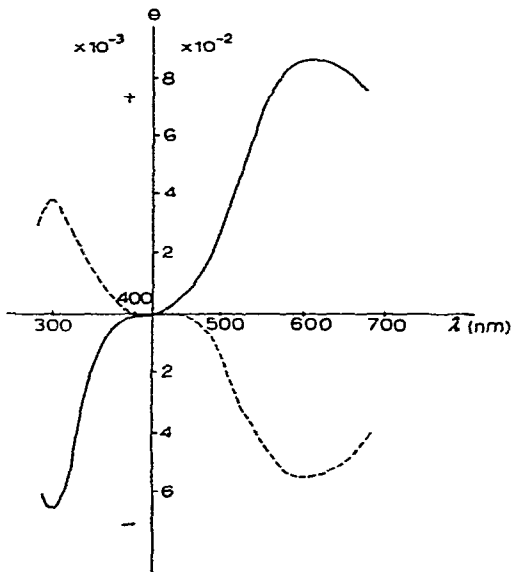

Fig.1. C.d. spectra of adenosine (- - -) and of methyl β-D-ribofuranoside (—) in Cupra A.

A study of the n.m.r. spectra of 2'- and 3'-*C*-methyladenosines[7] has suggested that the furanosyl ring in the former had a T_2^3 conformation, or in the latter, T_3^2. Cuprammonium solutions of these compounds★ showed positive and negative Cotton effects at *ca.* 600 nm, respectively, consistent with the n.m.r. findings.

Since there are several conformations of a furanoside ring which have the appropriate[1] geometry for complexing with cuprammonium, other conformations could give the results above. However, it is interesting that the results are in complete agreement with those from other methods.

Thus, it would appear that investigation of the circular dichroism of solutions of furanosides in cuprammonium could be useful for probing their conformation.

As expected, we have also shown that *lyxo* compounds gave Cotton effects, whereas *arabino* ones did not.

We thank the S.R.C. for financial support.

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★We thank Dr. E. Walton for supplying us with samples of 2'- and 3'-*C*-methyladenosine.

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Preliminary communication

The oxidation of 2-acetamido-2-deoxyaldoses. Two unsaturated lactones from 2-acetamido-2-deoxy-D-glucose, -D-mannose, and -D-galactose

N. PRAVDIĆ
Department of Organic Chemistry and Biochemistry, "Ruder Bošković" Institute, Zagreb (Yugoslavia)
and H. G. FLETCHER, JR.
National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, Maryland 20014 (U. S. A.)

(Received January 16th, 1970)

Although aqueous bromine has long been used for the conversion of simple aldoses into aldonic acids, the action of this oxidant on 2-acylamido-2-deoxyaldoses does not appear to have been investigated. We now report some aspects of the behavior of the three most readily accessible 2-acetamido-2-deoxyaldoses (**1**, **2**, and **3**) with this convenient reagent, and describe the transformation that occurs when the products thus obtained are treated with alkali.

