Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

SYNTHÈSE DE DÉRIVÉS PYRROLIDINIQUES. CYCLISATION INTRAMOLÉCULAIRE CONDUISANT AU 1,4; 2,5-DIANHYDRO-2,3,4-TRIDÉSOXY-2,4-BIS-(*p*-TOLUÈNESULFONAMIDO)-D-*thréo*-PENTITOL*

A. M. SEPULCHRE, J. CLEOPHAX, J. HILDESHEIM, ET S. D. GERO
Institut de Chimie des Substances Naturelles, C.N.R.S., 91-Gif-sur-Yvette (France)
(Reçu le 14 octobre, 1969)

ABSTRACT

The synthesis of pyrrolidine derivatives has been accomplished by nucleophilic displacement reactions of sulfonic esters of 2,5-anhydro-2,3-dideoxy-2-*p*-toluenesulfonamido (or 2-acetamido)-L-*erythro*-pentitol, using as reagent either sodium azide–*N*,*N*-dimethylformamide or sodium azide–acetonitrile. It has been found that the replacement of sulfonyloxy groups of the 1-benzoate-4-*p*-toluenesulfonate and 1,4-di-*p*-toluenesulfonate is fairly rapid. Catalytic hydrogenation of the monoazides at C-1 or C-4, followed by sulfonylation, afforded *in situ* 1,4:2,5-dianhydro-2,3,4-trideoxy-2,4-bis-(*p*-toluenesulfonamido)-D-*threo*-pentitol.

SOMMAIRE

La synthèse des dérivés pyrrolidiniques a été effectuée par déplacement nucléophile sur les esters sulfoniques du 2,5-anhydro-2,3-didésoxy-2-*p*-toluènesulfonamido (ou 2-acétamido)-L-*érythro*-pentitol. Le réactif employé est l'azothydrate de sodium soit dans la *N*,*N*-diméthylformamide, soit dans l'acétonitrile. Le déplacement des groupes sulfonyloxy pour les dérivés 1-benzoate-4-*p*-toluènesulfonate et 1,4-di-*p*-toluènesulfonate est une réaction rapide. L'hydrogénation catalytique des monoazides en position 1 ou 4, suivie d'une sulfonylation, conduit *in situ* au 1,4;2,5-dianhydro-2,3,4-tridésoxy-2,4-bis-(*p*-toluènesulfonamido)-D-*thréo*-pentitol.

INTRODUCTION

Les dérivés hétérocycliques analogues aux formes furanoside et pyranoside des glucides, où l'atome d'oxygène hémiacétalique est remplacé par un atome de soufre ou d'azote[2-4] sont des antimétabolites possibles; de plus, couplés à des bases pyrimidiques ou puriques, ils peuvent permettre la synthèse de « nucléosides modifiés » susceptibles de posséder une activité biologique[5,6].

Dans de nombreux travaux, des pentoses ou hexoses sont employés comme précurseurs pour la synthèse de dérivés hétérocycliques de ce type. Pour notre part,

*Une communication préliminaire a été présentée[1].

il nous a semblé intéressant de synthétiser des dérivés pyrrolidiniques à partir de l'alcool correspondant à la 4-hydroxy-L-proline[7]. Ce composé, qui peut être considéré comme un analogue azoté d'un système 2,5-anhydro-3-désoxy-pentitol, possède deux fonctions facilement modifiables; de plus, ne présentant pas un caractère hémi-acétalique, il est stable en milieu acide et non clivable par les enzymes.

Nous nous proposons, dans cet article, de décrire la synthèse de dérivés pyrrolidiniques et leur cyclisation intramoléculaire conduisant au 1,4;2,5-dianhydro-2,3,4-tridésoxy-2,4-bis-(*p*-toluènesulfonamido)-D-*thréo*-pentitol. Ces synthèses sont effectuées à partir du dérivé 2,5-anhydro-2,3-didésoxy-2-*p*-toluènesulfonamido-4-*O*-*p*-toluènesulfonyl-L-*érythro*-pentitol (**1**) facilement accessible par réduction de la 4-hydroxy-L-proline[7].

L'introduction d'une fonction amine, soit sur le cycle en position 4, soit sur la chaîne latérale, en vue d'une synthèse ultérieure de « nucléosides modifiés », nous a conduits, dans une première étape, à étudier la substitution nucléophile par l'ion azide d'un groupement sulfonyloxy situé sur l'une de ces positions.

Ce type de substitution sur un noyau pentagonal ou hexagonal dépend du degré de substitution du cycle. Pour le système pyrrolidinique envisagé **2** et **3** contenant deux carbones non substitués C-3 et C-5, une substitution nucléophile semble *a priori* facilement réalisable. Le benzoate **2** est préparé par action du chlorure de benzoyle dans la pyridine sur l'alcool **1**. La préparation du tri-*p*-toluènesulfonate **3** est déjà décrite dans la littérature[7].

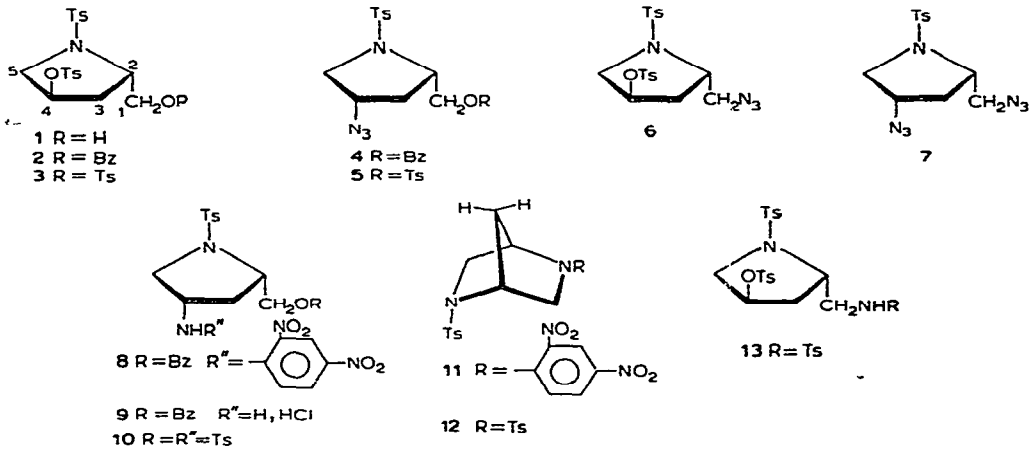
Par action de l'azothydrate de sodium dans la *N*,*N*-diméthylformamide à 120° sur le composé **2**, on introduit un groupement azide en position 4. L'azide **4** est isolé sous forme cristalline avec un rendement de 80%. L'amine correspondante est obtenue par réduction catalytique de l'azide **4** et caractérisée sous forme de dérivé *N*-2,4-dinitrophénylé **8** ou de chlorhydrate **9**.

Pour introduire, d'une manière sélective, une fonction azide sur la chaîne latérale, on fait agir l'azothydrate de sodium sur le dérivé tri-*p*-toluènesulfonate **3** dans l'acétonitrile à 80°. Deux produits cristallins sont isolés : le monoazide **6** (rdt. 25%) et le diazide **7** (rdt. 62%). Ce faible rendement en monoazide **6** indique qu'une substitution sélective sur un tel système ne peut être retenue comme méthode préparative. Ces résultats diffèrent de ceux obtenus dans le cas du 2,5-anhydro-1,3,4-tri-*O*-*p*-tolylsulfonyl-D-ribitol, où une substitution sélective du groupement tosyle relié au carbone primaire peut être effectuée avec un rendement quantitatif[10].

L'hydrogénation catalytique de l'azide **6** en présence d'oxyde de platine d'Adams ne conduit pas à l'amine correspondante. Le produit isolé sous forme de dérivé *N*-2,4-dinitrophénylé correspond au 4-amino-1,4;2,5-dianhydro-2,3,4-tridésoxy-2-*p*-toluènesulfonamido-D-*thréo*-pentitol (**11**). Sa structure a été confirmée par spectrométrie de masse (*m/e* 418) et spectrométrie de r.m.n. La cyclisation a eu lieu *in situ* lors de l'hydrogénation, les groupements aminométhylène et sulfonyloxy ayant une orientation *trans* favorable à la cyclisation.

En vue d'une corrélation avec le dérivé 1,4;2,5-dianhydro-2,3,4-tridésoxy-2,4-bis-(*p*-toluènesulfonamido)-D-*thréc*-pentitol (**12**) déjà décrit dans la littérature[9], nous

avons fait suivre l'hydrogénation catalytique de l'azide **6** d'une sulfonylation dans la pyridine. Il se forme deux produits. Le produit principal, isolé avec un rendement de 60%, a un point de fusion et un spectre r.m.n. identiques à ceux donnés dans la littérature pour **12**. Le produit mineur **13**, non isolé, traité par le méthylate de sodium



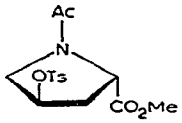
dans le méthanol, donne le produit bicyclique **12** déjà caractérisé. Ainsi par réduction catalytique, suivie d'une sulfonylation, on isole d'une part le produit bicyclique **12** et, d'autre part, le produit non cyclisé **13**, et cela dans un rapport de 3:1.

Il nous a paru intéressant de comparer le caractère nucléophile d'un groupement amine primaire situé sur un carbone secondaire à celui d'un même groupement relié à un carbone primaire. Dans cette perspective, l'azide **5** a été préparé par débenzoylation de l'azide **4** suivie d'une sulfonylation. L'hydrogénation catalytique de cet azide **5**, suivie d'une sulfonylation, conduit à des résultats analogues, mais dans ce cas le rapport entre le produit bicyclique **12** et le produit non cyclisé **10** est de 4:1.

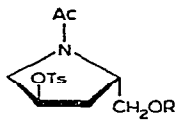
De ces deux expériences, il ressort que la cyclisation est plus aisée si le groupement nucléophile situé sur un carbone secondaire du cycle déplace un groupement sulfonyloxy situé sur la chaîne latérale que dans le cas inverse. Des résultats analogues ont été également observés dans le cas de systèmes 2,5-anhydropentitol convenablement substitués pour ce type de cyclisation intramoléculaire[11].

En outre, le fait que la cyclisation ait lieu à partir de l'azide **5** montre que la configuration est bien celle indiquée : la substitution par l'ion azide s'effectue avec inversion de configuration sur la position 4.

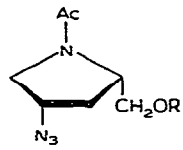
En conclusion, dans le cas de ce système pyrrolidinique, seuls les dérivés azidés en position 4 sont facilement accessibles par substitution nucléophile. Leur hydrogénation catalytique peut conduire aux dérivés soit du 4-amino-1,4;2,5-dianhydro-2,3,4-tridésoxy-2-*p*-toluènesulfonamido-D-*thréo*-pentitol **11** et **12**, soit de l'amine libre correspondante **8**, **9**, et **10**, suivant la nature du groupement fixé sur la chaîne latérale.



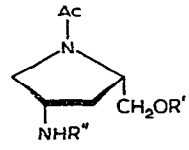
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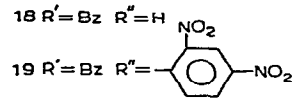
15 R=H
16 R=Bz



17 R=Bz



18 R'=Bz R"=H



Cependant, le clivage de la liaison N–Ts, nécessaire pour les réactions envisagées par la suite, est difficilement réalisable. Ce fait nous a amenés à protéger l'atome d'azote du cycle par un groupement acétyle. Nous avons réalisé la même suite de réactions à partir de l'ester méthylique[12] de la *N*-acétyl-4-*O*-*p*-toluènesulfonyl L-proline (**14**). L'alcool **15** est préparé par réduction de l'ester **14** par le borohydrure de lithium dans le 1,2-diméthoxyéthane et isolé sous forme cristalline. La benzoylation de cet alcool **15** par le chlorure de benzoyle dans la pyridine conduit au benzoate **16**. L'action de l'azothydrate de sodium dans la *N*,*N*-diméthylformamide sur le dérivé **16** permet de préparer l'azide **17** isolé sous forme de sirop incolore. L'amine correspondante **18**, obtenue par hydrogénation catalytique, a été caractérisée sous forme de dérivé *N*-2,4-dinitrophénylé **19**.

PARTIE EXPÉRIMENTALE

Conditions générales. — Les spectres de r.m.n. ont été mesurés dans le chloroforme-*d* sur un appareil Varian A-60 à la fréquence de 60 MHz. Les spectres de masse ont été mesurés sur un appareil MS 9. Les spectres i.r. ont été mesurés sur un appareil Perkin–Elmer 21. La détermination des pouvoirs rotatoires s'est faite à l'aide de l'appareil «Quick» de Roussel et Jouan. Les points de fusion sont corrigés. Les micro-analyses ont été effectuées au Laboratoire central du C.N.R.S.

*2,5-Anhydro-1-O-benzoyl-2,3-didésoxy-2-*p*-toluènesulfonamido-4-O-*p*-tolylsulfonyl-*L*-érythro-pentitol* (**2**). — Dans une solution de 3,6 g d'alcool[8] **1** dans 15 ml de pyridine anhydre, refroidie à −20° et maintenue sous agitation magnétique, on ajoute 2,5 ml de chlorure de benzoyle. Après une attente d'une h à −20°, le mélange réactionnel est conservé à 0° pendant 48 h. L'excès de chlorure de benzoyle est hydrolysé par addition d'eau glacée et on extrait ensuite au chloroforme. La phase organique est lavée plusieurs fois avec une solution d'acide chlorhydrique à 10%, puis une solution d'hydrogénocarbonate de sodium et enfin à l'eau jusqu'à neutralité. Elle est séchée sur sulfate de sodium. Après évaporation du chloroforme sous vide, on obtient 4,7 g de solide (rdt. 70%) qui recristallise de l'éthanol en paillettes blanches; p.f. 154–155°, $[\alpha]_D$ −31° (*c* 1,04, chloroforme).

Anal. Calc. pour $C_{26}H_{27}NO_7S_2$: C, 58,98; H, 5,10; N, 2,65; S, 12,10. Trouvé : C, 58,98; H, 5,24; N, 2,79; S, 11.83.

*2,5-Anhydro-4-azido-1-O-benzoyl-2,3,4-tridésoxy-2-*p*-toluènesulfonamido-D-thréo-pentitol* (**4**). — Le mélange de 265 mg de benzoate **2** et de 130 mg d'azothydrate

de sodium dans 4 ml de *N,N*-diméthylformamide est chauffé à 120° pendant 1 h 40 min sous agitation magnétique. Le mélange réactionnel est ensuite additionné d'eau glacée et extrait au chloroforme. La phase chloroformique est lavée à l'eau jusqu'à neutralité, puis séchée sur sulfate de sodium. Le produit obtenu après évaporation du solvant, est chromatographié sur gel de silice Davison. On recueille alors 149 mg (rdt. 75%) d'un produit qui cristallise dans l'éther. Le spectre i.r. montre une importante bande azide à 2100 cm^{-1}; p.f. 85–86°, $[\alpha]_D^{21}$ −47° (*c* 1,18, chloroforme).

Anal. Calc. pour $C_{19}H_{20}N_4O_4S$: C, 57,00; H, 5,00; N, 14,00; S, 8,00. Trouvé : C, 57,18; H, 5,06; N, 13,97; S, 8,11.

*2,5-Anhydro-4-azido-2,3,4-tridésoxy-2-*p*-toluènesulfonamido-1-*O*-p-tolylsulfonyl-*D*-thréo-pentitol* (**5**). — On ajoute à une solution de 545 mg d'azide **4** dans 10 ml de méthanol une quantité catalytique de sodium. Après agitation pendant 5 h, on neutralise par addition d'Amberlite IR-120 (H^+). La solution est ensuite filtrée et le filtrat est évaporé sous vide.

Le résidu obtenu précédemment est dissous dans 8 ml de pyridine anhydre et 855 mg de chlorure de tosyle sont ajoutés à 0°. Après une nuit à 0°, le produit est extrait suivant le processus habituel (*cf.* **2**). Le résidu solide obtenu après évaporation du solvant est lavé plusieurs fois à l'éther de pétrole à chaud et cristallisé dans un mélange acétate d'éthyle–éther de pétrole (rdt. 82%); p.f. 69–70°, $[\alpha]_D^{26}$ −55° (*c* 0,99, chloroforme).

Anal. Calc. pour $C_{19}H_{22}N_4O_5S_2$: C, 50,66; H, 4,92; N, 12,44; S, 14,21. Trouvé : C, 50,87; H, 5,08; N, 12,55; S, 14,46.

Action de l'azothydrate de sodium sur le dérivé **3**. — Le mélange réactionnel formé de 709 mg de tri-*p*-toluènesulfonate **3** et de 238 mg d'azothydrate de sodium en solution dans 5 ml d'acétonitrile est chauffé à 80° sous agitation magnétique pendant 48 h. Le mélange est ensuite versé sur de la glace et extrait au chloroforme. La phase organique est lavée à l'eau jusqu'à neutralité, puis séchée sur sulfate de sodium. Après évaporation du chloroforme, la chromatographie sur couche mince indique la présence de deux nouveaux produits en plus du produit de départ. Par cristallisation dans un mélange acétate d'éthyle–éther de pétrole, on isole un produit pur (rdt. 25%). L'analyse de ce dernier, ainsi que le spectre i.r. (bande azide à 2100 cm^{-1}) et le spectre de r.m.n., permettent d'établir qu'il s'agit du dérivé 2,5-anhydro-1-azido-1,2,3-tridésoxy-2-*p*-toluènesulfonamido-4-*O*-*p*-tolylsulfonyl-L-*érythro*-pentitol (**6**); p.f. 164–165°, $[\alpha]_D^{25}$ −16° (*c* 0,97, chloroforme).

Anal. Calc. pour $C_{19}H_{22}N_4O_5S_2$: C, 50,66; H, 4,92; N, 12,44; S, 14,21. Trouvé : C, 50,85; H, 4,98; N, 12,28; S, 14,17.

Après filtration du dérivé **6**, le filtrat est évaporé et chromatographié sur gel de silice Davison. On isole ainsi le 2,5-anhydro-1,4-diazido-1,2,3,4-tétradésoxy-2-*p*-toluènesulfonamido-D-*thréo*-pentitol (**7**, rdt. 62%) qui recristallise dans un mélange éther–éther de pétrole; p.f. 78–79°; $[\alpha]_D^{26}$ −38° (*c* 1,01, chloroforme).

Anal. Calc. pour $C_{12}H_{15}N_7O_2S$: C, 44,86; H, 4,67; N, 30,53; S, 9,97. Trouvé : C, 45,06; H, 4,66; N, 30,37; S, 10,03.

*4-Amino-2,5-anhydro-1-O-benzoyl-2,3,4-tridésoxy-2-*p*-toluènesulfonamido-D-*

thréo-*pentitol*. — L'azide **4** (200 mg) en solution dans 15 ml de méthanol est hydrogéné à pression et température ambiantes en présence de 40 mg de platine d'Adams. Après 4 h, la solution est filtrée sur Célite pour séparer le catalyseur. On isole après évaporation sous vide du solvant, un sirop incolore, homogène en c.c.m. révélée à la ninhydrine. L'amine ainsi obtenue a été caractérisée par préparation du dérivé *N*-2,4-dinitrophénylé **8** et du chlorhydrate **9**.

*2,5-Anhydro-1-*O*-benzoyl-2,3,4-tridésoxy-4-(2,4-dinitrophényl)amino-2-*p*-toluènesulfonamido-*D*-thréo-pentitol* (**8**). — L'amine (190 mg) est dissoute dans 4 ml de méthanol et additionnée de 1,5 g de carbonate de calcium et d'une solution de 150 mg de 1-fluoro-2,4-dinitrobenzène dans 4 ml de méthanol. Le mélange réactionnel est agité pendant une nuit à température ambiante. Après filtration sur Célite et lavage au méthanol, le filtrat est concentré sous vide et le résidu est repris au chloroforme. La phase chloroformique est lavée à l'eau jusqu'à pH neutre. Le produit obtenu après évaporation du solvant cristallise dans le méthanol en fines aiguilles jaunes; p.f. 120–122°, $[\alpha]_D^{24}$ +54° (*c* 0,57, chloroforme).

Anal. Calc. pour $C_{25}H_{24}N_4O_8S$: C, 55,56; H, 4,44; N, 10,37; S, 5,92. Trouvé : C, 55,61; H, 4,74; N, 10,15; S, 6,19.

*Chlorhydrate de 4-amino-2,5-anhydro-1-*O*-benzoyl-2,3,4-tridésoxy-2-*p*-toluènesulfonamido-*D*-thréo-pentitol* (**9**). — Une solution d'amine dans le méthanol est acidifiée par addition, goutte à goutte, d'une solution à 6% de gaz chlorhydrique dans le méthanol. Après évaporation du méthanol, le chlorhydrate cristallise dans l'acétone; p.f. 185–190°, $[\alpha]_D^{23}$ −56° (*c* 1, eau).

Anal. Calc. pour $C_{19}H_{23}N_2O_4SCl$: C,55,54; H, 5,63; N, 6,82; S, 7,78; Cl, 8,65. Trouvé : C, 55,50; H, 5,78; N, 6,60; S, 7,62; Cl, 8,78.

*Hydrogénation catalytique du 2,5-anhydro-1-azido-1,2,3-tridésoxy-2-*p*-toluènesulfonamido-4-*O*-p-tolylsulfonyl-*L*-érythro-pentitol* (**6**). — L'azide **6** (120 mg) en solution dans 10 ml de méthanol est hydrogéné à pression et température ambiantes en présence de 20 mg d'oxyde de platine. Après 4 h, la solution est filtrée sur Célite. Après évaporation du solvant, on recueille 114 mg d'un sirop incolore, homogène en c.c.m. révélée à la ninhydrine. Ce composé est caractérisé par la préparation du dérivé *N*-2,4-dinitrophénylé selon un procédé analogue à celui décrit pour le dérivé **8**. On isole par cristallisation dans le méthanol des cristaux jaunes de 1,4;2,5-dianhydro-2,3,4-tridésoxy-4-(2,4-dinitrophénylamino)-2-*p*-toluènesulfonamido-*D*-*thréo*-pentitol (**11**); p.f. 233–236°, $[\alpha]_D^{26}$ +662° (*c* 0,78, chloroforme).