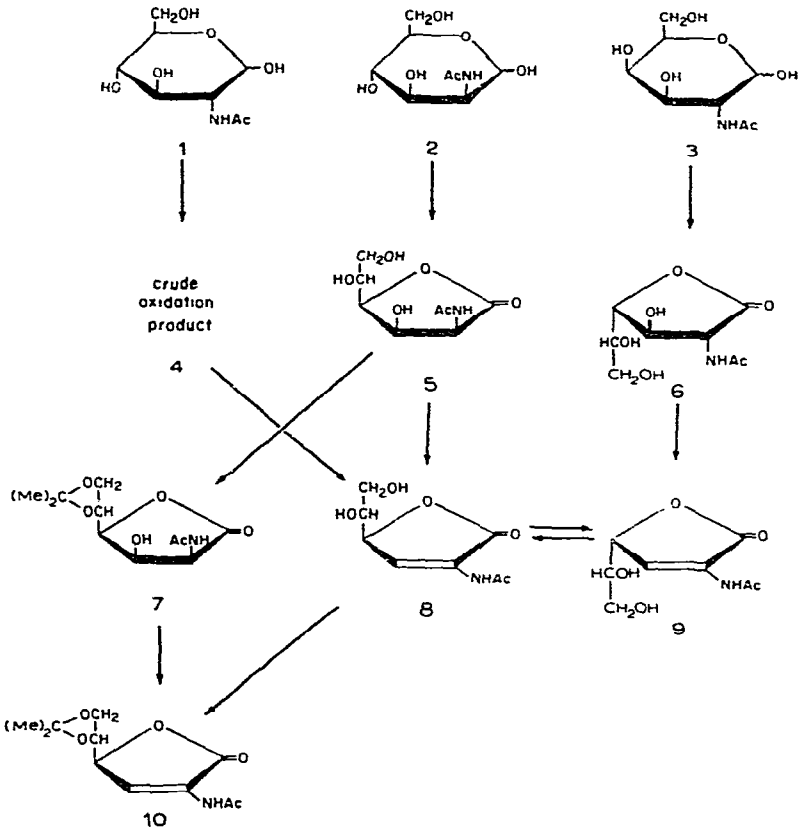
Each of the sugars (**1**–**3**) was subjected to the action of unbuffered bromine–water, and the reaction mixture was processed as described by von Euw and Reichstein[1]. The product from 2-acetamido-2-deoxy-D-glucose (**1**) was obtained as a hygroscopic foam: ν_{max}^{KBr} 1750 (C=O), 1660, and 1560 cm^{-1} (amide); paper chromatography showed the crude oxidation product (**4**) to be heterogeneous. Hydrolysis of a sample of **4** with hot 4 M hydrochloric acid gave material chromatographically indistinguishable from 2-amino-2-deoxy-D-gluconic acid[2]. With dicyclohexylamine, the oxidation product gave a crystalline salt: $C_{20}H_{38}N_2O_7$★, m.p. 191–194°, $[\alpha]_D$ −2.9° (*c* 0.95, water); ν_{max}^{KBr} 3200 (NH), 1780 (C=O), and 1630 and 1560 cm^{-1} (amide), indicating that 2-acetamido-2-deoxy-D-gluconic acid is one of the oxidation products obtained from **1**.

The oxidation of 2-acetamido-2-deoxy-D-mannose (**2**) with bromine–water led to the isolation in 51% yield of a product having m.p. 172–173° and $[\alpha]_D$ +87.4° (*c* 1.0, water); ν_{max}^{KBr} 3400 (OH), 3300 (NH), 1770 (C=O), and 1660 and 1550 cm^{-1} (amide); n.m.r. data (methyl sulfoxide-d_6) τ 1.92 (doublet, J 9.0 Hz, removed by D_2O exchange; NH), 4.40 (doublet, removed by D_2O exchange; OH), 5.02 (quartet, $J_{2,NH}$ 9.0, $J_{2,3}$ 4.5 Hz; H-2), 5.20 (doublet, OH), unresolved multiplets at 5.40–6.60, and 8.04 (NAc). The elemental analysis corresponded to $C_8H_{13}NO_6$, and the compound is assigned structure **5**, namely, 2-acetamido-2-deoxy-D-mannono-1,4-lactone.

With aqueous bromine, 2-acetamido-2-deoxy-D-galactose (**3**) gave the known[3,4]

★Acceptable elemental analyses were obtained for all compounds reported. Melting points are corrected values.

2-acetamido-2-deoxy-D-galactono-1,4-lactone (6): m.p. 162–166°, $[\alpha]_D$ −21.6° (*c* 1.09, water); ν_{max}^{KBr} 3350 (OH), 3250 (NH), 1780 (C=O), and 1650 and 1550 cm^{-1} (amide).



Treatment of 4 with methanolic potassium hydroxide at room temperature afforded a mixture of two crystalline products (*A* and *B*) in approximately equal proportions; similar treatment of the lactones 5 and 6 gave essentially the same mixture of products. By careful recrystallization of the mixture from methanol solution, one of the components (*A*) could be obtained in pure form: m.p. 186–188°, $[\alpha]_D$ +41.1° (*c* 1.04, water); ν_{max}^{KBr} 3350 (OH), 3200 (NH), 1750 (C=O), 1680 (C=C), 1630 and 1540 (amide), and 960 cm^{-1}. The elemental composition of the substance corresponded to $C_8H_{11}NO_5$; its n.m.r. spectrum (methyl sulfoxide-d_6) included signals at τ 0.05 (broad singlet, disappearing after D_2O exchange; NH), 2.50 (doublet, $J_{3,4}$ 2.0 Hz, vinyl proton; H-3), 4.75–5.0 (2H multiplet collapsing on D_2O exchange to a 1H quartet centered at 4.82, $J_{3,4}$ 2.0 Hz, $J_{4,5}$ 4.5 Hz; H-4), 5.27 (triplet, *J* 5.5 Hz, disappearing after D_2O exchange; primary OH), and 7.92 (NAc). The Legal test for unsaturated lactones was positive. Acetylation with acetic anhydride–pyridine gave a di-*O*-acetyl derivative which was obtained as a chromatographically homogeneous syrup having $[\alpha]_D$ +58.1° (*c* 0.86, chloroform) and an elemental composition corresponding to $C_{12}H_{15}NO_7$. I.r. absorption (neat) was observed at 3280 (NH), 1750 (C=O),

1720 (OAc), 1660 (C=C), and 1630 and 1540 cm^{-1} (amide); the n.m.r. spectrum (chloroform-*d*) included signals at τ 1.82 (broad singlet, NH), 2.51 (doublet, *J* 1.5 Hz; H-3), 4.65–4.90 (multiplet, 2H), 5.40–5.90 (multiplet, 2H), 7.77 (NAc), and 7.88 and 7.92 (OAc).

Although crystalline, the second component (*B*) of the mixture could not be obtained in pure form. The i.r. spectrum of this component very closely resembled that of *A*, except that it showed an absorption band at 915 cm^{-1} (not at 960 cm^{-1}). The n.m.r. spectrum of component *B* was also very similar to that of *A*, except that its low-field doublet of 2.0 Hz appeared at τ 2.58 (not at τ 2.50). These spectral characteristics clearly revealed the composition of the mixtures of the two components; in addition, the component *B* proved to be levorotatory, and mixtures rich in it were found to give values as low as $[\alpha]_D$ −97° (*c* 0.40, water).

The spectral data, as well as the fact that these two compounds are formed from both the D-*gluco* (**4**) and the D-*manno* compound (**5**), clearly indicate that they are diastereoisomeric 2-acetamido-2,3-dideoxyhex-2-enono-1,4-lactones; that they are also formed from the D-*galacto* lactone (**6**) suggests that they are epimeric at C-4. Such a proposal implies that the two components may be interconvertible in the presence of alkali and, indeed, this proved to be the case. Treatment of component *A* (available in pure form) with methanolic potassium hydroxide at room temperature caused a levomutarotation and the formation of a mixture such as had been separately obtained from **4**, **5**, and **6**; treated similarly, a fraction rich in component *B* showed a dextro-mutarotation.