Anal. Calc. pour $C_{18}H_{18}N_4O_6S$: C, 51,17; H, 4,34; N, 13,39; S, 7,65. Trouvé : C, 51,73; H, 4,37; N, 13,26; S, 7,94.

1,4;2,5-Dianhydro-2,3,4-tridésoxy-2,4-bis-(p-*toluènesulfonamido*)-*D*-thréo-*pentitol* (**12**). — (*a*) *A partir du composé* **6**. L'azide **6** (135 mg) est réduit en présence d'oxyde de platine et le composé bicyclique est isolé suivant le procédé habituel, puis sulfonylé par addition de 150 mg de chlorure de *p*-toluènesulfonyle dans 5 ml de pyridine. Après une nuit à 0°, le mélange réactionnel est versé sur de la glace et extrait au chloroforme. La phase organique est lavée avec une solution d'acide chlorhydrique à 10% jusqu'à pH acide, puis par une solution d'hydrogénocarbonate de sodium et enfin à l'eau

jusqu'à neutralité. Après évaporation du chloroforme, les deux produits obtenus sont séparés sur plaque préparative de gel de silice G.F.$_{254}$. Le composé bicyclique **12** cristallise dans le méthanol (rdt. 60%).

(*b*) *À partir du composé* **5**. — La même suite de réactions que dans le cas du dérivé **6**, effectuée sur l'azide **5**, permet d'isoler un dérivé cristallin identique en tout point au dérivé bicyclique **12** (rdt. 65%) précédemment décrit. Les caractéristiques du composé sont les suivantes : p.f. 123–124°, $[\alpha]_D^{23}$ −6°* (*c* 2,52, chloroforme). [Litt.[9] : $[\alpha]_D^{23}$—70° (*c* 2, chloroforme)].

Anal. Calc. pour $C_{19}N_{22}H_2O_4S_2$: C, 56,14; H, 5,46; N, 6,89; S, 15,76. Trouvé : C, 56,12; H, 5.50; N, 6.78; S, 15,76.

*2-Acétamido-2,5-anhydro-2,3-didésoxy-4-*O-p-*toluènesulfonyl-*L-érythro-*pentitol* (**15**). — L'ester méthylique[12] (3 g) de la *N*-acétyl-4-*O-p*-toluènesulfonyl-L-proline (**14**) est dissous dans 30 ml de 1,2-diméthoxyéthane et la solution est refroidie à 0°; 897 mg de borohydrure de lithium dissous dans 30 ml de 1,2-diméthoxyéthane sont ajoutés et le mélange est agité pendant 1 h à 0°, puis pendant 5 h à température ambiante. L'excès de borohydrure de lithium est détruit par addition d'acide acétique, puis d'eau, en refroidissant à 0°. Le produit est extrait au chloroforme. La phase organique est lavée avec une solution aqueuse d'hydrogénocarbonate de sodium, puis avec une solution saturée de chlorure de sodium; enfin elle est séchée sur sulfate de sodium. Après évaporation du chloroforme sous vide, on recueille un sirop incolore (2,3 g; rdt. 84%) qui, chromatographié sur gel de silice Davison, cristallise dans un mélange acétone–éther de pétrole; p.f. 116–118°, $[\alpha]_D^{23}$ −17,4° (*c* 1,09, chloroforme). Le spectre i.r. présente une bande à 3640 cm^{-1} (hydroxyle) et une bande à 1650 cm^{-1} (carbonyle; amide); la bande due au groupement ester méthylique est absente.

Anal. Calc. pour $C_{14}H_{19}NO_5S$: C, 53,67; H, 6,11; N, 4,47; S, 10,21. Trouvé : C, 53, 67; H, 6.17; N, 4,30; S, 10.29.

*2-Acétamido-2,5-anhydro-1-*O-*benzoyl-2,3-didésoxy-4-*O-p-*toluènesulfonyl-*L-érythro-*pentitol* (**16**). — L'alcool **15** (2,3 g) est benzoylé dans la pyridine par addition de 3,1 ml de chlorure de benzoyle à −20°. Le mélange est ensuite conservé une nuit à 0° puis extrait de la même manière que le composé **2**. Après chromatographie sur colonne de gel de silice Davison, le dérivé **15** est cristallisé dans un mélange acétone–éther; p.f. 116–117°, $[\alpha]_D^{23}$ −23° (*c* 1,06, chloroforme).

Anal. Calc. pour $C_{21}H_{23}NO_6S$: C, 60,42; H, 5,55; N, 3,36; S, 7,67. Trouvé : C, 60,56; H, 5,50; N, 3,43; S, 7,93.

*2-Acétamido-2,5-anhydro-4-azido-1-*O-*benzoyl-2,3,4-tridésoxy-*D-thréo-*pentitol* (**17**). — À 417 mg de benzoate **16** dissous dans 4 ml de *N,N*-diméthylformamide, on ajoute 260 mg d'azothydrate de sodium. Le mélange réactionnel est chauffé à 120°, sous agitation magnétique pendant 2 h. Après une extraction analogue à celle décrite pour l'azide **4**, on isole 270 mg de sirop incolore (rdt. 85%), homogène en c.c.m., mais qui n'a pu être cristallisé. Le spectre i.r. présente une forte bande d'absorption à

*Cette valeur a été obtenue dans les deux cas (*a*) et (*b*)

2100 cm^{-1} (azide) et la bande à 1600 cm^{-1} (tosyle) est absente; ceci est confirmé par spectrographie r.m.n.

2-Acétamido-4-amino-2,5-anhydro-1-O-benzoyl-2,3,4-tridésoxy-D-thréo-*pentitol* (**18**). — Pour obtenir cette amine **18**, l'azide **17** est hydrogéné dans les mêmes conditions que l'azide **4**. L'amine est caractérisée par la préparation du dérivé *N*-2,4-dinitrophénylé **19** par traitement avec le 1-fluoro-2,4-dinitrobenzène dans le méthanol. Après purification par chromatographie sur plaque préparative de gel de silice $G.F._{254}$, le produit cristallise à partir d'un mélange acétone–éther; p.f. 148–149°, $[\alpha]_D$ +6,2° (*c* 1,3, chloroforme).

Anal. Calc. pour $C_{20}H_{20}N_4O_7$: C, 56,07; H, 4,71; N, 13,08. Trouvé : C, 56,17; H, 4,58; N, 12,95.

REMERCIEMENTS

Cette étude a bénéficié d'une aide matérielle de la Ligue Nationale contre le Cancer. Nous remercions Monsieur le Professeur E. Lederer de l'intérêt qu'il a porté à ce travail et Monsieur D. Mercier pour ses conseils.

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THE CONFORMATIONS OF 1,2:4,6-DI-*O*-BENZYLIDENE-α-D-GLUCOPYRANOSE DERIVATIVES*

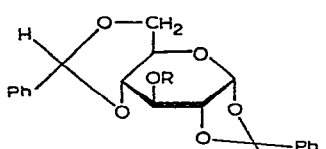
B. COXON
Institute for Materials Research, National Bureau of Standards, Washington, D. C. 20234 (U. S. A.)
(Received November 11th, 1969)

ABSTRACT

The conformations of a series of 1,2:4,6-di-*O*-benzylidene-α-D-glucopyranose derivatives in solution have been studied by p.m.r. spectroscopy and double-resonance techniques at 100 MHz. The coupling constants obtained by first-order analysis of the spectra were compared with those computed by iterative analysis. It was concluded that these compounds adopt conformations in which the *m*-dioxane and pyranoid rings have chair and flattened-chair forms, respectively.

INTRODUCTION

There is little doubt that the favored conformation of methyl 4,6-*O*-benzylidene-α-D-glucopyranosides and of other similar derivatives having the same configuration at C-4 is that in which the molecule has the $^4C_1$ (D) form. This has been amply demonstrated by i.r.[1] and p.m.r.[2–7] spectroscopy, and by chemical studies[8]. However, there has been controversy concerning the possible chair[9–12] or skew[13] conformations of 1,2-*O*-alkylidene-α-D-glucopyranose derivatives. The investigation of derivatives containing both 1,2- and 4,6-cyclic acetal substituents was therefore of interest, and the 1,2:4,6-di-*O*-benzylidene-α-D-glucopyranose (**1**) and 3-*O*-acetyl, 3-*O*-benzoyl, and 3-*O*-(methylsulfonyl) derivatives (**2**, **3**, and **4**) prepared by Fletcher *et al.*[14] have now been found to be suitable for study by p.m.r. spectrospcoy at 100 MHz.



1 R=H
2 R=Ac
3 R=Bz
4 R=Ms

*Presented, in part, at the 153rd National Meeting of the American Chemical Society, Miami Beach, Florida, April 12, 1967.

DISCUSSION

P.m.r. spectra of compounds **1–4** were obtained from their solutions in chloroform-*d* or pyridine, or in mixtures thereof. Although parts of the spectra were complex, because of overlap of multiplets, the signal multiplicities observed for the protons of the sugar chair were only those to be expected from coupling over two, or three, bonds. The spectral assignments for these protons were confirmed by spin-decoupling and spin-tickling, and by solvent shifts. Comparison of the chemical shifts of the benzylic protons (see Table I) with those of the 1,2-*O*-benzylidene-α-D-glucopyranoses[12,13] and 4,6-*O*-benzylidene-α-D-glucopyranosides[3] indicated that, in each case, the singlet at low field could be assigned to the 1,2 benzylic proton, and that at higher field, to the 4,6 benzylic proton. It has been observed[15] that the 1,2 benzylic protons of 1,2:3,5-di-*O*-benzylidene-α-D-glucofuranose derivatives resonate at lower field than the 3,5 benzylic protons, although for these compounds the chemical-shift difference between the benzylic resonances is appreciably smaller than for **1–4**.

The chemical shifts of the protons attached to the sugar chairs of **1–4** were sufficiently different for their spectra to be readily analyzable by first-order methods. However, an estimate of the accuracy of the parameters so derived, and of the validity of the analysis, was obtained by iterative analysis[16] of the spectrum (see Fig. 1a) of one of the compounds (**4**) as a seven-spin system (H-1–H-5, H-6*e*, and H-6*a*). From

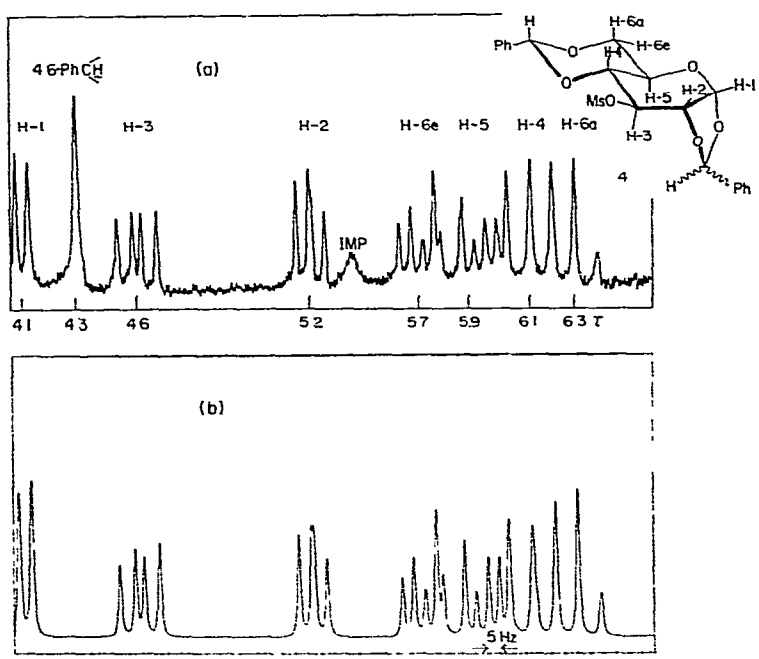


Fig. 1. Partial p.m.r. spectra of 1,2.4,6-di-*O*-benzylidene-3-*O*-(methylsulfonyl)-α-D-glucopyranose (a) Observed spectrum of solution in pyridine at 100 MHz, (b) computed spectrum after iterative analysis as a seven-spin system.

TABLE I

CHEMICAL SHIFTS (τ VALUES)[a] OF 1,2:4,6-DI-*O*-BENZYLIDENE-α-D-GLUCOPYRANOSE DERIVATIVES

Compound no.	*R-3*	*Solvent*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6e*	*H-6a*	*1,2-PhCH*	*4,6-PhCH*	*R-3*
1	H	C_5H_5N	4.14d[b]	5.34t	5.54q	6.22t	5.94sx	5.66q	6.28t	3.59	4.33	6.99[c]
1-*d*	D	$CDCl_3$–D_2O[d]	4.47d	5.77t	6.02q	6.58t	6.20sx	5.72q	6.42t	3.94	4.55	—
2	Ac	$CDCl_3$–C_5H_5N[e]	4.37d	5.69t	4.49q	6.36t	6.04o	5.71q	6.38t	3.74	4.54	8.00
3	Bz	C_5H_5N	4.02d	5.23t	3.86q	5 94t	5 75m	5.59q	6.20m	3.40	4.31	—[f]
4	Ms	C_5H_5N	4.105d[g]	5.229q[g]	4.555q[g]	6.076t[g]	5 858qi[g]	5.656q[g]	6.253t[g]	3.45	4.31	6.80

[a]Centers of multiplets, unless otherwise indicated [b]Multiplicities of signals are indicated by d(doublet, t(triplet), q(quartet), qi(quintet), sx(sextet), o(octet), and m(complex multiplet). [c]Solution in chloroform-*d*, broad singlet. [d]Overlayer. [e]Proportions 10:3 (v/v). [f]Obscured by solvent. [g]Refined value after seven iterations; computed probable error ≤0.0003 p.p.m.

TABLE II

PROTON COUPLING-CONSTANTS (Hz)[a] OF 1,2:4,6-DI-*O*-BENZYLIDENE-α-D-GLUCOPYRANOSE DERIVATIVES

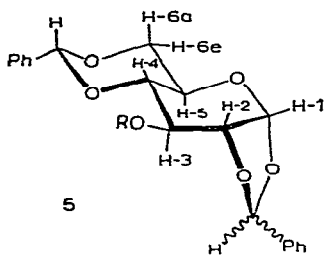
Compound no.	*R-3*	*Solvent*	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6e}$	$J_{5,6a}$	$J_{6e,6a}$
1	H	C_5H_5N	4.7	5.7	8.9	9.0	4.5	9.4	9.4
1-*d*	D	$CDCl_3$–D_2O[b]	4.8	6.2	9.2	9.2	3.9	9.5	9.1
2	Ac	$CDCl_3$–C_5H_5N[c]	4.7	5.7	9.5	9.3	4.4	9.7	9.7
3	Bz	C_5H_5N	4.8	5.7	8.9	9.0	4.5	8.9	~8.7
4	Ms	C_5H_5N	4.9	6.2	9.5	9.2	4.4	9.0	9 2
			4.97 ±0.03[d]	6.06 ±0.02[d]	10.03 ±0.02[d]	9.47 ±0.03[d]	5.03 ±0.03[d]	10.01 ±0.03[d]	10.15 ±0.02[d]

[a]First-order values (at 100 MHz), unless otherwise indicated [b]Overlayer. [c]Proportions 10:3 (v/v). [d]Refined value and computed probable error, after seven iterations. Coupling constants over more than three bonds were ≤0.6 Hz (unresolved).

spin-tickling experiments on **2**, it was established that $J_{6e,6a}$ is of opposite sign[17] to $J_{5,6e}$, and $J_{5,6a}$ and therefore $J_{6e,6a}$ was made negative in the iterative analysis of **4**. Support for the validity of the assignments is provided by the good agreement between the theoretical spectrum (Fig. 1b) computed from the refined parameters and the observed spectrum (Fig. 1a), and by the small error-parameters (see Experimental section). The chemical shifts and coupling constants of **1–4** are shown in Tables I and II, respectively. From the data for **4**, it was calculated that the maximum difference between the first-order and the refined value of each coupling constant is about 13% of the latter value, and that the corresponding mean difference for seven coupling constants is 6%.

The large values of $J_{3,4}$, $J_{4,5}$, and $J_{5,6a}$ (see Table II) are appropriate for pairs of protons having a *trans*-diaxial orientation, and the medium-sized values of $J_{5,6e}$ are typical of protons having a *gauche*-axial–equatorial orientation[3,5–7,8]. However, the values of $J_{1,2}$ are consistently ~1 Hz larger, and the values of $J_{2,3}$, ~3.6 Hz smaller, than those of methyl 4,6-*O*-benzylidene-α-D-glucopyranosides[3], indicating that the proton–proton dihedral angles $\phi_{1,2}$ and $\phi_{2,3}$ of **1–4** are smaller than those of the α-D-glucopyranosides. Application of the refined Karplus equation[19] to compounds **1–4** gave the approximate values $\phi_{1,2}$ 38–39° and $\phi_{2,3}$ 142–145°, which compare favorably with the values of 25° and 135°, respectively, measured on Dreiding molecular models.

The results are consistent with the conformation **5** in which the *m*-dioxane ring adopts a chair form, and in which the pyranoid ring has a flattened chair-form because of attachment of the 1,2-*O*-benzylidene group. As compound **1** was prepared[14] under acidic conditions, it is assumed that the phenyl group of the 4,6-*O*-benzylidene substituent is equatorially attached to the chair form of the *m*-dioxane



5

ring[20,21]. The configuration of the 1,2 benzylic carbon atom has not been definitely determined, but, on the basis of comparisons of the coupling constants of the diastereoisomers of 3,4,6-tri-*O*-acetyl-1,2-*O*-benzylidene-α-D-glucopyranose with respect to chair conformations, it has been suggested[12] that the single diastereoisomer obtained by Fletcher *et al.*[14] is that in which the phenyl group has the *endo* orientation. This compound is not the thermodynamically more stable diastereoisomer as, on treatment with equilibrating reagents, it gave a mixture that contained a major proportion (74%) of a second diastereoisomer. A similar mixture has been prepared recently[12] by reaction of tri-*O*-acetyl-1,2-*O*-(1-*exo*-ethoxyethylidene)-α-D-glucopyranose with benz-

aldehyde. As the 1,2-*O*-benzylidene derivative (Fletcher *et al.*[14]) was prepared by partial hydrogenolysis of **1** under neutral conditions, the configurational suggestion of Lemieux and Detert[12] implies that, for **1–4** also, the phenyl group of the 1,2-*O*-benzylidene substituent has the *endo* orientation.

Although study of molecular models revealed that the cyclic acetal substituents would not sterically prohibit the adoption of a skew shape by the pyranoid ring of the dibenzylidene derivatives were this energetically feasible, only a small value of $J_{2,4}$ (0.03 Hz) was obtained from the iterative analysis of compound **4**. Hence, there is no evidence for any conformational equilibrium involving the skew form of the pyranoid ring in which H-2 and H-4 and their intervening carbon atoms have an almost planar W-arrangement[22]. This arrangement is mandatory for the locked-skew conformation of 3-*O*-benzoyl-1,2,4-*O*-benzylidyne-α-D-ribopyranose, for which the value $J_{2,4}$ 2.45 Hz has been found[23].

In conclusion, the favored conformation of the 1,2:4,6-di-*O*-benzylidene-α-D-glucopyranose derivatives is that in which the pyranoid ring has a flattened-chair shape. Nevertheless, as the coupling constants $J_{2,3}$ and $J_{3,4}$ of these derivatives are in all cases significantly larger than those of 1,2-*O*-alkylidene[9,11,13] and -arylidene[11–13] -α-D-glucopyranoses, there is evidently less conformational distortion of the di- than of the mono-benzylidene acetals. This situation may be ascribed to the tendency of the 4,6-*O*-benzylidene substituent to increase the rigidity of the pyranoid ring.

EXPERIMENTAL

General. — Unless indicated otherwise, the p.m.r. spectra were recorded in the frequency-sweep mode at 100 MHz, with internal, field-frequency stabilization on the signal of tetramethylsilane (τ 10.00). Frequency-swept, spin-decoupling and spin-tickling experiments were performed after the connection of an external, audio oscillator to the spectrometer. The solutions used were either saturated, or contained 74–125 mg of solute in 0.5–1.0 ml of solvent. In most cases, chemical shifts were measured at the 1-kHz sweep-width; these shifts were then used for calibrating the sweep offset of the spectra recorded at the 250-Hz sweep-width, from which were obtained coupling constants and (for compound **4**) initial chemical-shifts for analysis by computer.

*Equilibration of the diastereoisomeric 3,4,6-tri-*O*-acetyl-1,2-*O*-benzylidene-α-D-glucopyranoses.* — A solution of crystalline 3,4,6-tri-*O*-acetyl-1,2-*O*-benzylidene-α-D-glucopyranose[14] (141 mg) in chloroform-*d* (0.38 ml) was examined by p.m.r. spectroscopy at 60 MHz. As reported previously[13], the spectrum showed only one benzylic-proton resonance (singlet, τ 3.57). Benzaldehyde (36.3 μl, 1 molar equivalent) was added to the solution, followed by *p*-toluenesulfonic acid (5 mg). The mixture was kept at 35°, and, after 17 h, its p.m.r. spectrum showed a second benzylic-proton singlet[12] at τ 4.13. The intensity of this signal slowly increased, while that of the singlet at τ 3.57 diminished. Formation of a diastereoisomeric compound[12] was

confirmed by observation of the changes in the spectra of the ring protons and acetyl methyl groups. The relative proportions of the diastereoisomers were measured periodically by 7–12 integrations of the benzylic resonances, including 2–4 planimetric measurements of spectra recorded at a sweep width of 250 Hz. At the following intervals of time (18, 64, 116, 164 h), the mean ratio of benzylic resonances was 1.4, 2.3, 2.8, 2.8 at high and low fields with standard deviations of 0.05–0.1.

At 35°, the equilibrium constant (2.8) corresponds to a free-energy change of −2.3 kJ/mole (−0.56 kcal/mole).

Spectral assignments. — *A. Compound* **1** *in chloroform*-d. As for other 1,2-*O*-alkylidene[9,11,13,24] and arylidene[11,13,15] derivatives, the doublet at low field (τ 4.47) was assigned to H-1. Irradiation at τ 4.47 caused the collapse to a doublet of the triplet at τ 5.78, which was therefore assigned to H-2. Conversely, irradiation at τ 5.78 resulted in collapse of the H-1 doublet to a singlet. Equilibration of the solution with deuterium oxide caused disappearance of the broad singlet at τ 6.99, which was therefore assigned to the hydroxylic proton.

B. Compounds **1–3** *in pyridine.* The application of a weak (spin-tickling) field at the frequency of the low-field line of the doublet at low field (H-1) caused the triplet at τ 5.2–5.5 to split into a sextet (therefore, this was due to H-2). For **1**, spin-tickling of H-2 at the frequency of its low-field line produced splitting of the H-1 doublet into a quartet. For **2**, strong-field irradiation at the frequency of the overlapping doublet and quartet at low field (H-1 and H-3) caused the triplet at τ 5.45 to be simplified to a singlet (H-2), and the quartet at τ 6.18 to collapse to a doublet (H-4). Alternatively, strong irradiation at τ 5.45 caused collapse of the multiplets at low field to a singlet (H-1) and a doublet (H-3). Other experiments, in which H-3 was spin-tickled, confirmed the assigment of the H-2 and H-4 signals. Weak-field irradiation at the frequency of the line of the H-6*e* quartet at lowest field caused splitting of the highest-field line of the H-5 octet and of the lowest-field of the H-6*a* triplet into doublets, thus indicating the assignments of H-5 and H-5*a*. Inspection of spin-state diagrams for H-5, H-6*e*, and H-6*a* showed that $J_{5,6e}$ and $J_{5,6a}$ have the same sign, and that $J_{5,6a}$ and $J_{6e,6a}$ have opposite signs.

Iterative spectral analysis. — The spectra of compounds **1–4** were analyzed initially by first-order methods. The parameters obtained from a solution of **4** in pyridine (see Tables I and II) were selected for refinement and were used in computing a trial theoretical spectrum for the seven-spin system composed of H-1–H-6*e*, and H-6*a*, by use of a modified[24] LAOCN 3 program[16] and a digital, incremental, x–y plotter, with Gaussian and Lorentzian line-widths of 1.0 Hz. The sign of the geminal coupling $J_{6e,6a}$ was taken as being the opposite of those of the vicinal couplings, as had been determined for compound **2**. By inspection of the trial spectrum, 96 of the 3003 theoretical transitions were assigned to 26 experimental line-frequencies (precision, ±0.1 Hz), and iterative variation of the seven chemical shifts and 21 coupling constants was allowed to proceed for four cycles, with root-mean-square (r.m.s.) errors of 0.828, 0.490, 0.333, and 0.362 Hz. The assignments of some of the lines were then adjusted or deleted, so as to minimize the frequency error ($\Delta\nu$), and

the *thrice*-iterated parameters were further refined by four more iterations of the program in which 76 transitions were assigned to 26 spectral lines. The r.m.s. errors were 0.11, 0.057, 0.056, and 0.057 Hz, and the computed probable errors of the refined parameters (see Tables I and II) were $\leqslant 0.04$ Hz, and $|\Delta\nu| \leqslant 0.16$ Hz.

ACKNOWLEDGMENTS

Thanks are due Dr. H. G. Fletcher, Jr., for compounds, Dr. K. A. McLauchlan for his help, and Dr. R. Schaffer for his support.

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Printed in Belgium

STRUCTURAL STUDIES OF THE O-SPECIFIC SIDE-CHAINS OF THE CELL-WALL LIPOPOLYSACCHARIDES FROM *Salmonella newport* AND *Salmonella kentucky*

CARL GUSTAF HELLERQVIST, BENGT LINDBERG, SIGFRID SVENSSON,
Institutionen för Organisk Kemi, Stockholms Universitet, Stockholm (Sweden)

TORD HOLME,
Bakteriologiska Institutionen, Karolinska Institutet, Stockholm (Sweden)

AND ALF. A. LINDBERG
Statens Bakteriologiska Laboratorium, Stockholm (Sweden)

(Received November 13th, 1969)

ABSTRACT

The structures of the O-specific side-chains of the cell-wall lipopolysaccharides (LPS) of *Salmonella newport* and *Salmonella kentucky* have been investigated. Methylation analyses of the original LPS, of the materials obtained on mild hydrolysis of the LPS with acid, and of a product obtained by acetalation of all of the free hydroxyl groups followed by deacetylation, have provided the essential information in this study. The mixtures of sugars obtained on acid hydrolysis of the different methylated products were analysed, as alditol acetates, by g.l.c–mass spectrometry. As a result of these studies, detailed structures of the repeating units of these side-chains are presented.

INTRODUCTION

The O-specific side-chains from *Salmonella* serogroup B, C_2, and C_3 contain the same sugar residues[1] (abequose, L-rhammose, D-mannose, D-galactose, and D-glucose). The structure of the O-specific side-chains of the serogroup B LPS has been extensively studied[2], but no similar studies on the LPS of groups C_2 or C_3 have been reported. Because of the different serological specificities between these groups, considerable differences in LPS structures may be expected. The present paper reports structural studies on the LPS from *S. newport* I.S.50 (6,8) of group C_2, and from *S. kentucky* I.S.98 (8,20) of group C_3.

The methods used were essentially those used previously in studies of other *Salmonella* LPS, and relied mainly on methylation analysis of the original and of chemically modified LPS[3].

ISOLATION OF LIPOPOLYSACCHARIDES

The bacteria were killed by γ-irradiation and disintegrated, and the cell-wall material was collected, and extracted by the phenol–water method[3]. In tests of

passive haemagglutination inhibition, the isolated LPS were shown to contain the same O-factors as the whole bacteria[3].

In order to minimize the destruction of abequose, acid hydrolysis was performed in two steps, with isolation of abequose after the first, mild hydrolysis as previously described[3]. The hydrolysate of the *S. newport* LPS contained abequose, L-rhamnose, D-mannose, D-galactose, D-glucose, and a heptose in the relative proportions 15:15:30:18:18:4. The corresponding figures for the *S. kentucky* LPS were 16:17:36:20:8:3. The first five sugars appear in the O-specific side-chains of the bacteria, whereas heptose together with D-galactose and D-glucose are found in the basal core. The sugars were analysed as their alditol acetates by gas–liquid chromatography[4] (g.l.c.), the identifications being confirmed by mass spectrometry[5] (m.s.). These sugars have been fully identified[1].

Both LPS showed a strong i.r.-absorption band at 1738 cm^{-1}, indicative of *O*-acetyl groups. This indication was corroborated by treating the LPS samples with methanolic hydrogen chloride and identification (g.l.c.–m.s.) of the methyl acetate formed.

METHYLATION ANALYSIS OF LIPOPOLYSACCHARIDES

The lipopolysaccharides, in methyl sulphoxide, were methylated by treatment[6] with methylsulphinyl sodium and methyl iodide. The fully methylated polysaccharides were hydrolysed, and the methylated sugars in the hydrolysates were converted into alditol acetates, and analysed by g.l.c.[7]–m.s.[8]. A typical g.l.c. (*S. newport*) is shown in Fig. 1. The results of the methylation analysis are given in Table I. All components were identified by their retention times (*T*-values) on g.l.c. and by m.s. Most of these methylated derivatives were the same as found[3] in the methylation analysis of group B LPS. Only the identification of components, not found in these studies, will be discussed below.

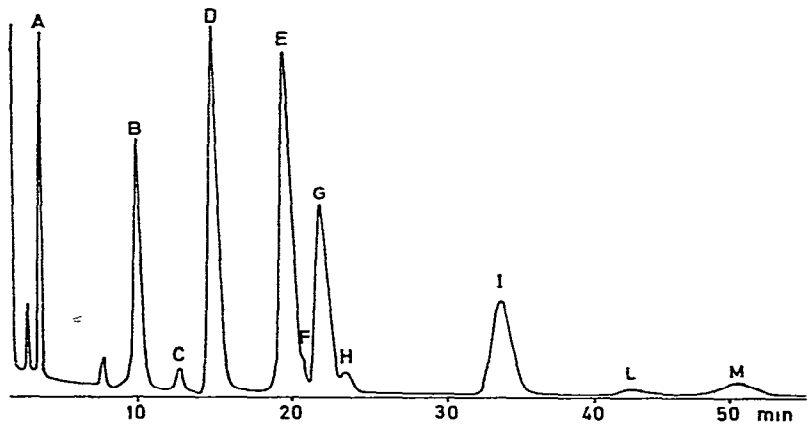


Fig. 1. G.l.c. separation of methylated sugars, as their alditol acetates, obtained from the hydrolysate of the fully methylated *S. newport* LPS

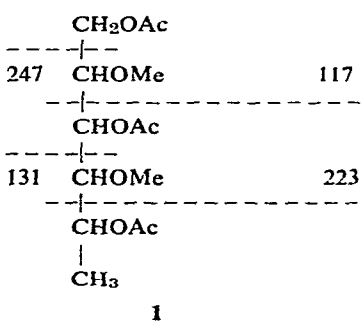
TABLE I

METHYL ETHERS FROM THE HYDROLYSATE OF METHYLATED LIPOPOLYSACCHARIDES

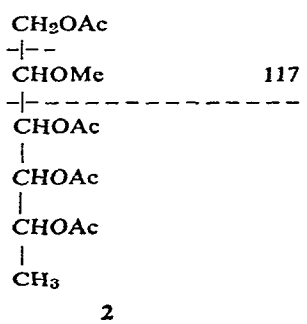
	Peak	T[a]	S. newport *mol %*[b]	S. kentucky *mol %*[b]
2,4-Di-*O*-methylabequose	A	0.32	11.9	11.4
2,3,4,6-Tetra-*O*-methyl-D-glucose	B	1.00	10.3	4.8
2,4-Di-*O*-methyl-L-rhamnose	B	0.94	1.0	1.0
2,3,4,6-Tetra-*O*-methyl-D-galactose	C	1.25	1.4	1.2
2-*O*-Methyl-L-rhamnose	D	1.52	14.1	16 0
3,4,6-Tri-*O*-methyl-D-mannose	E	1 95	21.3	34 4
3,4,6-Tri-*O*-methyl-D-glucose	F	2.11	1.3	1.2
2,4,6-Tri-*O*-methyl-D-galactose	G	2 29	13 7	13.3
3,4,6-Tri-*O*-methyl-D-galactose	H	2.50	0.4	1.2
4,6-Di-*O*-methyl-D-mannose	I	3 29	10 0	—
2,6-Di-*O*-methyl-D-galactose	K	3.65	—	3.5
3,6-Di-*O*-methyl-D-galactose	L	4 30	1.5	1 2
2,4-Di-*O*-methyl-D-glucose	M	5.10	2 7	2.2

[a]Retention times of the corresponding alditol acetates on the ECNSS-M column relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [b]As a considerable proportion of 2,4-di-*O*-methylabequose was lost during the methylation analyses, the % of methylated sugars are given relative to the sum of 2,4-di-*O*-methyl- and 2-*O*-methyl-L-rhamnose, which is assumed to be the same as the % of L-rhamnose in the original LPS

The m.s. of peak B (*T* 1.00) shows the presence of an alditol acetate derived from a 2,3,4,6-tetra-*O*-methylhexose. The corresponding tetra-*O*-methyl derivatives of D-glucose and D-mannose are not separated with a ECNSS-M column but were resolved (*T* 1.00 and 1.05, respectively) with a OS 138 (polyphenyl ether) column. By using the latter column, we found the D-glucose but not the D-mannose derivative. In addition to the fragments typical for the 2,3,4,6-tetra-*O*-methyl-D-glucose derivative, the mass spectrum also contained fragments having *m/e* 131, 233, and 247. These, together with *m/e* 117, are the primary fragments expected for the alditol acetate derived from a 6-deoxy-2,4-di-*O*-methylhexose (**1**). Since L-rhamnose is the only 6-deoxyhexose in the LPS, **1** must be derived from that sugar. No column was found on which a separation of the D-glucose and L-rhamnose derivatives could be obtained. The determination of L-rhamnose based on the intensities of relevant m.s. peaks must be regarded as a rough approximation.



1



2

The component in peak D (*T* 1.52) gave strong signals at *m/e* 117 and 275 which are primary fragments expected for the alditol acetate derived from a 6-deoxy-2-*O*-methylhexose (2). Although no authentic 2-*O*-methyl-L-rhamnose was available to verify this claim, the component must be derived from L-rhamnose, the only 6-deoxy sugar present.

The molar percentages of abequose, L-rhamnose, D-mannose, D-galactose, and D-glucose, calculated from the methylation analysis and assuming that the molar percentage of L-rhamnose is the same as in the original LPS, are 12:15:31:17:14 (*S. newport*) and 11:17:34:19:7 (*S. kentucky*). Except for abequose, there is good agreement between these figures and those obtained from the sugar analysis. It seems reasonable to assume, however, that part of the volatile 2,4-di-*O*-methylabequose and derivatives was lost during concentrations.

METHYLATION ANALYSIS OF THE PARTIALLY HYDROLYSED LIPOPOLYSACCHARIDES

The polysaccharides were subjected to mild hydrolysis with acid which, as anticipated, caused the preferential cleavage of all of the abequosidic and part of the L-rhamnosidic linkages. The oligo- and poly-meric material was recovered and subjected to methylation analysis as above. From these results (Table II), the mutual order of some sugar residues in the LPS may be deduced as discussed below.

TABLE II

METHYL ETHER FROM THE HYDROLYSATE OF PARTIALLY DEGRADED AND METHYLATED LIPOPOLYSACCHARIDES

	T[a]	S. newport *mol* %[b]	S. kentucky *mol* %[b]
2,3,4-Tri-*O*-methyl-L-rhamnose	0.46	4.5	2.8
2,3-Di-*O*-methyl-L-rhamnose	0.98	8.0	9.9
2,3,4,6-Tetra-*O*-methyl-D-glucose	1.00	6.3	5.0
2,3,4,6-Tetra-*O*-methyl-D-mannose	1.00	4.5	10.5
2,3,4,6-Tetra-*O*-methyl-D-galactose	1.25	0.5	0.8
3,4,6-Tri-*O*-methyl-D-mannose	1.95	24.2	25.8
2,4,6-Tri-*O*-methyl-D-galactose	2.29	13.7	13.3
4,6-Di-*O*-methyl-D-mannose	3.29	5.2	—

[a]See Table I. [b]Since no additional 2,3,4,6-tetra-*O*-methyl-D-galactose was formed, the percentages are given relative to 2,4,6-tri-*O*-methyl-D-galactose, assumed to be the same in the methylation analysis of both degraded and original LPS.

DETERMINATION OF THE POSITION OF THE *O*-ACETYL GROUPS

The LPS was treated with methyl vinyl ether[9], to protect all free hydroxyl groups as acetals, and the modified LPS was subjected to methylation analysis. A mixture of sugars and methylated sugars was obtained, the methoxyl groups in the latter marking the positions of the original *O*-acetyl groups.

By this treatment, the *S. newport* LPS afforded abequose, D-mannose, and D-galactose in the same proportions as were found in the original LPS. No L-rhamnose and only a small proportion of D-glucose was found. A g.l.c. peak was identified as the alditol acetate derived from 2-*O*-methyl-L-rhamnose. The size of this peak revealed a complete recovery of the initial amount of L-rhamnose. The components of two other peaks were identified, from their m.s. and *T*-values, as being derived from 2-*O*-methyl-D-glucose and 3-*O*-methyl-D-glucose. Their relative proportions were 3:2.

By the same treatment, the *S. kentucky* LPS yielded abequose, L-rhamnose, D-mannose, and D-galactose in the same proportions as found in the original LPS. Only a small proportion of D-glucose was detected; instead, 2-*O*-methyl- but not 3-*O*-methyl-D-glucose was found. The ratio of these two sugars was 1:5. Since the D-glucose residues are, in part, derived from the basal core, these values indicate that almost every D-glucose in the side chains is *O*-acetylated.

ACID HYDROLYSIS OF THE LIPOPOLYSACCHARIDES

A solution of the LPS in 0.05M sulphuric acid was kept at 80°, and the change in optical rotation was followed polarimetrically (Fig. 2). The shape of the curve is similar to that obtained on acid hydrolysis of the *S. typhi* and *S. enteritidis* LPS, and indicates, by the same arguments as used in the discussion of these results[10], that the abequose residues and the L-rhamnose residues are α-linked.

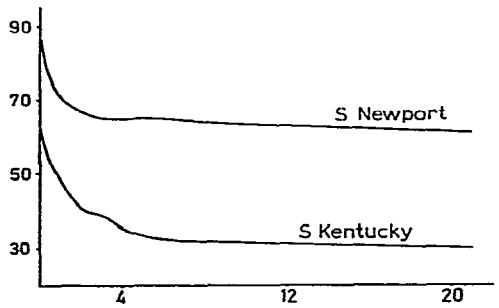


Fig. 2. Optical rotation *vs.* time on acid hydrolysis of the *S. newport* and *S. kentucky* LPS

DISCUSSION

It has been shown by biochemical and chemical methods that the O-antigenic side-chains of various *Salmonella* serogroups are built up of repeating units[1-5].

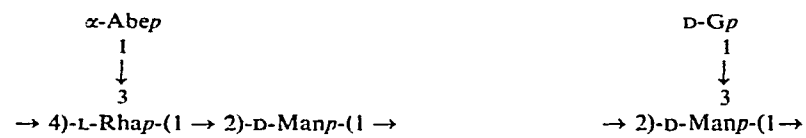
The results reported above support the assumption, used in the discussion below, that the O-antigenic side-chains of the LPS of serogroups C_2 and C_3 are also built up of repeating units.

S. newport. — From the sugar analysis it is evident that a repeating unit in the O-specific side-chains contains abequose, L-rhamnose, D-mannose, and D-galactose

residues in the relative proportions 1:1:2:1. The high percentage of D-glucose indicates that several of the repeating units also contain a D-glucose residue.

Several minor components found in the methylation analysis, *viz.*, 2,3,4,6-tetra-*O*-methyl-D-galactose, 3,4,6-tri-*O*-methyl-D-glucose, 3,4,6-tri-*O*-methyl-D-galactose, 3,6-di-*O*-methyl-D-galactose, and 2,4-di-*O*-methyl-D-glucose, have been found in methylation analysis of other *Salmonella* LPS[3,10,14] and most certainly derive from the basal core. The other components were those to be expected as derived from the O-specific side-chains. It is evident from the methylated derivatives that all of these residues are pyranosidic. Abequose and D-glucose residues are terminal. The D-galactose residues are linked through C-3, and most of the L-rhamnose residues are substituted at C-3 and C-4. All D-mannose residues are linked through C-2, but some are also linked through C-3. The percentage of the latter is equivalent to that of the terminal D-glucose residues.

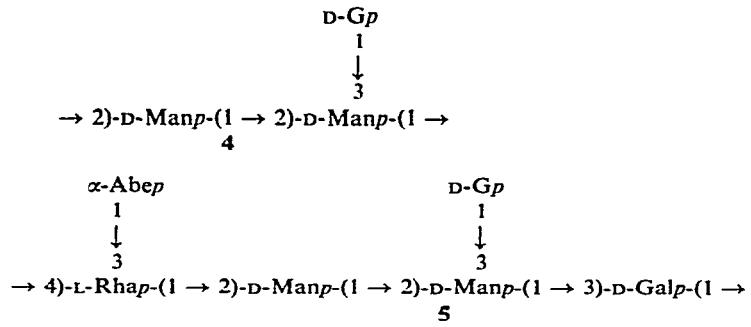
In the methylation analysis of the partially degraded LPS, neither abequose nor 2-*O*-methyl-L-rhamnose were present, but a high percentage of 2,3-di-*O*-methyl-L-rhamnose was observed. This finding demonstrates that abequose is linked to C-3 of L-rhamnose in the original LPS. The high percentage of 2,3,4,6-tetra-*O*-methyl-D-mannose, and the corresponding decrease of the other mannose ethers, demonstrates that the second most readily hydrolysed linkage, the L-rhamnosidic, is connected to C-2 of D-mannose. The absence of 2,4,6-tri-*O*-methyl-D-mannose in the hydrolysate of the modified LPS excludes the possibility that D-glucose is attached to the D-mannose residue to which L-rhamnose is linked at C-2. From these results, together with the assumption that the LPS is built up of repeating units, the partial structures **3a** and **3b** can be deduced.



3a **3b**

S. newport contains O-factor 6, which is also present in the group C_1 LPS. Recent results by Staub *et al.*[11], who studied the LPS from *S. colera suis*, suggest that this LPS contains chains of (1→4)-linked D-mannose residues intersected by 2-acetamido-2-deoxy-D-glucose residues. Some of the former carry terminal D-glucose residues, linked to C-3. Unpublished results by the present authors, on the LPS from the related *S. thompson* (6_2,6_2,7), demonstrate that all of the D-mannose residues are linked through C-2 and that some of them are substituted with a terminal D-glucose residue linked through C-3. Staub *et al.*[12] have also demonstrated, by immunological studies, that O-factor 6 is associated with the presence of the terminal α-D-glucose residue linked to a mannose oligosaccharide.

It seems reasonable, therefore, to assume that the structural element **4** is also present in the *S. newport* LPS, giving partial structure **5** for its repeating unit. From the methylation analysis of partially degraded LPS, it can be concluded, as shown above, that D-glucose is attached to the second D-mannose residue.



The low percentage of 2,4-di-*O*-methyl-L-rhamnose observed in the methylation analysis of the original LPS must derive from the abequosyl-rhamnose residue that terminates the O-specific side-chain. The percentage of this component, the determination of which was not very accurate (1.0 $\pm$0.2), indicates that each side-chain contains, on average, 20 repeating units.