As there is no reason to suppose that the configuration of C-5 has been affected, structures **8** and **9** may be assigned to the two products. Although optical rotatory considerations suggest that the pure isomer *A*, $[\alpha]_D$ +41.1°, is **8**, and the levorotatory isomer *B* is **9**, other evidence bearing on this point was sought. The saturated lactone **5** readily gave an isopropylidene acetal when treated with acetone and anhydrous copper(II) sulfate: m.p. 161–164°, $[\alpha]_D$ +75.8° (*c* 1.04, methanol); ν_{max}^{KBr} 3300 (OH), 3200 (NH), 1750 (C=O), 1650 and 1540 (amide), and 1380 and 1370 cm^{-1} (Me_2C); n.m.r. signals (acetone-d_6) at τ 7.05 (deleted by D_2O exchange; OH), 7.97 (NAc), and 8.61 and 8.68 (Me_2C); the elemental composition corresponded to $C_{11}H_{17}NO_6$. It is reasonable to assume that the isopropylidene group spans O-5–O-6 as in **7**; an analogous derivative of **6** was described by Karrer and Meyer[3]. An attempt was made to *p*-toluenesulfonylate the remaining hydroxyl group (at C-3) in **7** through the use of *p*-toluenesulfonyl chloride in pyridine solution. However, no trace of the expected derivative was detected, and the material isolated proved to be a sulfur-free, unsaturated compound having a composition corresponding to $C_{11}H_{15}NO_5$: m.p. 124–125°, $[\alpha]_D$ −29.1° (*c* 1.01, chloroform); ν_{max}^{KBr} 3300 (NH), 1780 (C=O), 1710 (C=C), 1660 and 1550 (amide), and 1385 and 1380 cm^{-1} (Me_2C); n.m.r. signals (chloroform-*d*) at τ 2.15 (broad singlet, NH), 2.41 (doublet, *J* 2.0 Hz; H-3), 5.06 (quartet, *J* 2.0 and 7.1 Hz; H-4), 5.85–6.10 (multiplet, 3H), 7.80 (NAc), and 8.55 and 8.67 (Me_2C).

The unsaturated lactone *A* (available in pure form) was condensed with acetone in the presence of anhydrous copper(II) sulfate to give a crystalline product **10**, identical in all respects with that obtained through the attempted sulfonylation of **7**. It is, therefore, evident that the dextrorotatory lactone is 2-acetamido-2,3-dideoxy-D-*erythro*-hex-2-enono-1,4-lactone (**8**), and that the levorotatory isomer is the corresponding D-*threo* compound

(9). In passing, it may be noted that **10** appeared to be unaccompanied by isomeric materials, regardless of whether it was prepared from **7** or **8**.

The investigation outlined here is being extended; further details, together with a discussion of the mechanistic features of some of the reactions involved, will be provided elsewhere.

ACKNOWLEDGMENTS

We acknowledge support provided through Agreement No. 719810 by the National Institutes of Health, Bethesda, Maryland, U. S. A., under P. L. 480. We are indebted to Mr. B. Danilov of the "Ruder Bošković" Institute for his skilful technical assistance.

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Carbohydrate Research
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Preliminary communication

A convenient route to derivatives of 3-amino-3-deoxy- and 3-deoxy-3-fluoro-L-idose

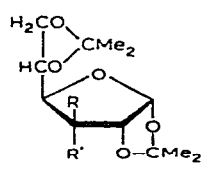
J. S. BRIMACOMBE★, (Miss) P. A. GENT
Chemistry Department, The University, P. O. Box 363, Birmingham 15 (Great Britain)
and J. H. WESTWOOD
Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London, S. W. 3 (Great Britain)

(Received January 2nd, 1970)

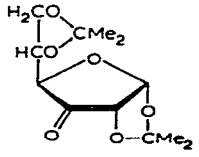
Bimolecular, nucleophilic displacements of *endo*-sulphonyloxy groups attached to trioxabicyclo[3.3.0]octane ring-systems have provided excellent syntheses of 3-amino-3-deoxy and 3-deoxy-3-fluoro sugars possessing the D-*gluco*[1–3] and D-*galacto* configurations[4,5]

1,2:5,6-Di-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-β-L-talofuranose (**4**) should, by analogy, undergo ready nucleophilic displacement of the *endo*-sulphonyloxy group to yield derivatives of L-idose.