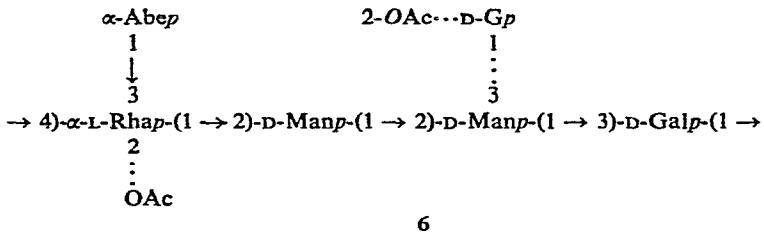
If the deduction above, based upon immunochemical evidence, is correct, partial structure **5** should therefore represent the biological repeating unit. This starts with D-galactose, by analogy with what has been demonstrated for group B LPS by biochemical[13] and chemical[14] methods.

Virtually all of the L-rhamnose and D-glucose residues are acetylated, the former in C-2 and the latter in both C-2 and C-3 (3:2). The percentage of D-glucose residues, acetylated in the 3-position, may not be structurally significant, but could be accounted for by *O*-acetyl migration, either in the bacteria or during the isolation of the LPS and subsequent reactions. Terminal D-glucose residues, acetylated in the 2-position, have been found[10] in the *S. typhi* LPS. Unpublished results indicate that the L-rhamnose residues in the *S. paratyphi A* LPS carry *O*-acetyl groups. The LPS from *S. newport* is, however, the first LPS in which the occurrence of *O*-acetyl groups in two different sugars has been demonstrated.

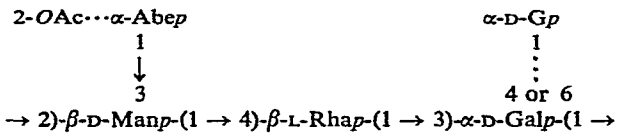
From the present results, including the results of the polarimetric studies, and from further arguments presented above, the partial structure **6** is proposed for the repeating unit of the O-specific side-chains in the *S. newport* LPS. For comparison, the corresponding structure (**7**) for the repeating unit in group B LPS is given. In these structures, a dotted line indicates a substituent that is not present in all repeating units. The finding of non-stoichiometrical amounts of D-glucose and *O*-acetyl groups in the repeating units in the *S. newport* LPS are expected from our studies on other *Salmonella* LPS. This is further in agreement with the finding, by Robbins *et al.*[15], that the trisaccharide of group E is biosynthesized, polymerized, and transferred to the basal core, and afterwards D-glucose and *O*-acetyl groups are attached.

A comparison of **6** and **7** reveals that they have several structural features in common. The differences are, however, sufficient to account for the different immunological properties of the corresponding LPS.

S. kentucky. — By similar arguments to those adduced above for the LPS of *S. newport*, the repeating unit of the *S. kentucky* LPS must contain structure element

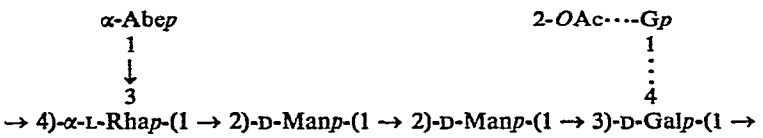


6



7

3a. In most respects, the LPS from these *Salmonella* strains are similar. An important difference between them is that the terminal D-glucose residues are attached to C-4 of D-galactose in the former, but to D-mannose residues at C-3 in the latter. Although no immunochemical evidence could be advanced to assign the mutual order of the D-galactose residue and the D-mannose residue not linked through C-2 to L-rhamnose, it is assumed that this order is the same as in the *S. newport* LPS. Therefore, structure **8** is proposed for the biological repeating unit of the *S. kentucky* LPS. From the percentage of 2,4-di-*O*-methyl-L-rhamnose, the average number of repeating units in the side-chains is estimated to be approximately 20.



8

Immunochemical implications — The O-antigen carrying factor 8 is present in serogroups C_2 and C_3. It is most likely associated with the presence of the α-abequose residue, linked (1→3) to L-rhamnose. The fact that all L-rhamnose residues are acetylated in the *S. newport* LPS, and none in the *S. kentucky* LPS, suggests the possibility that serological differences between the two strains may be found.

The O-factor 6, present in sero groups C_1 and C_2, has been divided[11,12] into subfactors 6_1 and 6_2. In the LPS of *S. cholera suis* (6_1,6_2,7) of group C_1, the 6_1 factor has been shown to be associated with the presence of a terminal α-D-glucose residue linked to D-mannose through C-3. The LPS of *S. newport* reacted with an 06,14 antiserum, but not with the anti 14 serum, which shows that O-factor 6 is present in this strain. No inhibition of homologous anti 6_1 or 6_2 passive hemagglutination systems could be demonstrated (Tables III and IV) for the LPS or a deacetylated preparation. These findings suggest that O-factor 6 must be further subdivided.

O-Factor 20, in the *S. kentucky* LPS, is most probably associated with the presence of the terminal 2-*O*-acetyl-D-glucose residue, linked to D-galactose at the

TABLE III

HEMAGGLUTINATION INHIBITION TESTS[a]

LPS	*O-factor antisera*				
	06_1	06_2	*08*	012_2	*020*
S. newport (6.8)	>256	>256	16	>256	>256
Deacetylated *S. newport*	>256	>256	8	>256	>256
S. kentucky (8,20)	>256	>256	4	>256	<1
Deacetylated *S. kentucky*	>256	>256	2	>256	2
S. thompson (6_1,6_2,7)	8	<1	>256	>256	>256

[a]Data recorded represent minimal concentration of LPS (μg/ml) dry weight which causes a macroscopic inhibition of the hemagglutination system. Hemagglutination systems: 06_1, *S. cholerae suis* var. kunzendorff I.S.36 LPS/anti 6_1 serum; 06_2, *S. cholerae suis* var. kunzendorff I.S.37 LPS/anti 6_2 serum; 08, *S. newport* I S.50 LPS/anti 08 serum; 012_2, *S. typhimurium* 395 MS LPS/*S. typhi* T4 I.S.58 anti 12_2 serum and 020 *S. kentucky* I.S.98 LPS/anti 20 serum.

TABLE IV

TUBE AGGLUTINATION TEST[a]

Antigen	*O-factor antisera*	
	06_1	06_2
S. cholerae suis I.S. 36 (6_1,7)	1280	80
S. cholerae suis I.S. 37 (6_2,7)	<10	1280
S. newport I.S.50 (6,8)	<10	80

[a]An undiluted, heat-killed, bacterial suspension (0.5 ml; 10^{10} bacteria/ml) of each strain was added to 0.5 ml of absorbed monospecific sera, in twofold-dilution series, and incubated for 18 h at 37°. The last tube showing visible agglutination was recorded.

4-position. Terminal α-D-glucose residues, linked to D-galactose at the 4-position, are found in groups B and D LPS and are associated with the presence of O-factor 12_2. Even if the D-glucose is α-linked in the *S. kentucky* LPS, which seems most probable, its structural environment is sufficiently different from that in the LPS of groups B and D to account for a new O-factor, different from 12_2.

EXPERIMENTAL

The experimental methods were those used[3] in the investigation of *S. typhimurium* 395 MS. A Perkin–Elmer 900 gas chromatograph with flame-ionisation detector was used. For g.l.c.–m.s., the mixtures of alditol acetates dissolved in chloroform were injected into a Perkin–Elmer 270 mass spectrometer. The mass spectra were recorded at a manifold temperature of 200°, ionisation potential of 60 eV, ionisation current of 80 μamp, and an ion-source chamber temperature of 80°.

ACKNOWLEDGMENTS

We are indebted to Pharmacia AB for a fellowship to one of us (S.S.). The skilled technical assistance of Miss Mary Andersson, Miss Berit Lindholm, and Mrs. Kerstin Norrgren is also acknowledged. This work was supported by grants from the Swedish Medical Research Council (to T.H. and A.A.L., No. B 68-48x-656-03; to B.L., No. B 69-13x-2522-01) from the Swedish Natural Research Council, from Harald Jeanssons stiftelse, and from Emil and Vera Cornells stiftelse.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

ANALYSIS OF DISACCHARIDES AS PERMETHYLATED DISACCHARIDE ALDITOLS BY GAS–LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

JORMA KÄRKKAINEN
Department of Medical Chemistry, University of Helsinki, Helsinki (Finland)

(Received October 6th, 1969; in revised form, November 14th, 1969)

ABSTRACT

Gas–liquid chromatography (g.l.c.) and combined gas–liquid chromatography–mass spectrometry (g.l.c.–m.s.) have been used for the analysis of disaccharides containing simple hexoses and amino sugars. The disaccharides were reduced to the corresponding alditols with borodeuteride, methylated, and separated by g.l.c. The molecular weight of the monosaccharide units and the position of the glycosidic linkage could be determined on the basis of the mass spectra. Differentiation of (1→4)- and (1→3)-linked alditols was not possible without deuterium labelling, whereas (1→6)-linked alditols could be distinguished by the presence of a fragment containing carbon atoms 1–4 of the alditol moiety.

The glycosidic linkage was cleaved by methanolysis after isolation of the disaccharide alditol methyl ethers by preparative g.l.c., and the products were identified by g.l.c.–m.s.

Methylation (methyl iodide in the presence of methylsulphinyl carbanion) of disaccharide alditols containing *N*-acetylated amino sugars resulted in the formation of a derivative in which the nitrogen atom carried both a methyl and an acetyl substituent.

INTRODUCTION

Conversion of oligosaccharides into volatile and thermally stable derivatives, *e.g.*, methyl or trimethylsilyl (TMS) ethers, permits the application[1–6] of g.l.c. and/or m.s. in their separation and analysis. Reduction of disaccharides, followed by conversion of the resulting alditols into their TMS derivatives[7], simplifies both the g.l.c. analysis and the interpretation of the mass spectra, as compared to the analysis of TMS derivatives of intact disaccharides. Introduction of a deuterium atom into the molecule in the reduction stage[8] is needed for the assignment of the position of the glycosidic linkage.

This paper describes an analogous study of permethylated disaccharide alditols. Methyl ethers are more volatile and have significantly smaller molecular weights than the corresponding TMS derivatives. Moreover, the acid stability of methyl ethers makes it possible to assign the position of the glycosidic bond by identification of the

monosaccharide components after cleavage of the glycoside linkage, in addition to the analysis by m.s. It is hoped that this work will provide a basis for g.l.c.–m.s. studies of higher oligosaccharides.

EXPERIMENTAL

Reference disaccharides[7], lactose, cellobiose, maltose, laminaribiose, gentiobiose, melibiose, 2-acetamido-2-deoxy-4-*O*-(β-D-galactopyranosyl)-D-glucose (*N*-acetyl-lactosamine), and 4-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-D-galactose were converted[7] into disaccharide alditols with potassium borohydride or sodium borodeuteride. The alditols were permethylated as follows [9–11]. The samples (0.2 mg) were dissolved in dry, distilled methyl sulphoxide (0.7 ml) in a Teflon-lined, screw-cap, test tube, and a solution (0.7 ml) of 2M methylsulphinyl carbanion in methyl sulphoxide was added. After ultrasonication for 30 min, methyl iodide (0.7 ml) was slowly added with simultaneous cooling with ice–water. Ultrasonication was continued until a clear solution was obtained (20 min). All of the above procedures were carried out under a nitrogen atmosphere. Chloroform (5 ml) was added, and the chloroform phase was washed twice with water, dried (Na_2SO_4), and evaporated under nitrogen. The methylated alditols were dissolved in a suitable volume of acetone before analysis by g.l.c. Peaks occasionally present, and additional to those of the main derivatives, were due to permethylated products of the dehydrated parent alditol (identification based on m.s.). Peaks due to products of incomplete methylation were not observed.

Analytical and preparative g.l.c., and combined g.l.c.–m.s. were carried out as previously described[7].

RESULTS AND DISCUSSION

Analysis by g.l.c. — The relative retention times of the methylated disaccharide alditols in g.l.c., using four different stationary phases, are shown in Table I. Methyl ethers are more volatile than the corresponding TMS derivatives; the retention time of the TMS ether of melibiitol on SE-30 is approximately six times greater than that of the methylated sugar.

The two alditols containing amino sugars were well separated from each other and from the simple alditols with all of the stationary phases employed. However, methyl ethers of simple alditols were more poorly resolved than the corresponding TMS derivatives. Of the liquid phases examined, 1% OV-22 was the most suitable for the separation of permethylated simple alditols.

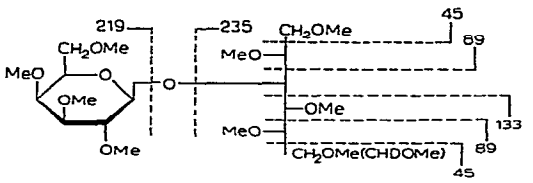
Analysis of simple disaccharide alditols with g.l.c.–m.s. — To facilitate the interpretation of the mass spectra, both borohydride and borodeuteride were used for the reduction of the disaccharides in this study. For routine analyses, however, only the deuterated alditol needs to be prepared. Table II shows the partial mass spectra of the permethylated disaccharide alditols.

TABLE I

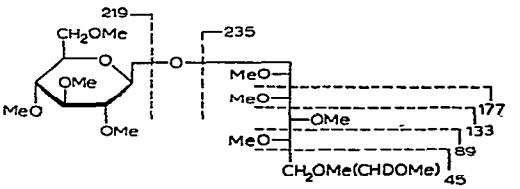
RELATIVE RETENTION TIMES OF PERMETHYLATED DISACCHARIDE ALDITOLS IN G.L.C.

Compound	*Stationary phase*			
	2.2% SE-30 (222°)	*3% QF-1 (194°)*	*3% XE-60 (200°)*	*1% OV-22 (197°)*
Lactitol	0.70	0.66	0.78	0.70
Cellobiitol	0.71	0.65	0.66	0.55
Maltitol	0.71	0.63	0.62	0.61
Laminaribiitol	0.74	0.65	0.61	0.61
Gentiobiitol	1.08	1.04	1.00	0.92
Melibiitol	1.00	1.00	1.00	1.00
N-Acetyl-lactosaminitol	2.18[a]	5.33[b]	4 41[c]	3.06[d]
D-GalNAc*p*-(1→4)-*β*-D-Gal-ol	1.89[b]	4.02[b]	3.66[c]	2.52[d]

[a]Column temperature 250°; [b]225°; [c]230°; [d]221°.



1



2

In general, the fragmentation pattern of methyl ethers of disaccharide alditols was similar to that of TMS derivatives[7], and the primary fragmentations are exemplified in **1** and **2**. The molecular ion was not present in any of the spectra, and the base peak was at *m/e* 88. The ions at *m/e* 219, 187 (219−32), and 155 (187−32) formed through cleavage of the hexose unit from the molecule[4,5], followed by elimination of one and two molecules of methanol, respectively, were unchanged in the spectra of alditols reduced with borodeuteride. The corresponding fragments (*m/e* 235, 203, and 171) arising from the ionized alditol unit, and the ion at *m/e* 295 (analogous to the ion *m/e* 279 in the spectra of dihexose methyl ethers) appeared at the next higher *m/e* for the products obtained by borodeuteride reduction. The presence of these ions allows the determination of the molecular weights of the monosaccharide components and, indirectly, of the whole molecule. Another indirect way of calculating the molecular weight is on the basis of ions (M−45, M−89, and M−133) formed through primary cleavage of the C–C bonds in the alditol moiety. Fragments analogous to all those mentioned above were present in the spectra of the corresponding TMS derivatives[7].

In the spectra of (1→6)-linked disaccharide alditols, the ions at *m/e* 425 (M−45) and 381 (M−89) were very weak or totally absent, but an ion at *m/e* 177 was present (5–8% of base peak), and the intensity of the ion at *m/e* 145 (177−MeOH) was increased. These ions evidently arise through primary cleavage of the C-4–C-5 bond of the alditol moiety and thus cannot be formed from alditols with (1→3)- and (1→4)-

TABLE II

PARTIAL MASS SPECTRA OF PERMETHYLATED DISACCHARIDE ALDITOLS

m/e	*Lactitol*	*Cellobiitol*	*Maltitol*	*Laminaribiitol*	*Gentiobiitol*	*Melibiitol*	N-*Acetyl-lactosaminitol*	D-*GalNAcp*-(1→4)-β-D-*Gal-ol*
45	80	68	83	57	60	60	74	100
71	51	43	48	33	43	43	33	65
75	43	33	47	28	30	40	5	4
88	100	100	100	100	100	100	100	20
89	45	43	43	33	38[a]	33[a]	19	45
101	87	76	90	67	76	81	85	70
111	38	30	43	25	20	23	50	14
129	3	2	3	2	2	2	10	54
130	0.5	0.5	0.5	—	0.5	0.5	41[a]	8
133	10[a]	7[a]	10[a]	4	11[a]	10[a]	—	5[a]
145	7	7	9	6	23[a]	18[a]	9	5
155	6	4	15	5	7	4	9	3
171	15[a]	10[a]	10[a]	11[a]	10[a]	9[a]	—	18[a]
174	—	—	—	—	—	—	30[a]	—
177	—	—	0.5	—	7[a]	6[a]	—	—
187	43	56	77	37	32	28	55	2
203	2[a]	1[a]	2[a]	1[a]	3[a]	3[a]	—	0.9[a]
219	15	5	15	5	6	11	16	12
228	—	—	—	—	—	—	0.5	15
235	41[a]	30[a]	34[a]	20[a]	23[a]	22[a]	—	23[a]
260	—	—	—	—	—	—	—	17
276	—	—	—	—	—	—	45[a]	—
295	4.0[a]	2.2[a]	3.8[a]	5.1[a]	5.8[a]	7.5[a]	—	1.8[a]
336	—	—	—	—	—	—	0.9[a]	—
337	0.2[a]	0.1[a]	0.4[a]	0.5[a]	2.0	1.6	—	9.0[a]
349	1.8	1.0	0.5	1.2[a]	—	—	3.0	—
381	1.7	3.0	1.2	1.4[a]	—	—	1.9	—
425	0.6[a]	1.0[a]	1.4[a]	0.5[a]	—	—	—	—
466	—	—	—	—	—	—	12	—

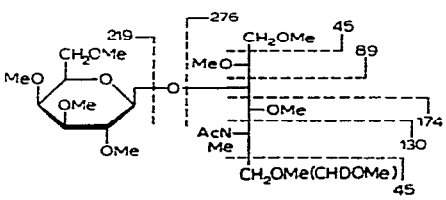
[a]In the deuterated derivative, >50% of the ion is converted into the next higher *m/e*.

linked structures. The corresponding ions (*m/e* 178 and 146) in the spectra of deuterated derivatives further support the proposed mechanism. A fragment of 177 mass units can also be cleaved from disaccharide alditols possessing a (1→2)-linkage, but this ion should not contain deuterium in the spectrum of the deuterated derivative. In the spectra of TMS derivatives, the ion at *m/e* 409, analogous to the ion at *m/e* 177, was of very weak intensity.

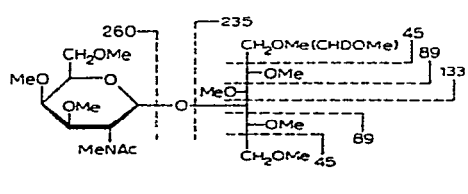
By using borohydride in the reduction step, (1→6)-linked alditols could be differentiated from (1→4)- and (1→3)-linked derivatives, but deuteration was necessary to distinguish the last two types. The ion at *m/e* 133 arising through primary cleavage of the bond between C-3 and C-4 in the alditol moiety (**1**) differentiates alditols with (1→3)-bonds (no deuterium effect) from (1→4)- and (1→6)-linked alditols. It is therefore possible to assign the position of the glycosidic linkages next to the alditol on the basis of the two ions at *m/e* 177 and 133. This is important in the interpretation of the spectra of higher oligosaccharide alditols, in which the intensity of fragments of high *m/e* is weak[12].

The ion at *m/e* 337 (M−133), which like the analogous ion M−307 in the spectra of TMS derivatives is *not* formed through primary cleavage of the C-3–C-4 linkage, was almost completely converted into *m/e* 338 in the spectra of deuterated (1→4)- and (1→3)-linked alditols but remained unchanged in the case of (1→6)-linked derivatives. The distribution of deuterium in the ions arising from the primary cleavage of C-2–C-3 and C-4–C-5 linkages of the alditol moiety was also in accordance with the earlier results with TMS derivatives.

Analysis of amino sugar-containing disaccharide alditols with g.l.c.–m.s. — The peak with the highest intensity in the spectrum of permethylated *N*-acetyl-lactosaminitol was *m/e* 88 (the primary cleavages are shown in **3**). The ions at *m/e* 219 and 187, arising through cleavage of the galactose moiety from the molecule, were present in relatively high intensity. The ion containing the intact alditol unit (*m/e* 276) had an intensity of 45% of that of the base peak. This fragment is formed from an amino sugar alditol unit in which five hydrogen atoms are replaced by methyl groups and one by an acetyl group. The presence of the intense ions at *m/e* 130 (131 after deuteration) and *m/e* 174 (175), as well as of the ion at *m/e* 466 (no deuterium effect), further showed that the fifth methyl group must be attached to the nitrogen atom. Methylation using methyl iodide in the presence of methylsulphinyl carbanion thus differs from the more conventional methylation procedures[13], which do not *N*-methylate acetamido groups. Similar results have been obtained by Lindberg[14].



3



4

As the ion at *m/e* 88 is predominantly formed from fragments containing C-2 of the hexose unit, its intensity was greatly diminished in the spectrum of 4-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-D-galactitol (the primary cleavages are shown in **4**), where it was only 20% of the base peak (*m/e* 45). The presence of an ion at *m/e* 129 (88+41) having an intensity of 54% of the base peak showed that, in this case also, the amino group carried both a methyl and an acetyl substituent. The peaks containing the monosaccharide unit (see above) were correspondingly shifted to 260 and 228. The absence of the ion at *m/e* 177 and the change of the ion at *m/e* 133 to 134 in the deuterated derivative were consistent with the results obtained above in the case of simple (1→4)-linked alditols.

Identification of the monosaccharide components by g.l.c.–m.s. — Methyl ethers of maltitol and melibiitol (*ca.* 20 μg), both containing deuterium, were subjected to preparative g.l.c. on 1% OV-22, followed by methanolysis with M methanolic HCl for 3 h at 70°. After neutralization of the acid, the methanol was evaporated under nitrogen at 0° to prevent the loss of the volatile methyl ethers. G.l.c.–m.s. with the 1% OV-22 stationary phase disclosed the presence of three peaks in both cases; two of these were identified as the anomers of the fully methylated hexose unit, and the third peak (with the greatest retention time) was due to a methylated monosaccharide alditol with one free hydroxyl group. The position of the free hydroxyl group could be determined on the basis of ions formed through primary fragmentation of the alditol chain.

Since stereoisomeric sugars generally produce identical mass spectra, complete identification of the monosaccharide components would require the synthesis and g.l.c. analysis of all different stereoisomers of the mono-unsubstituted monosaccharide alditols. Conditions for the separation of the completely methylated common hexoses by g.l.c. have already been described[2,14]. In the case of maltitol and melibiitol, the monosaccharide alditols, as well as the anomers of fully methylated D-glucose and D-galactose, were all well separated from each other in g.l.c. with the OV-22 stationary phase.

ACKNOWLEDGMENTS

I am grateful to Dr. H. Adlercreutz and Dr. T. Luukkainen for permission to use the g.l.c.–m.s. instrument at the Department of Clinical Chemistry, University of Helsinki.

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Carbohydrate Research
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METABOLISM OF THE RESERVE POLYSACCHARIDE OF *Streptococcus mitis*. PROPERTIES OF GLYCOGEN SYNTHETASE

JANET E. BUILDER AND GWEN J. WALKER
Institute of Dental Research, 2 Chalmers Street, Sydney (Australia 2010)
(Received September 26th, 1969)

ABSTRACT

The presence of glycogen synthetase has been demonstrated in cell extracts of five strains of *S. mitis*. The purified enzyme was free from transglucosylase, α-(1→6)-D-glucosidase, pullulanase, phosphorylase, and 5′-adenylate kinase, but contained a trace of branching enzyme.

The enzyme transferred D-glucosyl residues from ADPG to the acceptor with the formation of α-(1→4)-D-glucosidic linkages. The preferred acceptors were highly branched polysaccharides. The efficiency (relative to *S. mitis* glycogen) of various acceptors, present in excess in activity digests, was as follows: glycogen phosphorylase limit-dextrin (107), glycogen (100), amylopectin phosphorylase limit-dextrin (78), amylopectin (65), amylopectin β-amylase limit-dextrin (30), and amylose (23).

For branched oligosaccharide and polysaccharide acceptors, the side chain was the favoured site of D-glucosyl transfer. The enzyme displayed an absolute requirement for three or more D-glucosyl residues in the side chain of branched oligosaccharides of low molecular weight.

INTRODUCTION

Gibbons and Socransky[1] have suggested that some oral bacteria may contribute to the caries process by storing polysaccharides within the cell, and then metabolizing this reserve carbohydrate to lactic acid when the exogenous sugar supply is no longer available. About 80% of the cultivable flora of human dental plaque produced abundant amounts of iodophilic polysaccharides. Whereas cariogenic, rodent streptococci were strong and stable producers of polysaccharide, polysaccharide synthesis by non-cariogenic streptococci *in vivo* was subject to a high degree of variability[2]. Thus, one difference between a cariogenic and a non-cariogenic flora might be a difference in the stability of polysaccharide production of the organisms in dental plaque.

A strain of *Streptococcus mitis* (S3) isolated from human, dental plaque was shown to be cariogenic by monocontamination into gnotobiotic rats[3]. This streptococcus has a high ability to store intracellular, iodophilic polysaccharide when grown in the presence of excess D-glucose or maltose. The properties of the polysaccharide

indicate that it is glycogen; these include the wavelength of peak absorption of the iodine-stained polysaccharide (λ_{max} = 490 nm)[4], the average chain-length ($\overline{CL}$ = 12)[4], the degree of β-amylolysis (46%)[4], and the degree of phosphorolysis (30%)[5].

Some of the enzymes responsible for the metabolism of *S. mitis* glycogen have been studied[4–8]. In the present work, the purification of ADPG: (1→4)-α-D-glucan α-4-D-glucosyltransferase (glycogen synthetase) is described, and the properties of the enzyme are compared with those of certain other bacteria that store glycogen. In contrast to the glycogen synthetase of rabbit muscle, the enzyme transfers D-glucosyl residues preferentially to the side chains of branched oligosaccharides and polysaccharides.

MATERIALS AND METHODS

Carbohydrates. — *S. mitis* glycogen[5] and pullulan[8] were prepared as previously described. Oyster glycogen was obtained from Calbiochem. (U. S. A.), and rabbit-liver glycogen was a gift from Professor D. J. Manners. Maize, waxy-maize, and potato starches were prepared by the method of Schoch[9]. Waxy-maize starch β-dextrin was prepared by the action of sweet-potato β-amylase (crystalline) on waxy-maize starch. The phosphorylase limit-dextrins of *S. mitis* glycogen, oyster glycogen, and waxy-maize starch were prepared by the action of rabbit-muscle phosphorylase on the appropriate polysaccharide. Potato amylose, maltodextrins, and 6^2-α-maltotriosylmaltose were prepared as described by Walker[6]; isomaltose, panose, and α-amylase limit-dextrins were prepared as by Walker and Builder[7].

[^{14}C]-α-D-Glucopyranosyl phosphate was obtained from International Chemical and Nuclear Corporation (U. S. A.). All nucleotides and nucleotide sugars were purchased from Sigma Chemical Company (U. S. A.). Adenosine 5-(D-glucopyranosyl dihydrogen pyrophosphate) (ADPG) was also synthesized by treating AMP piperidate with the tri-*n*-octylammonium salt of α-D-glucopyranosyl phosphate in anhydrous pyridine. ADP-[^{14}C]-G was synthesized in a similar manner.

Enzymes. — Sweet-potato β-amylase was purchased from Worthington Biochemical Corp. (U. S. A.). Human, salivary α-amylase was prepared as described by Fischer and Stein[10], the purification being taken as far as the dialysis stage. Rabbit-muscle phosphorylase was isolated[11] from freshly excised, rabbit muscle. Pullulanase[12] was prepared from *Aerobacter aerogenes*, pyruvate kinase was obtained from Sigma Chemical Company, and D-glucose oxidase (pure) and horseradish peroxidase were purchased from the Nutritional Biochemicals Corp. (U. S. A.), Egg-white lysozyme (B grade) was obtained from Calbiochem. Streptomyces enzyme refers to the muralytic enzyme obtained from *Streptomyces albus*[13].

Concanavalin A[14], the hemagglutinin of Jack bean, was a gift from Dr. I. J. Goldstein.

Strains of S. mitis. — The sources of the organisms have previously been described[6]. Strain S33, a mutant of strain S3, was unable to store intracellular polysaccharide.

Preparation of cell extracts. — The organisms were grown anaerobically (N_2+CO_2, 95:5) for 20 h at 39°. The medium contained 2% of trypticase, 0.4% of dipotassium hydrogen phosphate, 0.1% of potassium dihydrogen phosphate, 0.2% of sodium chloride and, unless otherwise indicated, 0.1% of D-glucose. The cells were centrifuged at 2° for 10 min at 4,000 *g*, the clear, supernatant solution was retained, and the residue was washed twice with 0.05M Tris-HCl buffer (pH 7.9). The cells were resuspended in the same buffer (12 ml for 2 litres of medium), placed in a cooling device[15], and disrupted for 5 min with a Branson Sonifier S-75 at full power. The cell debris was removed by centrifuging at 27,000 *g* for 20 min at 2°, and the supernatant solution was dialysed for 16 h against 0.05M Tris-HCl buffer (pH 7.9).

The cell-free medium was tested for extracellular glycogen synthetase. Protein precipitated with 80% saturated ammonium sulphate contained 2% of the glycogen-synthetase activity released from the cells by sonication.

Glycogen-synthetase activity was assayed in digests (0.2 ml) that contained phosphate buffer (2.0 μmoles, pH 6.5), EDTA (750 nmoles), ADPG (128 nmoles), and *S. mitis* glycogen (400 μg) and enzyme. The digest and appropriate controls were incubated for 15 min at 35°, and the reaction was terminated by heating to 100° for 30 sec. Glycine buffer (4 μmoles, pH 9.5) was added to raise the pH to a suitable value for pyruvate kinase action, phospho-enol pyruvate (sodium salt) in 0.4M KCl (12.5 μl) was then added, followed by pyruvate kinase (0.25 μmolar unit) in 0.1M $MgSO_4$ (12.5 μl). After 15 min at 35°, 0.1% dinitrophenylhydrazine in 2N HCl (75 μl) was added, and incubation was continued for 5 min at 35° before the addition of 10N NaOH (0.1 ml). After 30 sec, 95% ethanol (0.55 ml) was added, and the digests were centrifuged to remove the precipitated glycogen and other insoluble material. The extinctions of the supernatant solutions were read at 520 nm in 1-cm cells. The amount of ADP released by glycogen synthetase was determined by including standard ADP estimations with all activity determinations.

Tests for ADPG glucohydrolase and ADPG phosphorylase in cell extracts. — Enzymes that could cause release of ADP from ADPG by hydrolysis or phosphorolysis were shown to be absent in cell extracts. When an extract from strain S3 was incubated for 18 h at 35° with ADPG (128 nmoles), D-glucose could not be detected with the D-glucose oxidase reagent under conditions where 1.5 nmoles could easily have been detected. In a similar experiment with ADP-[^{14}C]-G, labelled D-glucose or maltodextrin was not seen by radioautography.

Neither dialysis of a cell extract to remove inorganic phosphate nor addition of phosphate to a digest for glycogen-synthetase activity made any difference to the liberation of ADP. It was concluded that *S. mitis* cell-extracts do not contain ADPG glucohydrolase or ADPG phosphorylase, and that glycogen-synthetase activity could therefore be accurately measured by the liberation of ADP from ADPG.

Isolation of purified, glycogen synthetase from cell extracts. — All operations were carried out at 1–4°.

Step 1. A saturated solution of ammonium sulphate (pH 7.0) was added dropwise to an undialyzed cell-extract to give 40% saturation. After 1 h, the suspension

was centrifuged at 12,000 *g* for 10 min, and ammonium sulphate was added to the supernatant solution to give 60% saturation. The precipitate was collected by centrifuging, redissolved in 0.05M Tris-HCl buffer (pH 7.9), and dialysed against the same buffer.

Step 2. The enzyme was treated with NaCl and diluted with the above buffer until the E_{280nm} of a 10-fold dilution was 0.27, and the concentration of sodium chloride was 0.1M. DEAE-Cellulose was equilibrated with the same buffer, excess buffer was removed by suction, and the partially dried material was added to the enzyme solution (*ca.* 5 g dry weight/100 ml) until the mixture was very thick, but mixing by inversion was still possible. After 20 min of intermittent mixing, DEAE-cellulose was removed by centrifuging at 27,000 *g* for 10 min. The residue was washed once with 0.05M Tris-HCl buffer (pH 7.9) containing 0.1M NaCl, and the mixture was centrifuged again. Traces of DEAE-cellulose were removed from the supernatant solutions by passing through a grade 3 sintered-glass filter, and the filtrate was dialyzed overnight against 0.05M Tris-HCl buffer (10 vol., pH 7.9) to reduce the concentration of sodium chloride. The enzyme solution was freeze-dried, redissolved in distilled water (0.05 vol.), and dialyzed overnight against a buffer containing 0.05M phosphate and 0.001M EDTA (pH 6.5).

RESULTS

Glycogen-synthetase activity of cell extracts. — Sonicated cells of *S. mitis* strain S3 grown on 1% D-glucose were separated into fractions by differential centrifugation (Table I). A cell-free extract was obtained by centrifuging at 1,200 *g* for 15 min. The supernatant solution was then fractionated to give a 27,000 *g* supernatant solution, a 105,000 *g* particulate fraction, and a 105,000 *g* supernatant solution. The 27,000 *g* supernatant solution, which contained 87% of the glycogen-synthetase activity, was chosen for subsequent studies of the enzyme in cell extracts.

TABLE I

DISTRIBUTION OF GLYCOGEN SYNTHETASE IN FRACTIONS OF CELL EXTRACTS

Extract	*Procedure*	*Fraction*	*Activity (μmoles of ADP/total vol.)*
1	(*a*) Sonicated cells centrifuged for 15 min at 1,200 *g*	1,200 *g* supernatant solution	411
	(*b*) Supernatant solution from (*a*) centrifuged for 15 min at 27,000 *g*	27,000 *g* supernatant solution	356
2	(*a*) Sonicated cells centrifuged for 20 min at 27,000 *g*	27,000 *g* supernatant solution	290
	(*b*) Supernatant solution from (*a*) centrifuged for 1 h at 105,000 *g*	105,000 *g* particulate fraction	98
		105,000 *g* supernatant solution	145

Comparison of glycogen-synthetase activity in strains of S. mitis. — Cell extracts were prepared as described in the Materials and Methods section, except that 0.05M phosphate buffer (pH 6.5) containing mM EDTA was used to improve the stability of the enzyme and to avoid the inhibition caused by Tris-HCl buffer. Glycogen-synthetase activity was determined one day after harvesting, and the results are shown in Table II.

TABLE II

GLYCOGEN-SYNTHETASE ACTIVITY OF *S. mitis* STRAINS WITH DIFFERENT ABILITIES TO STORE INTRACELLULAR POLYSACCHARIDE

Strain	D-*Glucose in broth* (%)	*Activity (nmoles of ADP/mg of protein N)*	D-*Glucose content*[a] *of acid-hydrolysed cells* (%)
S3	1.0	26.9	37.1
S3	0.1	26.2	2.6
S33	1.0	29.7	—
439	1.0	27.8	10.1
RB1633	1.0	15.7	2.8
FW251	1.0	6.3	1.3

[a]Data from Ref. 6

Incorporation of [^{14}C]-α-D-*glucose from ADP-*[^{14}C]-D-*glucose into glycogen.* — Evidence that glycogen synthesis occurred during the production of ADP by cell extracts was obtained by comparing the liberation of ADP with the incorporation of [^{14}C-α-D-glucose into glycogen. Portions of the 105,000 *g* particulate and supernatant fractions described above were tested in the usual activity digests for liberation of ADP from ADPG, and at the same time identical digests were incubated with ADP-[^{14}C]-D-glucose (0.52 μCi/μmole). The reaction was terminated by the addition of 75% methanol containing 1% of KCl (10 vol.), the mixture was centrifuged, and the glycogen precipitate was washed twice with the methanol–KCl solution (10 vol.). After dissolution of the glycogen in water, a portion of the solution was counted with Scintipak SP2 in a liquid scintillation counter (Model N 530 G, Ekco Electronics Ltd.) connected to a six-decade scaler. The production of ADP by the particulate fraction was equal to the [^{14}C]-D-glucose transferred, whereas the 105,000 *g* supernatant solution liberated 1.52 μmoles of ADP for every μmole of [^{14}C]-α-D-glucose transferred.

The radioactive glycogen products were digested at 35° with β-amylase (36 units[34]) at pH 4.8 in the presence of glutathione (0.5 mM) and bovine serum albumin (0.05%). After 1 h, the digests were boiled to inactivate the enzyme, deionized with Bio-Deminrolit, and chromatographed on Whatman No. 3MM paper by descending irrigation with 10:4:3 ethyl acetate–pyridine–water. ^{14}C-Labelled carbohydrates were detected by radioautography, and maltodextrin standards were stained with silver nitrate–NaOH reagent. [^{14}C]-Maltose was released by β-amylase digestion of the

[^{14}C]-glycogen solutions; thus glycogen synthetase in both 105,000 *g* particulate and supernatant fractions formed new α-(1→4)-D-glucosidic linkages during the incorporation of [^{14}C]-α-D-glucose into glycogen.

Dependence of the glycogen-synthetase reaction on the presence of primer. — Cell extracts from five strains of *S. mitis* were prepared in phosphate–EDTA buffer, and incubated with ADPG in the presence and absence of added glycogen. When excess glycogen was present, a linear relationship was obtained between enzyme concentration and release of ADP. The non-linear relationship observed in the absence of added glycogen (Fig. 1) indicated the presence of primer in the cell extracts, although there was no correlation between the total glucan content of each extract (Table III) and

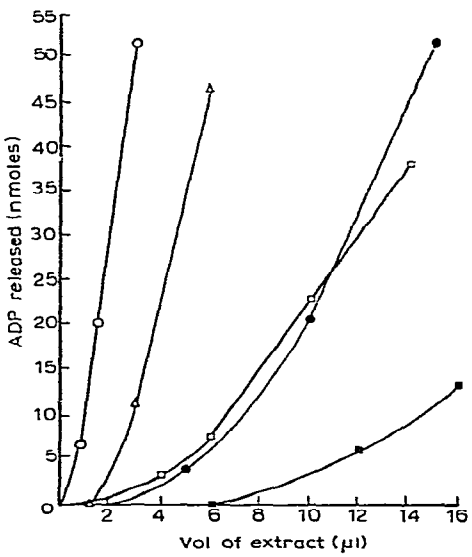


Fig. 1. Effect of increasing enzyme concentration on the release of ADP, in the absence of added glycogen primer, in activity digests containing cell extracts of strain S3 (○), S33 (●), 439 (△), RB1633 (■), and FW251 (□). For strain FW251, the volume of extract plotted in the figure is one tenth of the volume used for the assay.

TABLE III

GLYCOGEN SYNTHETASE-ACTIVITY AND GLUCAN[a] CONTENT OF *S. mitis*[b] CELL-EXTRACTS

Strain	*ADP released*[c] (*nmoles/µl*)	*Total glucan* (*µg/µl*)
S3	22.5	3.35
S33	7.7	0.08
439	28.0	0.62
RB1633	7.0	0.53
FW251	0.7	0.01

[a]Glucan in cell extracts was determined by the cysteine–H_2SO_4 method[33] for hexose. [b]The cells were grown in medium containing 1% of D-glucose. [c]The activity digests contained an excess of glycogen primer.

the release of ADP in the absence of added primer. Only some of the glucans are likely to be primers for glycogen synthetase.

Demonstration of endogenous primer in a cell extract. — The most likely explanation for the rapid rise in ADP release shown in Fig. 1 was that the increased volume of cell extract was providing extra primer as well as extra enzyme. To demonstrate the presence of an endogenous primer for glycogen synthetase in a cell extract, the boiled extract was added to an activity digest that contained no exogenous glycogen. The results (Table IV) showed that the liberation of ADP by a cell extract from strain RB1633 was increased by 25% when a boiled extract of strain 439 was included in the digest.

TABLE IV

THE EFFECT OF BOILED CELL-EXTRACT ON THE GLYCOGEN SYNTHETASE-ACTIVITY[a] OF A CELL EXTRACT OF *S. mitis*

Cell extract	*Glucan in extract added to digest (μg)*	*ADP released without added glycogen (nmoles)*	*ADP released with excess glycogen (nmoles)*
439	2.1	68.3	127
RB1633	1.4	24.9	47.8
RB1633+439	3.5	92.8	—
RB1633+boiled 439	3.5	31.4	—

[a]Activity digests contained twice the usual concentration of ADPG.

Incorporation of [^{14}C]-α-D-glucose from ADP-[U-^{14}C]-D-glucose into endogenous primer. — A dialysed cell-extract (0.02 ml) from strain 439 was incubated with ADP-[^{14}C]-D-glucose (128 nmoles, 0.13 μCi/μmole), EDTA (130 nmoles), and glycine buffer (3.8 μmoles, pH 8.5) in a total volume of 0.035 ml. After 14 h at 35°, the reaction was terminated by boiling, *S. mitis* glycogen (100 μg) was added as a carrier, and then the glycogen was precipitated and washed, and its radioactivity was measured as described above. It was found that 53% of [^{14}C]-α-D-glucose had been transferred from ADP-[^{14}C]-D-glucose to a methanol-insoluble product.

In a similar experiment, a dialysed cell-extract from strain FW251 (0.175 ml) was incubated with phosphate buffer (2.0 μmoles, pH 6.5), EDTA (750 nmoles), and ADP-[^{14}C]-D-glucose (128 nmoles) in a total volume of 0.20 ml. After 14 h at 35°, the reaction was terminated by boiling, and the digest was divided into two equal portions. One portion was incubated at 30° with β-amylase (36 units) at pH 5.1 in the presence of glutathione (0.5mM) and bovine serum albumin (0.05%). The second portion was treated similarly, except that pullulanase (0.1 unit[34]) was also added to hydrolyse the α-(1→6)-D-glucosidic linkages of glycogen. The reaction mixtures were treated for chromatography as described above, and examination of the radioautograph revealed that [^{14}C]-maltose had been released by β-amylase. The simultaneous action of β-amylase and pullulanase on the labelled product gave an increased amount of [^{14}C]-maltose, together with some [^{14}C]-maltotriose. The presence of labelled malto-

triose indicated that branching enzyme was also present in the cell extract and had acted on the freshly elongated chains so that the label became incorporated near the new branch linkages.