The starting sulphonate **4**, m.p. 135–137°, $[\alpha]_D$ +84° (*c* 0.53, chloroform), was prepared by oxidation of 1,2:5,6-di-*O*-isopropylidene-β-L-idofuranose[6] (**1**) with acetic anhydride–methyl sulphoxide[7] to the ketone **2**, m.p. 87.5–89° (after sublimation), $[\alpha]_D$ +207° (*c* 0.4, dry chloroform), ν_{max} 1780 cm^{-1} (C=O), which, on reduction with sodium borohydride in aqueous ethanol, afforded exclusively 1,2:5,6-di-*O*-isopropylidene-β-L-talofuranose (**3**), m.p. 85–86°, $[\alpha]_D$ +31° (*c* 0.5, chloroform); [lit.[8] (D enantiomer), m.p. 85–86°, $[\alpha]_D$ −25 ±1° (*c* 0.47, chloroform)]. Treatment of the diacetal **3** with toluene-*p*-sulphonyl chloride in pyridine then gave the sulphonate **4**.



1 R = OH, R′ = H
3 R = H, R′ = OH
4 R = H, R′ = OTs
5 R = OBz, R′ = H
6 R = N_3, R′ = H
7 R = NHAc, R′ = H
8 R = F, R′ = H



2

The *endo*-toluene-*p*-sulphonyloxy group of **4** was displaced smoothly with benzoate ion in *N,N*-dimethylformamide at 140° to give 3-*O*-benzoyl-1,2:5,6-di-*O*-isopropylidene-β-L-idofuranose (**5**), m.p. 108–109.5°, $[\alpha]_D$ −22° (*c* 0.46, chloroform), ν_{max} 1720 cm^{-1} (benzoate), which was identical with the product obtained by direct

★Present address: The Chemistry Department, The University, Dundee, DD1 4HN, Great Britain.

benzoylation of the diacetal **1**. By analogy, the product resulting from the reaction of the sulphonate **4** with sodium azide in boiling *N,N*-dimethylformamide was assigned the structure 3-azido-3-deoxy-1,2:5,6-di-*O*-isopropylidene-β-L-idofuranose (**6**), b.p. 135–140° (bath)/0.5 mmHg, $[\alpha]_D$ −80° (*c* 0.5, chloroform), ν_{max} 2100 cm^{-1} (N_3). The azide **6** was converted into 3-acetamido-3-deoxy-1,2:5,6-di-*O*-isopropylidene-β-L-idofuranose (**7**), m.p. 158.5–159.5°, $[\alpha]_D$ −18.5° (*c* 0.6, chloroform), following hydrogenation over 10% palladised calcium carbonate and acetylation of the resulting amine. Finally, treatment of the sulphonate **4** with tetra-*n*-butylammonium fluoride in boiling acetonitrile gave, after chromatography on silica gel, 3-deoxy-3-fluoro-1,2:5,6-di-*O*-isopropylidene-β-L-idofuranose (**8**), b.p. 65–70° (bath)/0.05 mmHg, $[\alpha]_D$ −39 ±2° (*c* 0.6, chloroform).

The diacetal **1** can be prepared from 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose in five steps[6,9], and the reactions described above should therefore provide a practical route to derivatives of 3-amino-3-deoxy- and 3-deoxy-3-fluoro-L-idose.

New compounds had satisfactory elemental analyses, and their n.m.r. spectra were in agreement with the structures assigned.

The authors thank Professors M. Stacey, F. R. S., and A. B. Foster for their interest, and the Science Research Council for a grant (to P. A. G.).