Attempts to prepare primer-free cell extract. — It became apparent during studies on the purification of glycogen synthetase that the enzyme could not be completely separated from primer by centrifugation at 105,000 *g*, by ammonium sulphate fractionation, or by column chromatography on DEAE-cellulose or DEAE-Sephadex. Several possibilities of preparing a cell extract containing enzyme without primer were considered. First, D-glucose was replaced with D-galactose (0.1%) in the growth medium for strain S3. Then *S. mitis* FW 251, a strain that does not store significant amounts of carbohydrate, was grown in a trypticase–soy medium without carbohydrate. In both cases, the cell extracts contained endogenous primer for glycogen synthetase. The amounts of carbohydrate added with the enzyme to the activity digests were extremely low (540 and 70 ng, respectively), yet they were sufficient to give 100% (strain S3) and 11% (strain FW251) of the ADP released when excess glycogen primer was added. It is shown below (Fig. 6) that 100 μg of *S. mitis* glycogen was needed to provide excess primer in activity digests containing purified glycogen synthetase from strain S3.

Information on the location of glycogen synthetase in the cells was obtained by studying enzyme release during the preparation of cell lysates and spheroplasts. Enzyme present external to the cytoplasmic membrane was expected to be free from glycogen primer. It was found that glycogen synthetase could be prepared only from lysed spheroplasts, and its activity was not dependent on the presence of added glycogen.

Attempts to remove primer from glycogen synthetase. — Concanavalin A, which interacts with glycogen in solution to form an insoluble precipitate[16], was stirred for 10 min at 22° with cell extracts of strain S3 and 439, at a final concentration of 2 mg/ml of 0.1M NaCl. Determinations of total hexose and glycogen-synthetase activity in the supernatant solutions (obtained after centrifuging) indicated that the enzyme was precipitated as rapidly as glycogen by concanavalin A, and the precipitated enzyme was active in the resuspended residue of strain 439.

An attempt to destroy the endogenous primer (840 μg) in a cell extract of strain S3 treated with ammonium sulphate (Step 1, Methods) by oxidation of the solution (2 ml) with periodate (10 μmoles, 0.2 ml) also led to great losses in activity of the enzyme.

The possibility of removing endogenous primer by digestion of a cell extract of strain S3 with salivary α-amylase was tested with three different concentrations of the enzyme. The amylase was assayed as described by Walker and Whelan[35]. The lowest amount added (0.002 unit/ml) would have been sufficient to degrade all the polysaccharide associated with the enzyme in 2 h at 4° if it were present as free amylopectin. Accordingly, the incubation was continued for 20 h at 4°, and, after removal of α-amylase by adsorption on starch granules, the synthetase was precipitated with ammonium sulphate, dissolved in 5 mM Tris-HCl buffer (pH 7.9), and dialyzed against

the same buffer. The yield of glycogen synthetase was 88%, and its primer dependence was down to 50% of the original. On repeating the experiment with a 100-fold increase in α-amylase concentration, no further degradation of primer occurred.

Finally, by incubating glycogen synthetase with a higher concentration of α-amylase (0.375 unit/ml) for 15 min at 35°, the endogenous primer was completely removed. The amount of α-amylase required degraded a similar weight of amylopectin to the achroic state in 0.15 sec.

Adsorption of glycogen synthetase on polysaccharides. — (*a*) *Glycogen.* The presence of glycogen synthetase in the 105,000-*g* particulate fraction of a cell extract was considered to be due to adsorption of the enzyme on glycogen. When this fraction was resuspended in 0.05M Tris-HCl buffer (pH 7.9), centrifuged again, and resuspended in the same buffer, 30% of the original glycogen-synthetase activity was recovered together with 4% of branching-enzyme activity. Transglucosylase and maltodextrin phosphorylase were completely removed by this procedure.

The adsorption on glycogen of the synthetase in the 105,000-*g* supernatant solution was determined by treating portions with *S. mitis* glycogen (5 mg/ml) and with oyster glycogen (5 mg and 25 mg/ml). After being stirred for 5 h at 2°, the solutions were centrifuged at 105,000 *g* for 10 min, and the pellets were resuspended in 0.05M Tris-HCl buffer (pH 7.9). In a second experiment, a dialysed 105,000-*g* supernatant solution was treated with oyster glycogen at pH 5.2, as described in method 2 of Villar-Palasi *et al.*[17] for muscle-glycogen synthetase. Table V shows the recovery of glycogen synthetase in the particulate fractions from the two experiments. Adsorption of the enzyme under all conditions was limited.

TABLE V

ADSORPTION OF GLYCOGEN SYNTHETASE ON GLYCOGEN

Glycogen	*Glycogen concentration (mg/ml)*	*pH*	*Mixing time (h)*	*Particulate fraction* (g)	*Glycogen recovered* (%)	*Glycogen synthetase recovered* (%)
S mitis	5	7.9	5	105,000	72	15
Oyster	5	7.9	5	105,000	25	7
Oyster	25	7.9	18	105,000	—	4
Oyster	1	5.2	0	27,000	—	0

(*b*) *Amylose.* A cell extract (1 ml) from strain S3 was treated with retrograded amylose (2 mg) in 0.01M Tris-HCl (0.6 ml). The mixture was stirred for 20 min at 2°, and the amylose was removed by centrifuging. The enzyme was fully recovered in the supernatant solution, showing that *S. mitis* glycogen synthetase, unlike the soluble-starch synthetase of glutinous rice[18], does not form a complex with amylose.

(*c*) *Starch granules.* A cell extract of strain S3 (1.5 ml), 0.4M phosphate buffer (pH 6.95, 0.5 ml), and defatted, maize-starch granules (200 mg) were stirred together for 30 min at −1°, and then centrifuged at 12,000 *g* for 10 min at −1°. The supernatant solution contained all the original glycogen-synthetase activity.

Removal of endogenous primer from glycogen synthetase by digestion with salivary α-amylase, followed by adsorption of the amylase on starch granules. — The aim was to insert the α-amylase digestion procedure between steps 1 and 2 in the preparation of purified glycogen synthetase from a cell extract (30 ml) of strain S3. The protein that precipitated between 40 and 60% saturated ammonium sulphate was dissolved in 50mM phosphate buffer (pH 6.5)–mM EDTA, and dialyzed against the same buffer. The solution contained 85% of the original synthetase activity, and the glucan content was 778 μg/ml, equivalent to 31% of the original weight. The enzyme was then incubated for 1 h at 35° with a high concentration of salivary α-amylase (0.375 unit/ml) in 10mM NaCl. A test for glycogen-synthetase activity at this stage was possible despite the presence of α-amylase. The synthetase had become completely dependent on the presence of added glycogen.

The enzyme (24 ml) was stirred for 30 min at −1° with defatted maize-starch granules (2.4 g). These were removed by centrifuging, and then a second treatment with starch granules was given. The final, supernatant solution contained only 0.1% of the original α-amylase activity, but no glycogen-synthetase activity remained. It was concluded that, once the enzyme had lost the primer normally associated with it, adsorption on starch granules could occur. No other method was successful in removing all of the α-amylase, but the use of an insoluble derivative of α-amylase is being investigated.

Properties of partially purified, glycogen synthetase. — The synthetase preparation from strain S3 was free from transglucosylase, α-(1→6)-D-glucosidase, pullulanase, phosphorylase, and 5′-adenylate kinase, and contained only a trace of branching-enzyme activity. When the synthetase (50 μl) was added to a branching-enzyme activity digest, the extinction of iodine-stained amylose fell by only 4% during 1 h. The studies of primer specificity on glycogen synthetase, which were carried out with 1 μl or less of enzyme, were therefore not affected by the trace of branching enzyme present. The use of low concentrations of enzyme also ensured that synthetase activity due to endogenous primer was almost negligible.

The enzyme was incubated in activity digests at various pH values. After 15 min, the reaction was terminated, and the pH was adjusted to within the range 7 to 9.5 before measurement of the release of ADP. The optimal pH for synthetase activity was between 6.5 and 7.5 (Fig. 2). The enzyme was incubated for 1.5 h at 35° in 0.1M buffer at various pH values, and then the activity was determined in digests at pH 7.0. Glycogen synthetase was most stable between pH 6.0 and pH 7.0 (Fig. 3).

The optimal temperature for a reaction of 15 min at pH 6.5 was 40° (Fig. 4), but when the enzyme was incubated in 50mM phosphate containing mM EDTA (pH 6.5) for 1.5 h at 35, 40, and 45°, the losses in activity were 40, 60, and 89%, respectively. Digests for glycogen-synthetase activity were therefore incubated at 35° for all experiments.

Increasing volumes of enzyme were incubated in activity digests with and without added glycogen (Fig. 5a). With excess glycogen, the relationship between enzyme concentration and activity was linear until 50% of the D-glucosyl residues had

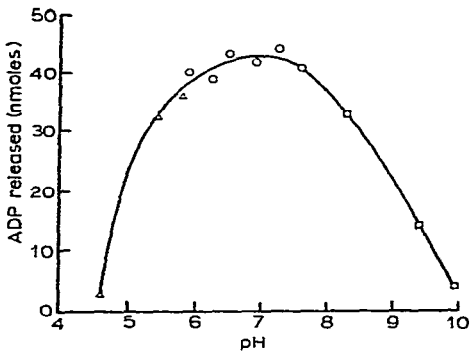


Fig. 2

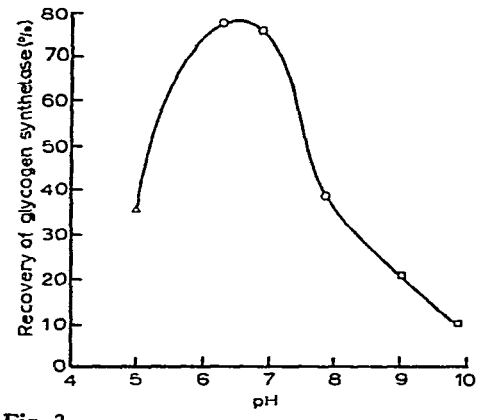


Fig. 3

Fig. 2. Effect of pH on glycogen-synthetase activity. The digests contained 16 μmoles of acetate (Δ), phosphate (○), or glycine buffer (□).

Fig. 3. Effect of pH on the stability of glycogen synthetase. The enzyme was incubated for 1.5 h at 35° in acetate (Δ), phosphate (○), or glycine buffer (□).

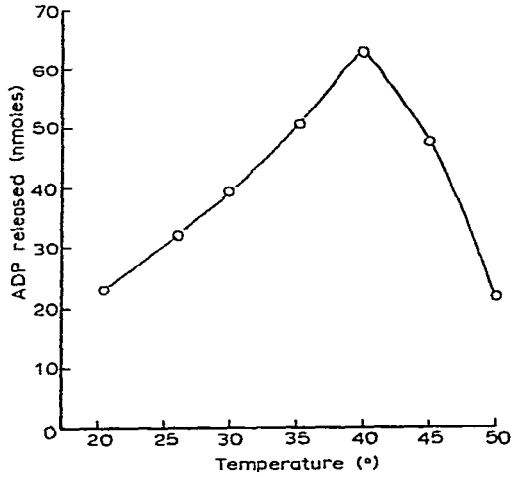


Fig 4. Effect of temperature on glycogen-synthetase activity.

been transferred from ADPG (64 nmoles of ADP released). In the absence of added glycogen, the relationship was similar to that obtained with cell extracts (Fig. 1). The results in Fig. 5b show that ADP-release in activity digests was linear for 60 min.

Specificity of sugar nucleotide donor. — Uridine 5-(D-glucopyranosyl dihydrogen pyrophosphate) was tested as a possible donor in the glycogen-synthetase reaction. UDP was not released under conditions where the release of ADP from ADPG was 46 nmoles. When the amount of UDPG was increased to 1.2 μmoles and the concentration of the enzyme was increased 30-fold, there was still no significant release of UDP.

Glycogen-synthetase activity was measured in digests containing increasing concentrations of ADPG. A Lineweaver–Burk plot gave a K_m value of 3.45×10^{-4}M.

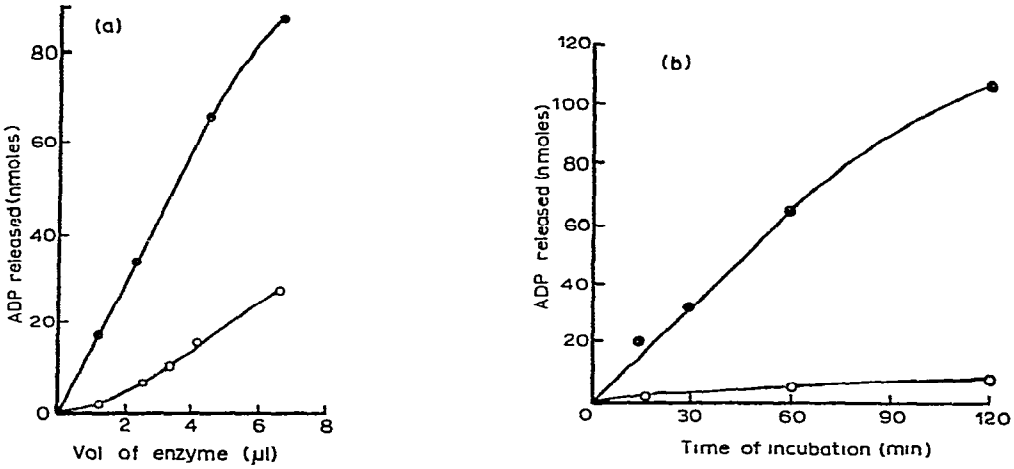


Fig. 5. (*a*) Effect of enzyme concentration on the activity of purified glycogen-synthetase, in the presence (●) and absence (○) of added glycogen primer. (*b*) Linearity of glycogen synthetase-activity with time. The purified enzyme (1·2 µl) was incubated with (●) and without (○) added glycogen.

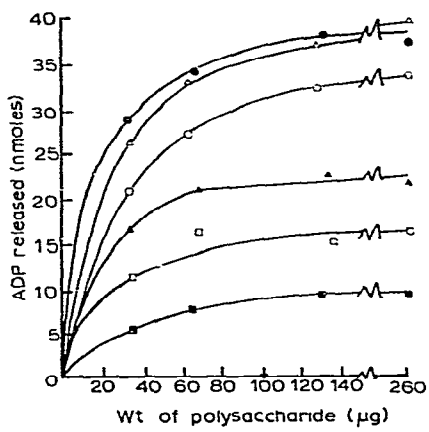


Fig. 6

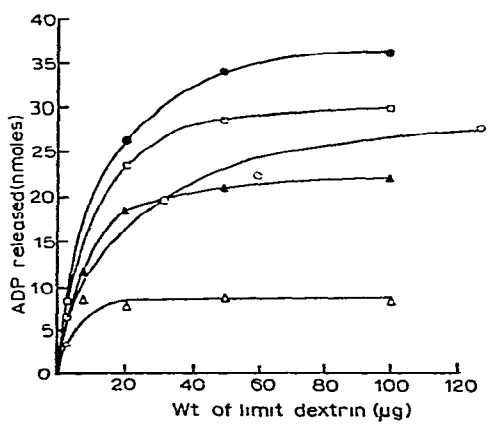


Fig. 7

Fig. 6. Effect of various polysaccharide acceptors on glycogen-synthetase activity. The digests contained increasing weights of oyster glycogen (●), rabbit-liver glycogen (△), *S. mitis* glycogen (○), amylopectin (▲), potato starch (□), and amylose (■). The pullulan digest (▼) was incubated for 18 h.

Fig. 7 Determination of the accepting efficiency of limit dextrins Various weights of untreated *S. mitis* glycogen (○), the phosphorylase limit-dextrins of oyster glycogen (●), *S. mitis* glycogen (□) and amylopectin (▲), and the β-amylase limit-dextrin of amylopectin (△) were tested in activity digests

Specificity of primer for glycogen synthetase. — (*a*) *Polysaccharides.* The enzyme (0.6 µl) was incubated with various weights of each primer for 1 h. The results for the liberation of ADP (Fig. 6) indicated the preference of glycogen synthetase for the

more highly branched polysaccharides. Amylose was a poor primer compared with glycogen, and pullulan, an unbranched polysaccharide containing a high proportion of α-(1→6)-D-glucosidic linkages, was not an acceptor.

(*b*) *Limit dextrins.* When excess primer was present in the digests similar to those described above, the phosphorylase limit dextrins of oyster glycogen, *S. mitis* glycogen, and amylopectin were 14, 7, and 16% more efficient, respectively, than the corresponding polysaccharides (Fig. 7). Amylopectin β-amylase limit-dextrin was only 46% as efficient as amylopectin. Hydrolysis of the limit dextrins with pullulanase revealed that at least 80% of the side chains of the phosphorylase limit-dextrins consisted of four D-glucosyl residues, whereas those of the β-dextrin contained two or three residues.

(*c*) *Maltodextrins.* Release of ADP occurred only in the presence of maltopentaose when glycogen synthetase (1.0 μl) was incubated for 1 h in activity digests containing various maltodextrins (130 nmoles, equivalent to the non-reducing chain-ends in 250 μg of glycogen). When the weight of maltodextrins was increased to 2.7 μmoles, the relative rate of ADP release in digests containing D-glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose was 0, 10, 15, 22, 23, 12, and 11, respectively (glycogen = 100).

The products of the transfer of D-glucosyl residues to maltopentaose and maltohexaose were identified after incubating the enzyme (1 μl) with the dextrins (2.7 μmoles) and ADP-[^{14}C]-D-glucose for 17 h. The maltodextrins were separated by paper chromatography, and a radioautograph showed that the main product of transfer to maltopentaose was maltohexaose with traces of maltoheptaose. Similarly, transfer to maltohexaose had produced mainly maltoheptaose and a small proportion of malto-octaose.

(*d*) *Branched oligosaccharides.* Addition of branched oligosaccharides to glycogen-synthetase activity digests caused a fall in the release of ADP. This effect became more marked as the concentration of dextrin was increased, and was possibly due to competitive inhibition of the reaction between the enzyme and endogenous primer. To test whether the small amount of ADP released in these digests was due to transfer of D-glucose to the branched dextrins or to endogenous primer, the enzyme (1.0 μl)

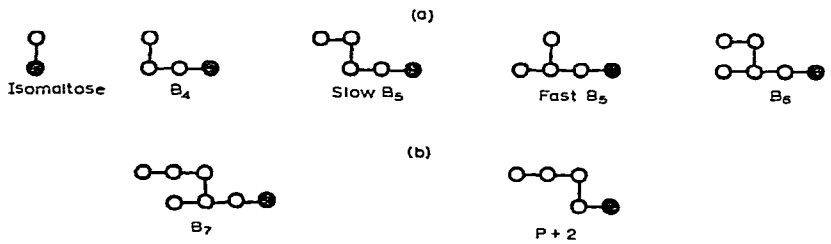


Fig 8. Structure of branched dextrins tested as primers for glycogen synthetase (*a*) Dextrins that were not acceptors. (*b*) Acceptors for glycogen synthetase ○, D-glucose unit; ●, reducing D-glucose unit; ——, α-(1→4)-D-glucosidic link; |, α-(1→6)-D-glucosidic link B_4, 6^3-α-D-glucosylmaltotriose, Slow B_5, 6^3-α-maltosylmaltotriose; Fast B_5, 6^3-α-D-glucosylmaltotetraose; B_6, 6^3-α-maltosylmaltotetraose; B_7, 6^3-α-maltotriosylmaltotetraose; P+2, 6^2-α-maltotriosylmaltose.

was incubated for 15 h with ADP-[^{14}C]-D-glucose and isomaltose, 6^2-α-maltotriosylmaltose, 6^3-α-D-glucosylmaltotriose, 6^3-α-maltosylmaltotriose, 6^3-α-maltosylmaltotetraose, and 6^3-α-maltotrioslymaltotetraose (0.9 μmole), and with 6^3-α-glucosylmaltotetraose (3.6 μmoles). Labelled dextrins were formed only from 6^3-α-maltotriosylmaltotetraose and 6^2-α-maltotriosylmaltose (Fig. 8). These were branched dextrins containing eight and six D-glucosyl units, respectively.

Site of D-*glucosyl transfer to branched primers.* — The reaction products of the transfer of [^{14}C]-D-glucose to 6^3-α-maltotriosylmaltotetraose (B_7) and to amylopectin β-dextrin were examined in order to determine whether glycogen synthetase transferred D-glucosyl residues to the side chains or main chains of branched acceptors. The branch linkage in the branched octasaccharide (B_8) obtained from B_7 was hydrolysed with pullulanase (0.1 unit), and the products were separated by paper chromatography. A radioautograph showed that hydrolysis of B_8 had released [^{14}C]-maltotetraose and a faint trace of [^{14}C]-maltopentaose. This proved that glycogen synthetase transferred D-glucosyl residues mainly to the side-chain of 6^3-α-maltotriosylmaltotetraose to form 6^3-α-maltotetraosylmaltotetraose.

A similar experiment was carried out, in which amylopectin β-dextrin (2 and 0.05 mg) was incubated with glycogen synthetase (1.0 μl) and ADP-[^{14}C]-D-glucose for 2 and 16 h, respectively. Degradation of the products with pullulanase (0.35 unit) released labelled maltodextrin containing three or more D-glucosyl residues. The products from the higher concentration of limit dextrin (2 mg) were eluted from the chromatogram, dried on aluminium planchets, and counted by gas flow. The percentage of the total radioactivity (incorporated into the limit dextrin) that was recovered in labelled maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, malto-octaose, and in a mixture of maltononaose and maltodecaose was 2, 28, 12, 6, 5, 3, and 6, respectively. Thus, at least 62% of the labelled D-glucose transferred to amylopectin β-dextrin was added to the side chains. The low yield of labelled maltotriose compared with maltotetraose indicated again the preference of the enzyme for side chains that are more than two units in length.

Activators and inhibitors. — Various substances were added to standard activity-digests (Table VI). The inorganic ions were without significant effect on glycogen synthetase activity with the exception of K^+, which caused slight activation, and Ca^{2+}, which produced slight inhibition.

No effect was observed when D-glucose, D-glucose 6-phosphate, D-ribose 5-phosphate, D-fructose 6-phosphate, D-fructose 1,6-diphosphate, or D-glyceraldehyde 2,3-diphosphate were added to activity digests at a concentration of 5mM.

Stimulation of synthetase activity by potassium chloride, glutathione, and bovine serum albumin has been reported previously for the glycogen synthetase of *E. coli*[19], and *Arthrobacter* sp.[20], and for the starch synthetase of *Chlorella pyrenoidosa*[21].