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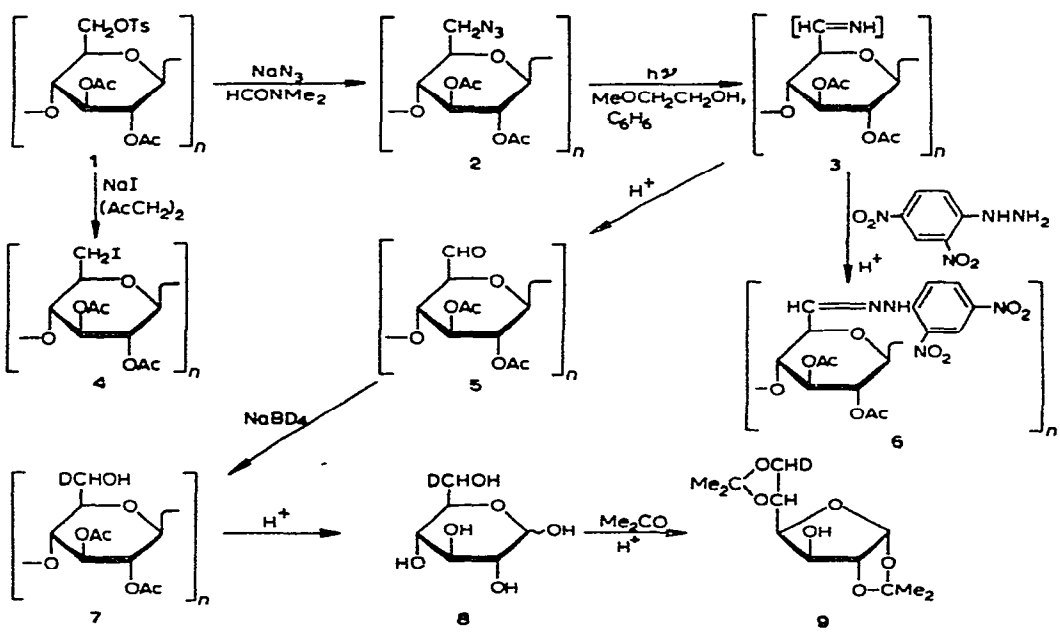
Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

Preliminary Communication

Synthesis of the 6-aldehydo derivative of cellulose, and a mass-spectrometric method for determining degree of substitution★

D. M. CLODE and D. HORTON
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210 (U.S.A.)
(Received December 17th, 1969)

A 6-azido-6-deoxycellulose acetate (**2**) has been prepared which, on photolysis, gives 6-aldehydocellulose acetate (**5**) of the same degree of substitution (d.s.). The d.s. was determined by formation of the 6-(2,4-dinitrophenyl)hydrazone (**6**), or by reduction with sodium borodeuteride followed by hydrolysis, acetonation, and determination by mass spectrometry of the extent and position of incorporation of deuterium in the resultant 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**9**).



★Supported in part by the Agricultural Research Service, U.S. Department of Agriculture, Grant No. 12-14-100-9201(71) (The Ohio State University Research Foundation Project 2573), administered by the Northern Utilization Research and Development Division, Peoria, Illinois, U.S.A.

Cellulose acetate[1] having d.s. 2.8 (Ac, 43.0) was *p*-toluenesulfonylated in pyridine[2] to give 6-*O*-*p*-tolylsulfonylcellulose acetate (**1**), having d.s. 0.25 by the *p*-tolylsulfonyl group and d.s. 2.7 by acetyl groups (Found: C, 49.96; H, 5.31; Cl, 1.17; S, 2.68; d.s. values computed by the method of Horton and Pardoe[3]), which was almost exclusively sulfonylated at O–6, as, with sodium iodide in 2,5-hexanedione for 6 h at 120°, it gave 6-deoxy-6-iodocellulose acetate (**4**) having d.s. 0.24 by the iodo group and d.s. 2.7 by acetyl groups (Found: C, 45.50, H, 5.70; I, 10.58; S, 0.70). A solution of **1** (5 g) in *N,N*-dimethylformamide was stirred for 90 h at 110° with an excess of sodium azide, to give (after dialysis) 6-azido-6-deoxycellulose acetate (**2**), having d.s. 0.25 by the azido group and d.s. 2.7 by the acetyl group, as an ash-free, white, fluffy solid; yield 2.13 g, λ_{max}^{KBr} 4.73 μm (N_3) (Found: C, 47.2; H, 5.65; N, 3.35; S, 0.67).