The inhibition caused by citrate was of interest in connection with the stimulatory and stabilizing effect that citrate ions have on the branching enzyme of *S. mitis*[4]. Since these are the enzymes involved in the synthesis of glycogen, it is feasible that

the concentration of citrate, by regulating the activity of glycogen synthetase and branching enzyme, could control the degree of branching of the product.

TABLE VI

EFFECT OF VARIOUS COMPOUNDS ON GLYCOGEN-SYNTHETASE ACTIVITY

Addition	*Concentration* (mM)	*Activity* (%)	*Addition*	*Concentration* (mM)	*Activity* (%)
None		100	Glutathione	10	147
KCl	65	119	Tris	26	47
NaCl	65	96	Citrate	40	9
$MgCl_2$	5	95	ADP	0.15	90
$CaCl_2$	10	88	AMP	2.5	80
Na_2SO_4	5	100	*p*-Chloromercuribenzoate	0.01	84
	15	104	Glycerol	600	93
NaF	2.6	95	Bovine serum albumin	0.05%	131

DISCUSSION

Glycogen synthetase was present in all strains of *S. mitis*, and the activity in strain S3 was the same whether or not the organism was grown under conditions suitable for carbohydrate storage. The synthetase is therefore a constitutive enzyme. The glycogen-synthetase activity in strain S33, the non-carbohydrate storing mutant of strain S3, was the same as the activity in the parent organism. It is therefore unlikely that the variability of polysaccharide synthesis displayed by some oral streptococci is due to changes in production of glycogen synthetase.

The glycogen synthetase in cell extracts was partly bound to native glycogen, 40% of the enzyme being found in the particulate fraction. Luck[22] similarly suggested that the presence of glycogen synthetase in the glycogen pellet of rat-liver homogenates was due to its binding to glycogen rather than to structural elements. The distribution of *S. mitis* glycogen-synthetase in fractions of cell extract resembled that observed in *Rhodospirillum rubrum*, whereas other bacterial glycogen-synthetases were found mainly in the soluble or particulate fractions of crude extracts[23].

The formation of specific complexes of enzyme protein with substrate has been described for several D-glucosyltransferases. The soluble-starch synthetase[18] of glutinous rice grains forms a complex with amylose, and yeast glycogen-synthetase[24] can be adsorbed on retrograded amylose, and eluted with glycogen solution. *S. mitis* glycogen-synthetase from the soluble fraction was not adsorbed to any appreciable extent on either glycogen or retrograded amylose. The possible explanation for this is that the enzyme was already in a complex with some of the glucan of low molecular weight which remained in the 105,000-*g* supernatant solution. Small amounts of carbohydrate remained bound to glycogen synthetase after various stages in the isolation of partially purified enzyme. The weight of glucan, sometimes only 0.5 μg, added with the enzyme into activity digests, made the enzyme completely independent

of added primer. Since the weight of normal glycogen that must be added to an activity digest to give excess of primer was 100 μg, it was clear that the primer associated with the enzyme must be far superior in priming efficiency to any of the polysaccharides tested.

Our results suggest that a model primer must be highly branched, with low, internal chain-length, and with side chains 3 or more units long. Such a molecule would not easily be degraded with α-amylase, an enzyme that hydrolyzes maltotriose only with difficulty. This proposed primer might be similar to α-macrodextrin[25], a highly branched dextrin produced by the action of α-amylase on glycogen. An amylolytic enzyme[26] was found with the glycogen pellet obtained on centrifuging sonicated *S. mitis* cells, and an intracellular α-amylase is also present in *E. coli*[27]. The function of these amylases may be to provide suitable indestructible primers that are always available in the cell Even cell extracts of *S. mitis* strains that did not store polysaccharide contained small amounts of primers suitable for glycogen synthetase.

Studies with polysaccharides showed that phosphorylase limit-dextrins were more efficient primers than the parent polysaccharides. The most effective primer would therefore have an outer chain of more than 3 D-glucose residues, but less than in native glycogen. Since the phosphorylase limit-dextrin of glycogen was more efficient as a D-glucosyl acceptor than the limit dextrin of amylopectin, the length of the inner chains is also important. Glycogen synthetase in cell extracts of *A. aerogenes* showed a decrease in K_m with increased chain-length of the primer[28], and rabbit-muscle glycogen synthetase showed a marked decrease in V_{max} with phosphorylase limit-dextrin when compared with the parent glycogen[29]. These enzymes are unlike the *S. mitis* enzyme in their preference for longer, outer chains. The *S. mitis* enzyme also preferred low molecular weight maltodextrins of intermediate chain length. Maltopentaose was the most effective oligosaccharide primer, the activity of the enzyme in the presence of excess pentasaccharide being 23% of the activity with excess glycogen. The enzyme showed a progressive decrease in affinity for dextrins of lower or higher molecular weight. Similarly, the synthetase of potato-starch granules displayed a maximum affinity for maltotetraose[30].

Branched oligosaccharides of low molecular weight were poor primers for *S. mitis* glycogen-synthetase when compared with maltodextrins. The absolute requirement for at least 3 D-glucosyl units on the side chains of the branched dextrins revealed a greatly different specificity from the mammalian, muscle enzyme[29], which is the only other synthetase that has been investigated in this manner. The mammalian enzyme could transfer D-glucosyl residues to the main chains of branched dextrins that have a D-glucosyl unit attached to the main chain peripheral to the branch point. The length of the side chain could be one (fast B_5), two (B_6), or three (B_7). Conversely, transfer of D-glucosyl residues by *S. mitis* glycogen-synthetase to the side chains occurred irrespective of the presence of a peripheral D-glucose residue on the main chain of the acceptor.

Hers and Verhue[31] have found that mammalian enzyme attached at least 30% of transferred D-glucosyl residues to the side chains of limit dextrins. The results

presented in this paper show clearly that *S. mitis* glycogen-synthetase transfers D-glucosyl residues mainly to the side chains of branched oligosaccharide and polysaccharide acceptors.

Since these differences of specificity exist between the mammalian and bacterial synthetase, it would be expected that the specificity of the branching enzymes might vary accordingly. Brown and Brown[32] found that muscle branching-enzyme transferred maltodextrinyl residues chiefly from the main chains to nearby side chains.

ACKNOWLEDGMENT

This investigation was supported by a grant from the Dental Board of New South Wales.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

THE EXISTENCE OF OLIGOSACCHARIDES IN BOVINE AND OVINE SUBMAXILLARY MUCINS*

MAURICE BERTOLINI† AND WARD PIGMAN
Department of Biochemistry, New York Medical College, New York, New York 10029 (U.S.A.)
(Received July 21st, 1969; in revised form, November 17th, 1969)

ABSTRACT

Treatment of bovine and ovine submaxillary mucins with alkaline sodium borohydride converted all of the carbohydrate side-chains into dialyzable fragments. However, 10% and 22% of the hexosamine of native ovine submaxillary mucin (OSM) and bovine submaxillary mucin (BSM), respectively, was not reduced to aminodeoxyhexitol by alkaline sodium borohydride after 120 h. Most of the hexosamine that was resistant to alkaline borohydride was present in newly described oligosaccharides. The β-elimination of the carbohydrate side-chains from the serine and threonine residues of the protein core was shown to be 95% quantitative when applied to BSM and OSM.

INTRODUCTION

The principal carbohydrate side-chain of ovine[1] and bovine[2] submaxillary mucins‡ is reported to be the disaccharide: *N*-acetylneuraminyl-(2→6)-2-acetamido-2-deoxy-D-galactose, with the latter residue *O*-glycosidically bound to serine and threonine[3–5]. A recent study by Murty and Horowitz[6] demonstrated that about 80% of the sialic acid of OSM was present as part of this disaccharide. The total hexosamine and sialic acid of BSM and OSM has not been accounted for in previous studies. In addition, both mucins contain small proportions of L-fucose and D-galactose, which suggest the occurrence of other oligosaccharide side-chains.

Prolonged treatment of OSM and BSM with alkaline sodium borohydride caused the liberation of 90 to 95% of the constituent sialic acid and hexosamine as dialyzable fragments. However, only 75% of the total hexosamine of BSM was reduced to the corresponding aminodeoxyhexitol. For OSM only 85% of the total hexosamine was reduced to aminodeoxyhexitol. In both cases, the unreduced hexosamine appeared in a bound form in the dialyzable fragments[7].

*Supported by a grant AM-04619 from the National Institutes of Health, U.S. Public Health Service, and is a portion of a thesis submitted for the Ph.D. degree. We thank the New England Enzyme Center, Tufts University of Medicine, Boston, Mass. for assistance in the preparation of the mucins.
†Present address: National Institutes of Health, National Institute of Arthritis and Metabolic Diseases, Laboratory of Chemistry, Bethesda, Maryland 20014, U.S.A.
‡Abbreviations used: BSM, bovine submaxillary mucin; OSM, ovine submaxillary mucin

We undertook this investigation to determine if the unreduced hexosamine present in alkaline borohydride-treated BSM and OSM represented an alkali-stable linkage to the peptide core (that is, glycosylamine linkage) and/or new oligosaccharide side-chains. It was also possible that the unreduced hexosamine might be present in *O*-glycosidic linkages to serine and/or threonine. Jones *et al.*[8] have demonstrated that *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-serine was quite stable to alkali. Thus, as the protein core of these mucins is hydrolyzed by alkali to smaller peptides, the *O*-glycosidic linkages may become progressively less labile to alkali.

We also wished to determine whether the β-elimination reaction could be used as a quantitative measurement of the *O*-glycosidic linkages to serine and threonine. This could only be accomplished after the nature of the hexosamine, resistant to reductive cleavage by alkaline borohydride, was elucidated.

EXPERIMENTAL PROCEDURE

Materials. — BSM and OSM were prepared in this laboratory by the method described by Tettamanti and Pigman[9]. The major mucin fraction, which was not adsorbed onto hydroxyapatite, was used. The carbohydrates and amino acids used as standards were commercial preparations obtained from Calbiochem Inc. and Beckman Co., respectively. 2-Acetamido-2-deoxy-D-glucitol and 2-acetamido-2-deoxy-D-galactitol had melting points noted in an earlier report[6]. Sephadex G-10 was purchased from Pharmacia Fine Chemicals Inc. Ion-exchange resins were purchased from Bio-Rad Laboratories.

Methods. — Total hexosamines were determined by the Elson–Morgan method as modified by Boas[10]. Samples were hydrolyzed with 6M hydrochloric acid for 1 h at 110° in sealed tubes. 2-Amino-2-deoxy-D-glucose and -galactose were each determined with an amino acid analyzer (Beckman Model 120).

2-Acetamido-2-deoxyhexitols were determined by gas–liquid chromatography according to the method described by Murty and Horowitz[6] after *N*-acetylation of the hydrolyzates and standards according to the procedure of Roseman *et al.*[11]. In this study, a 6-ft glass column containing 0.5% of ethylene glycol adipate coated on acid-washed Celite was used. The trimethylsilyl derivatives were prepared by the method of Sweeley *et al.*[12]. Hexosamines and aminodeoxyhexitols were also determined simultaneously on an amino acid analyzer by the method reported by Donald[13].

Sialic acid was measured by the resorcinol method of Svennerholm[14] as modified by Miettinen *et al.*[15]. L-Fucose and D-galactose were determined by the methods described by Dische *et al.*[16,17].

Periodate consumption was measured by iodometric titration as reported by Fleury and Lange[18]. Periodate oxidation was performed at room temperature, in the dark, in the presence of acetate buffer of pH 4.5. Formaldehyde was determined, after periodate oxidation, with chromotropic acid[19]. *N*-Acetylneuraminic acid and 2-acetamido-2-deoxy-D-galactitol were used to obtain a standard curve. Both materials gave the same molar optical densities.

High-voltage paper electrophoresis was conducted for 2 h with a Savant High Voltage Electrophoresis apparatus equipped with a cooling plate. A voltage of 55 $V.cm^{-1}$ was applied to Whatman 3MM paper. An aqueous 1% solution of sodium tetraborate (pH 9.1) was used as buffer. Carbohydrates were detected by the periodate–benzidine staining technique[20].

N-Dansylation* of samples was performed essentially by the procedure of Gray[21–23]. The dansylated samples were hydrolyzed for 1 h at 110° in 6M hydrochloric acid. The brief conditions of hydrolysis were used to assure maximal recovery of 2-dansylamido-2-deoxyhexose and 2-dansylamido-2-deoxygalactitol. 2-Dansylamido-2-deoxy-D-galactitol, 2-dansylamido-2-deoxy-D-glucose, and 2-dansylamido-2-deoxy-D-galactose were prepared from the appropriate amino sugars. T.l.c. was performed on silica gel with solvent systems (*A*) 5:1:5 butyl alcohol–acetic acid–water (upper layer), and (*B*) 70:1:30 chloroform–acetic acid–*tert*-amyl alcohol. The latter system was used at 5°.

Hydrolysis of bovine and ovine submaxillary mucins with alkaline sodium borohydride. — Both BSM and OSM were treated with 0.3M sodium borohydride in 0.1M sodium hydroxide for 120 h. The hexosamine content of both mucins remained constant from 10 h of treatment to 120 h. The content of ninhydrin-positive materials increased progressively from 2 h up to 120 h of treatment. Approximately every 48 h, 50 mg of sodium borohydride was added to the reaction mixture. The initial concentration of both mucins was 1%.

After 120 h the reaction was stopped by the addition of M hydrochloric acid to a pH of 5.5. The resultant solution was promptly adjusted to pH 6.6 with 0.2M sodium hydroxide and concentrated to approximately one-third of the initial volume.

A column (5 x 34 cm) of Sephadex G-10 was prepared in the usual manner and thoroughly washed with water. Portions (25 ml) of the alkaline borohydride-treated BSM or OSM were layered over the surface of the gel and eluted with water at a flow-rate of 16 ml/h. The fractions containing sialic acid and hexosamine were collected and lyophilized The carbohydrate composition of the latter materials, referred to as GP-120-BSM and GP-120-OSM is given in Table I.

Fractionation of the GP-120 materials on Dowex-1 resin. — A solution of 200 mg of GP-120-BSM or -OSM was adjusted to pH 7.5 with 0.05M sodium hydroxide and placed on a column of Dowex-1 resin (acetate form). The first 100 ml was eluted with water. Thereafter, a linear gradient of 500 ml of 0.1M sodium acetate in the reservoir chamber, and 500 ml of 5 mM sodium acetate in the mixing chamber was used to elute the material from the column. The flow-rate was 24 ml/h and 6-ml fractions were collected.

Both GP-120-BSM and -OSM contained two fractions which were incompletely separated. These fractions, P_I and P_{IA}, (see Figs. 1 and 2) were combined, desalted on Sephadex G-10, lyophilized, and subsequently assayed for amino acids and carbohydrates.

*Dansyl = *N*-(5-dimethylamino-1-naphthalenesulfonyl).

TABLE I

THE CARBOHYDRATE COMPOSITION OF BOVINE AND OVINE SUBMAXILLARY MUCINS BEFORE AND AFTER TREATMENT WITH ALKALINE SODIUM BOROHYDRIDE AND FRACTIONATION ON SEPHADEX G-10

	Untreated BSM	*GP-120-BSM*	*% Recovery*
	(mg/g untreated BSM)		
N-Acetylneuraminic acid	285	265	93
2-Acetamido-2-deoxyhexose	228	51	22
D-Galactose	20	11	55
L-Fucose	11	5	45
	Untreated OSM	*GP-120-OSM*	*% Recovery*
	(mg/g untreated OSM)		
N-Acetylneuraminic acid	300	290	97
2-Acetamido-2-deoxyhexose	230	22	9.6
D-Galactose	7	4	57
L-Fucose	4	2.4	60

Fraction P_{II}, observed to occur only in GP-120-BSM, was desalted on Sephadex G-10, lyophilized and assayed for amino acids and carbohydrates. The formaldehyde formed by periodate oxidation, and the periodate consumption, were also measured. Fraction P_{II} was subjected to high-voltage paper electrophoresis.

Fraction P_{III} was found in both GP-120 materials (see Figs. 1 and 2) and contained approximately 60% and 75% of the sialic acid of the original BSM and OSM, respectively. These fractions were concentrated, desalted on Sephadex G-10 and lyophilized. Fraction P_{III} was assayed for carbohydrates and also subjected to high-voltage paper electrophoresis and t.l.c. (on Silica Gel G).

Fraction P_{IV} occurred only in GP-120-BSM and was apparently free sialic acid.

Fractionation of P_I and P_{IA} on Dowex-50 resin. — Fractions P_I and P_{IA} (10 mg) from GP-120-BSM or -OSM were dissolved in 1 to 2 ml of 2% acetic acid and applied to a Dowex-50 column. The first 60 ml was eluted with 2% acetic acid at pH 3.2. Thereafter, a linear gradient of 250 ml of 4% acetic acid (adjusted to pH 4.4 with pyridine) in the reservoir chamber and 250 ml of 2% acetic acid (at pH 3.2) in the mixing chamber was started. The flow-rate was 36 ml/h and 6-ml fractions were collected. Fig. 3 shows the elution pattern of sialic acid of P_I and P_{IA} from GP-120-BSM. The same elution pattern was observed for GP-120-OSM material. The fraction containing 85–90% of sialic acid and hexosamine of the P_I and P_{IA} material is referred to as D_I-50-BSM from the BSM material and D_I-50-OSM from the OSM material. The D_I-50 fractions were evaporated to dryness and the residues dissolved in 0.5 ml of water. The solutions were applied to Whatman 3MM paper and subjected to high-voltage electrophoresis.

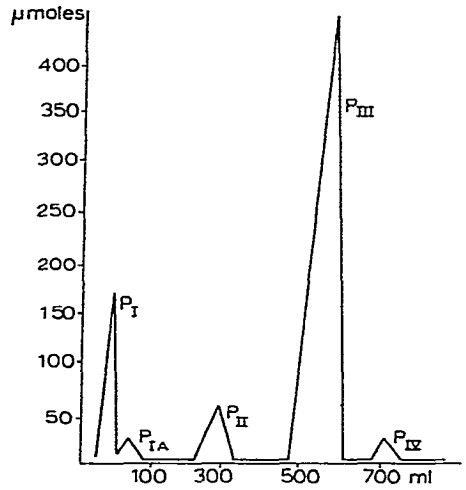


Fig. 1

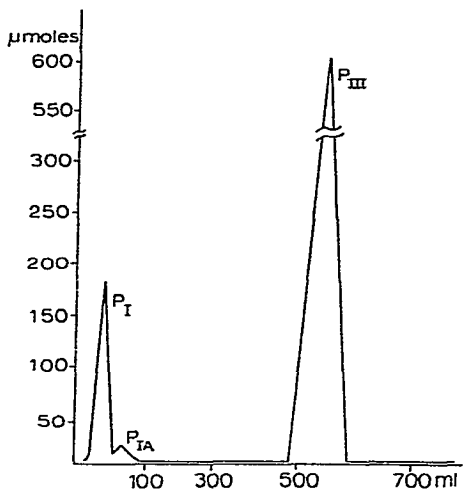


Fig. 2

Fig. 1. The elution pattern of GP-120-BSM from a Dowex-1 column. The pattern is based upon sialic acid determinations.

Fig. 2. The elution pattern of GP-120-OSM from a Dowex-1 column. The pattern is based upon sialic acid determinations.

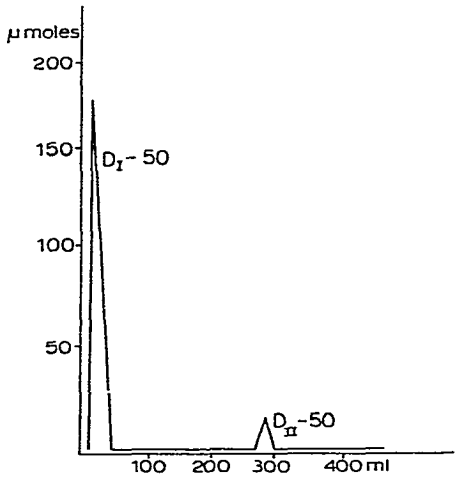


Fig. 3

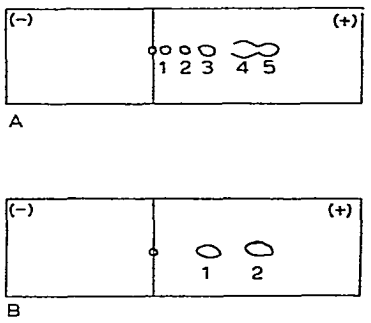


Fig. 4

Fig. 3. The elution pattern of P_I and P_{IA} (from GP-120-BSM) from a Dowex-50 column. The pattern is based upon sialic acid determinations.

Fig. 4. The high-voltage paper electrophoretic resolution of D_I-50-BSM (A) and D_I-50-OSM (B), respectively, in the presence of 1% sodium tetraborate, pH 9.1.

High-voltage electrophoresis resolved D_I-50-BSM into five components, as shown in Fig. 4 (A), while D_I-50-OSM was resolved into two components as shown in Fig. 4 (B). Each fraction was eluted from the paper, filtered, and concentrated to

a known volume. These fractions were then assayed for their amino acid and carbohydrate composition.

RESULTS

Treatment of BSM and OSM with alkaline sodium borohydride. — Table I shows the carbohydrate composition of BSM and OSM before and after 120 h of treatment with alkaline sodium borohydride. After 120 h approximately 22% and 10% of the original hexosamine of BSM and OSM, respectively, was resistant to alkaline borohydride. About one-half of the L-fucose and D-galactose of the native BSM and OSM remained intact.