A solution of **2** in 5:1 2-methoxyethanol–benzene was photolyzed[4] under nitrogen with unfiltered light from a mercury arc for 6 h at 20°, to give, after treatment with water and dialysis, a white, fluffy, ash-free solid (Found: N, 0.52) that gave a positive Fehling test; by analogy with previous work[5,6] this material contained a polymeric product derived from the imine **3**, in admixture with the aldehyde **4**. Treatment of this product in 2-methoxyethanol with (2,4-dinitrophenyl)hydrazine and a small proportion of hydrochloric acid gave, after dialysis, an orange, ash-free solid formulated as the (2,4-dinitrophenyl)hydrazone (**6**) of the acetylated 6-aldehydocellulose; it had d.s. 0.20 by the Schiff-base group (Found: N, 3.71).

Photolysis of 0.5 g of the azide **2** as before, but with treatment of the photolyzed product with M aqueous hydrochloric acid for 1.2 h at 25° before dialysis, gave 6-aldehydocellulose acetate (**5**) as a white, ash-free solid that reduced Fehling solution and contained no nitrogen. Reduction of this product with an excess of sodium borodeuteride in water, with subsequent dialysis, gave a Fehling-negative product, formulated as 6-deuteriocellulose acetate (**7**), that had deuterium incorporation at C-6 equivalent to the original d.s. of the aldehyde group in the precursor **5**.

Hydrolysis of the deuterated product **7** in 0.5M sulfuric acid for 20 h at 90° gave a partially deuterated D-glucose (**8**) that was purified by charcoal-column chromatography and then acetonated, to give crystalline 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose-*6-d* (**9**), which was purified by chromatography on silica gel and examined by mass spectrometry. Ions having *m/e* 245, 187, and 101, known[7] to contain the C-6 moiety in non-deuterated 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose, were observed in **9**, but were also accompanied by less-intense peaks one mass unit higher (*m/e* 246, 188, and 102) corresponding to the 6-monodeuterated fragments. From the ratio of the peak areas for the deuterated and nondeuterated **9** (after correction for the natural abundance of ^{13}C, ^{2}H, and ^{17}O), the d.s. by deuterium in the parent polymer **7** was calculated to be 0.2. The *m/e* 159 fragment (loss of the C-5–C-6 moiety in **9**) had at *m/e* 160 a small satellite peak whose relative intensity reflected only natural isotopic abundances, indicating that no deuterium was incorporated except into the C-4 side chain of **9**.

The foregoing reactions provide a position-specific method for introducing aldehyde functionality into cellulose without the adverse effects on the stability of the polymer that are engendered by the use of glycol-cleavage reagents. The reaction can be performed at various levels of d.s. to afford polyfunctional, linear polymers of potential practical utility; mild oxidation can be performed to yield the corresponding D-glucuronan.

These products, and such labeled derivatives as those obtainable by reduction of the aldehyde with sodium borotritiide, may be useful in studies on cellulolytic enzymes. The mass-spectrometric method for determining d.s., although not of high precision, is nevertheless applicable on a very small scale for measuring position and extent of substitution, and it could be extended for use with other carbonyl derivatives of polysaccharides.

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CORRIGENDA

Carbohyd. Res., 11 (1969) page 314: delete lines 28 and 29.

Carbohyd. Res., 12 (1970) page 32: footnotes [a] and [b] of Table II should read:
[a] Relative to oligosaccharide **1**, acetate buffer, pH 4. [b] Relative to oligosaccharide **1**, solvent system *a*.