Fractionation of the alkaline borhydride-treated BSM (GP-120-BSM) and OSM (GP-120-OSM). — Figs. 1 and 2 show the elution pattern of the constituent sialic acid of GP-120-BSM and -OSM, respectively, from a Dowex-1 column. Fractions P_I and P_{IA} obtained from GP-120-BSM contained approximately 50–55% of the intact, unreduced hexosamine. Similarly, P_I and P_{IA} obtained from GP-120-OSM contained about 85% of the unreduced hexosamine. Essentially all of the L-fucose and D-galactose was recovered in the P_I and P_{IA} fractions. The combined amino acid and carbohydrate composition of P_I and P_{IA} from the BSM and OSM material is given in

TABLE II

THE AMINO ACID AND CARBOHYDRATE COMPOSITION OF THE P_I AND P_{IA} FRACTIONS FROM GP-120-BSM AND GP-120-OSM

Component	*P_I and P_{IA} from GP-120-BSM*	*P_I and P_{IA} from GP-120-OSM*
	(*μmoles/mg*)	
Lysine	09	.21
Histidine	.03	.07
Arginine	.07	.06
Aspartic acid	.08	10
Threonine	.05	08
Serine	.08	.10
Glutamic acid	.17	.18
Proline	.61	.60
Glycine	.49	.75
Alanine	.92	1.07
2-Aminobutyric acid	.42	.55
Valine	.41	.46
Isoleucine	.08	.12
Leucine	16	.16
N-Acetylneuraminic acid	84	.71
2-Amino-2-deoxygalactose	.28	.25
2-Amino-2-deoxyglucose	.26	.04
D-Galactose	.19	.16
L-Fucose	.09	.09
2-Amino-2-deoxygalactitol	.55	.47
2-Amino-2-deoxyglucitol	trace	trace

Table II. The amino sugars and 2-amino-2-deoxygalactitol are present as free bases, not the *N*-acetylated forms. Dansylation of P_I and P_{IA}, and subsequent t.l.c. analyses on silica gel in solvent system (*B*) indicated the presence of *N*-deacetylated amino sugars (R_F 0.45) and 2-amino-2-deoxygalactitol (R_F 0.68).

With the exception of 2-amino-2-deoxyglucose, which is present only in trace amounts in native OSM, the molar ratios of the carbohydrates (with L-fucose taken as unity) are quite similar for P_I and P_{IA} materials in BSM and OSM. The presence of serine and threonine suggests the occurrence of glycopeptide material in addition to the possible presence of oligosaccharides.

Fraction P_{II} (see Fig. 1) was present in GP-120-BSM and contained 30% of the intact hexosamine remaining after 120 h of reductive treatment. The amino acid and carbohydrate composition of P_{II} is given in Table III. The molar ratios of the carbohydrates and the observance of two carbohydrate fractions by high-voltage paper electrophoresis, strongly suggest that P_{II} contains two trisaccharides. The P_{II} fraction consumed 4.6 μmole of periodate per μmole of constituent 2-acetamido-2-deoxygalactitol and produced 3 μmoles of formaldehyde per μmole of 2-acetamido-2-deoxyhexose. These data suggest the possible structures of the trisaccharides as given in Table VI.

TABLE III

THE CHEMICAL COMPOSITION OF P_{II} (OBTAINED FROM THE DOWEX-1 FRACTIONATION OF GP-120-BSM)

Component	*μmoles/mg*	*Component*	*μmoles/mg*
Lysine	.02	Alanine	.082
Histidine	.006	2-Aminobutyric acid	.027
Arginine	—	Valine	.031
Aspartic acid	.007	Isoleucine	.003
Threonine	003	Leucine	.007
Serine	.009	*N*-Acetylneuraminic acid	1.20
Glutamic acid	019	2-Acetamido-2-deoxygalactose	.60
Proline	.035	2-Acetamido-2-deoxyglucose	.60
Glycine	.051	2-Acetamido-2-deoxygalactitol	1.10

Fraction P_{III} (see Figs. 1 and 2) was present in both GP-120 materials and appeared to be the same material. 2-Acetamido-2-deoxygalactitol was the only amino sugar present in both materials, and sialic acid was present in an approximately equimolar amount. The P_{III} fractions represent the reduced disaccharide: *N*-acetylneuraminyl-(2→6)-2-acetamido-2-deoxygalactitol, the main carbohydrate side-chain of BSM[1] and OSM[2].

Fractionation of P_I and P_{IA} on Dowex-50. — Fig. 3 shows the elution pattern of the sialic acid of P_I and P_{IA} (from GP-120-BSM) from a Dowex-50 column. About 85-90% of the sialic acid and hexosamine was not retained by the resin, and all of the L-fucose and D-galactose was found in this fraction, referred to as D_I-50-BSM. Fractions P_I and P_{IA} from GP-120-OSM showed a similar elution pattern and D_I-50-OSM refers to the analogous fraction described above for BSM.

High-voltage paper electrophoresis of the D_I-50 fractions. — Fraction D_I-50-BSM was resolved into five subfractions as seen in Fig. 4 (A), while D_I-50-OSM was resolved into two sub-fractions (see Fig. 4, B). The carbohydrate and partial amino acid composition of each of these fractions are given in Table IV and Table V for the

TABLE IV

THE CARBOHYDRATE AND AMINO ACID COMPOSITION OF THE D_I-50-BSM FRACTION AFTER RESOLUTION BY HIGH-VOLTAGE PAPER ELECTROPHORESIS

	Electrophoretic fraction no.				
	1	*2*	*3*	*4*	*5*
	(*μmoles/mg*)				
Aspartic acid	—	.008	.010	.008	.004
Threonine	—	.010	.007	.004	.003
Serine	—	.042	.020	.020	.005
Glutamic acid	—	.010	.010	.005	.010
Proline	trace	.020	.030	—	.020
Glycine	.02	.050	.037	.014	.055
Alanine	.02	.078	.031	.007	.067
2-Amino-2-deoxygalactitol	.036	—	.080	.180	.200
2-Amino-2-deoxyglucose	.011	.009	.050	.030	.010
2-Amino-2-deoxygalactose	.025	.045	.040	—	—
N-Acetylneuraminic acid	—	.020	.037	.200	.300
D-Galactose	—	.020	.080	.010	—
L-Fucose	—	—	.040	—	—

TABLE V

THE CARBOHYDRATE AND AMINO ACID COMPOSITION OF THE D_I-50-OSM FRACTION AFTER RESOLUTION BY HIGH-VOLTAGE PAPER ELECTROPHORESIS

Component	*Electrophoretic fraction no.*	
	1	*2*
	(*μmoles/mg*)	
Aspartic acid	.008	—
Threonine	.011	trace
Serine	.032	trace
Glutamic acid	.005	—
Proline	.020	—
Glycine	.055	.01
Alanine	.067	.02
2-Amino-2-deoxygalactitol	—	.31
2-Amino-2-deoxyglucose	.009	trace
2-Amino-2-deoxygalactose	.16	.02
N-Acetylneuraminic acid	.17	.38
D-Galactose	.08	—
L-Fucose	.04	—

materials from D_1-50-BSM and D_1-50-OSM, respectively. Only sub-fraction 2 from the BSM material and sub-fraction 1 from the OSM material appear to contain glycopeptides. The molar ratios of the carbohydrates present in the other fractions suggest the presence of oligosaccharides of various sizes.

Tables VI and VII summarize the results of this study and show the probable composition of the various fractions obtained by treatment of BSM and OSM with alkaline borohydride.

TABLE VI

SUMMARY OF THE COMPOSITIONS OF THE FRACTIONS OBTAINED BY TREATMENT OF BOVINE SUBMAXILLARY MUCIN WITH ALKALINE SODIUM BOROHYDRIDE

Fraction (from Dowex-1)	*Probable composition*[a]	*Recovery of hexosamine*	
		Of total bound, %	*Of intact mucin, %*
IV	NANA	—	—
III	NANA-(2→6)-GalNac-ol	—	60
II A	NANA-(2→6)-GalNac-(1→4)-GalNac-ol	15	3.3
B	NANA-(2→6)-GluNac-(1→6)-GalNac-ol	15	3.3
I and I_A	Mixture of oligosaccharides and glycopeptides	55	12
Sub-Fractions of I and I_A			
1	GalNac-GalNac-ol GluNac-GalNac-ol	8 8	2
2	Glycopeptides; hexosamine bound to serine and threonine	13.2	2.6
3	L-Fuc→[D-Gal, $GluNH_2$]-$GalNH_2$-ol NANA→[D-Gal, $GalNH_2$]-$GalNH_2$-ol	22	4.4
4 & 5	Mainly reduced disaccharides of NANA-$GalNH_2$-ol (composition of hexosamine not known)	10	2.4

[a]NANA, *N*-acetylneuraminic acid; GalNac, 2-acetamido-2-deoxy-D-galactose. GluNac, 2-acetamido-2-deoxy-D-glucose. L-Fuc, L-fucose; $GalNH_2$, 2-amino-2-deoxy-D-galactose; $GluNH_2$, 2-amino-2-deoxy-D-glucose; GalNac-ol, 2-acetamido-2-deoxy-D-galactitol; $GalNH_2$-ol, 2-amino-2-deoxy-D-galactitol.

DISCUSSION

The purpose of this study was to elucidate the nature of the hexosamine resistant to reductive cleavage by alkaline sodium borohydride, and to determine if the β-elimination reaction could be used as a quantitative measurement of the *O*-glycosidic linkages of the carbohydrate side-chains to the serine and threonine residues in BSM and OSM. The results obtained clearly show that the aforementioned hexosamine is present mainly in oligosaccharide side-chains more complex than the main disaccharide reported previously[1,2].

TABLE VII

SUMMARY OF THE COMPOSITIONS OF THE FRACTIONS OBTAINED FROM THE ALKALINE SODIUM BOROHYDRIDE TREATMENT OF OVINE SUBMAXILLARY MUCIN

Fraction (from Dowex-1)	*Probable composition*	*Recovery of hexosamine*	
		Of total bound, %	*Of intact mucin, %*
IV	None detected	—	—
III	NANA-(2→6)-GalNac-ol	—	75
II	None detected	—	—
I and I_A	Oligosaccharides bound to serine and threonine. Reduced disaccharides: NANA-(2→6)-$GalNH_2$-ol	85	9
Sub-Fractions of I and I_A			
1	(Ser. +Thr.)$_1$ ($GalNH_2$)$_4$ (D-Gal)$_2$ (L-Fuc)$_1$ (NANA)$_4$	66	6
	(Ser. +Thr.)$_1$ ($GluNH_2$)$_3$ (NANA)$_3$	4	0.4
2	Reduced disaccharides: NANA-(2→6)-$GalNH_2$-ol	7.5	1

NANA = *N*-acetylneuraminic acid. GalNac-ol = 2-acetamido-2-deoxygalactitol. L-Fuc = L-fucose. Ser = serine. Thr = threonine $GluNH_2$ = 2-amino-2-deoxy-glucose. $GalNH_2$ = 2-amino-2-deoxy-galactose. $GalNH_2$-ol = 2-amino-2-deoxy-galactositol.

A previous study[4] from this laboratory showed that when BSM was treated with alkaline sodium borohydride at 5°, there was an initial rapid loss of hexosamine which was greater than the loss of serine and threonine. This rapid loss was thought to reflect the possible presence of an alkali-labile glycosidic linkage (*O*-glycosidic ester linkages) previously reported to occur in BSM and OSM[24,25]. However, subsequent investigations demonstrated that *O*-glycosidic ester linkages were not present in ovine, bovine, or porcine submaxillary mucins[7]. The results obtained herein offer an alternative explanation for the loss discussed above. A portion of the hexosamine content of BSM is present in oligosaccharide side-chains containing more than one hexosamine residue. The loss (on a molar basis) would be greater than the loss of serine and threonine during the course of the β-elimination reaction.

The newly described oligosaccharides help to explain the occurrence of some of the L-fucose and D-galactose, which occur in small proportions in both mucins. Approximately one-third of the hexosamine residues in native BSM is 2-amino-2-deoxy-D-glucose, whereas OSM contains only trace amounts of 2-amino-2-deoxy-D-glucose. The near absence of 2-amino-2-deoxyglucitol in alkaline borohydride-treated BSM and OSM indicate that the majority of the 2-amino-2-deoxy-D-glucose residues are not involved in *O*-glycosidic linkages to serine and threonine.

The β-elimination reaction has been used quite extensively in structural studies on glycoproteins[3–5]. The results presented here indicate that the β-elimination reaction,

as applied to BSM and OSM, is about 95% quantitative. The loss of hexosamine during the course of the reactions reaches a maximum value of 75% for BSM and 85% for OSM after 10 h of treatment with alkaline borohydride at 45°, the optimal conditions[7]. The β-elimination reaction is completed in 10 h of treatment, under the conditions described, and the treatment for 120 h was employed only to cleave the peptide core to smaller units.

About one-half of the L-fucose and D-galactose residues were lost after treatment of the mucins with alkaline borohydride. The isolation and purification procedure for both mucins requires a treatment with hydroxyapatite. Part of the mucin is adsorbed on the hydroxyapatite, and this fraction is enriched in L-fucose and D-galactose. The treatment may not have removed all of this minor fraction, which appears to have oligosaccharide side-chains of different composition than those found in the main mucin fraction (which is not adsorbed on hydroxyapatite).

Whether the oligosaccharides discussed herein arise from the same mucin molecule or from mucins having different blood-group activities is presently not known, since the materials used in this study were obtained from pooled glands.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

THE STRUCTURE OF THE OLIGOSACCHARIDES SYNTHESISED BY A STRAIN OF *Claviceps purpurea*

F. ARCAMONE, W. BARBIERI, G. CASSINELLI, AND C. POL
Istituto Ricerche Farmitalia, Milano (Italy)
(Received November 24th, 1969)

ABSTRACT

The structures of a disaccharide, a trisaccharide, and a tetrasaccharide produced by a strain of *C. purpurea* grown on a sucrose–ammonium citrate medium in aereated fermenters have been elucidated. The disaccharide is identical to 6-*O*-β-D-fructofuranosyl-D-glucose; the two, new oligosaccharides are homologous β-D-fructofuranosyl derivatives of the disaccharide, and their structures have been determined by chemical as well as n.m.r. studies.

INTRODUCTION

Three reducing oligosaccharides and D-fructose accumulated in the medium when *Claviceps purpurea* (Stevens et Hall strain 275) was grown aerobically on a sucrose–ammonium citrate medium in shake-flasks or in stirred fermenters[1]. The oligosaccharides (*A*, *B*, and *C*, in order of decreasing R_F values on paper chromatograms) were characterized as follows: *A*, $[\alpha]_D$ +1.0° (water, equilib.), a disaccharide containing D-glucose and D-fructose residues; *B*, $[\alpha]_D$ −15.5° (water, equilib.), a trisaccharide containing one D-glucose and two D-fructose residues; *C*, $[\alpha]_D$ −22.5° (water, equilib.), a tetrasaccharide containing one D-glucose and three D-fructose residues. We now report on the identification of compounds *A*, *B*, and *C*.

RESULTS AND DISCUSSION

In all three oligosaccharides, D-glucose is the reducing unit as shown by the titration of aldose groups (one aldose group per mole of oligosaccharide) and by the formation of D-fructose and D-glucitol upon acid hydrolysis of the reduced oligosaccharides (Table I). The specific rotations of the hydrolysis products are in agreement with the number of D-fructose units (Table I). The oligosaccharides were also completely hydrolyzed by yeast invertase, which is a typical β-D-fructofuranosidase[2], thus establishing that the fructose units are present as β-D-fructofuranosyl groups.

The results of periodate oxidation (Table I) of the alcohol derived from *A* indicated that *A* is 6-*O*-β-D-fructofuranosyl-D-glucose (**1**) which is a known product of the action of *A. levanicum* levansucrase on sucrose[3], and of yeast invertase on a

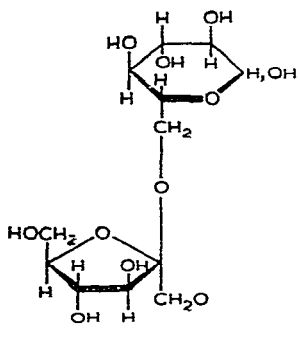
TABLE I

PROPERTIES OF THE ALCOHOLS OBTAINED ON REDUCTION OF THE OLIGOSACCHARIDES WITH BOROHYDRIDE

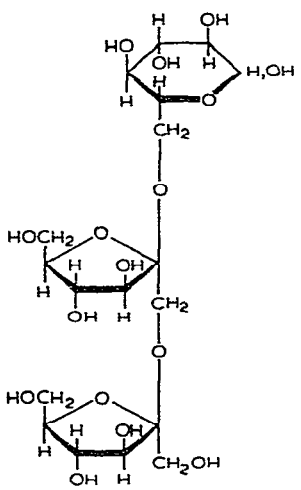
Parent compound	*Composition*[a]	$[\alpha]_D$ *of the hydrolysate*		*Periodate consumed (moles per mole)*
		Calc.	*Found*	
A	1F, 1G	−46°	−44°	5.37
B	2F, 1G	−61°	−58°	6 04
C	3F, 1G	−69°	−66°	7 05

[a]F = fructose; G = D-glucitol.

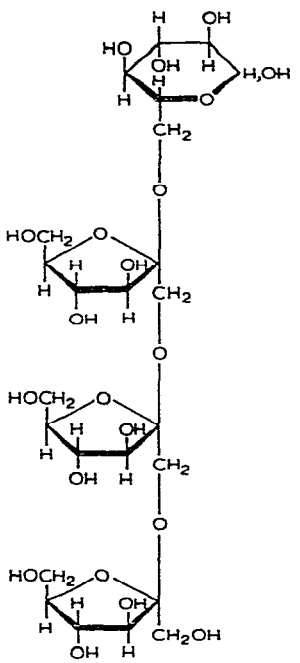
mixture of sucrose and D-glucose[4]. Direct chromatographic comparison of disaccharide *A* with an authentic sample of **1** confirmed the identity of the products.



1



2

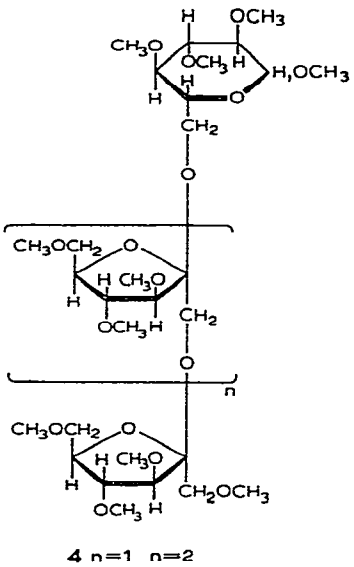


3

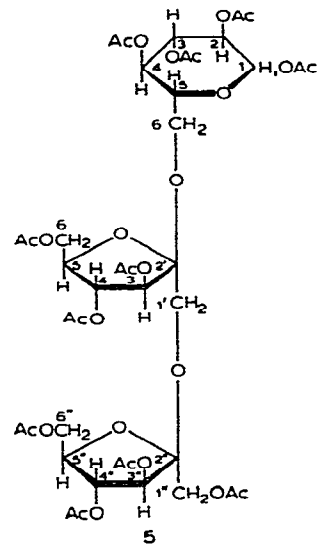
Partial enzymic hydrolysis of trisaccharide *B* gave, in addition to D-glucose and D-fructose, a compound showing the same chromatographic behaviour as **1**. The alcohol derived from *B* consumed one mole of periodate per mole more than did 6-*O*-β-D-fructofuranosyl-D-glucitol (Table I), thus indicating that the second β-D-fructofuranosyl unit is attached to one of the primary alcoholic groups of the β-D-fructofuranosyl unit of **1**. Fully methylated trisaccharide *B* gave, on selective acid hydrolysis of the β-D-fructofuranosyl linkages, the following sugar derivatives: methyl 2,3,4-tri-*O*-methyl-D-glucoside (2,3,4-tri-*O*-methyl-D-glucose was formed when acid hydrolysis was complete), 1,3,4,6-tetra-*O*-methyl-D-fructose, and 3,4,6-tri-*O*-methyl-

D-fructose which was identified on the basis of its mobility during electrophoresis in borate buffer[4]. Therefore, structure **2** can be assigned to the trisaccharide.

Similarly, partial enzymic hydrolysis of tetrasaccharide *C*, and periodate oxidation of the corresponding alcohol (Table I), indicated that *C* was a β-D-fructofuranosyl derivative of **2**, with the third D-fructose residue linked to one of the primary alcoholic groups of **2**. Hydrolysis of the fully methylated tetrasaccharide *C* gave a mixture of methylated sugars which was qualitatively identical (t.l.c.) with that obtained by the same procedure from **4** ($n = 1$); the spot corresponding to the tri-*O*-methyl derivative of fructose appeared to be more intense, and di-*O*-methyl derivatives were not detected. One component of the mixture was separated by preparative t.l.c., and analysis by electrophoresis in borate buffer showed it to be homogeneous and identical with 3,4,6-tri-*O*-methyl-D-fructose. Therefore, structure **3** is assigned to tetrasaccharide *C*.



4 n=1 n=2



5

Nuclear magnetic resonance studies. — The n.m.r. spectra of the peracetate **5** in different solvents (chloroform-*d*, acetone-d_6, benzene-d_6, and methanol-d_4) provided confirmation of structure **2**. In addition to the absorptions near δ 2.1 (acetyl protons), three well-defined groups of signals were obtained: (*1*) an 8-proton multiplet between δ 5–6, assigned to the methine protons of the C*H*–OAc groups (*i.e.*, H-1, H-2, H-3, H-4, H-3′, H-4′, H-3″, H-4″); (*2*) a broad absorption at δ 4.33 (9 H), assigned to the protons of the three CH_2–OAc groups (CH_2-6′, CH_2-1″, CH_2-6″), and to those of the three methine groups C*H*–OR (H-5, H-5′, H-5″); and (*3*) a group of signals in the range δ 3.65–3.87 (4 H) due to the two methylene bridges of **5**.

These assignments are in agreement with those reported[5] for peracetates of other oligosaccharides related to sucrose. In order to confirm the position of attach-

ment of the second D-fructofuranosyl unit, the signals of the highest-field groups (δ 3.60–3.80) were examined at 60 and 100 MHz (Fig. 1).

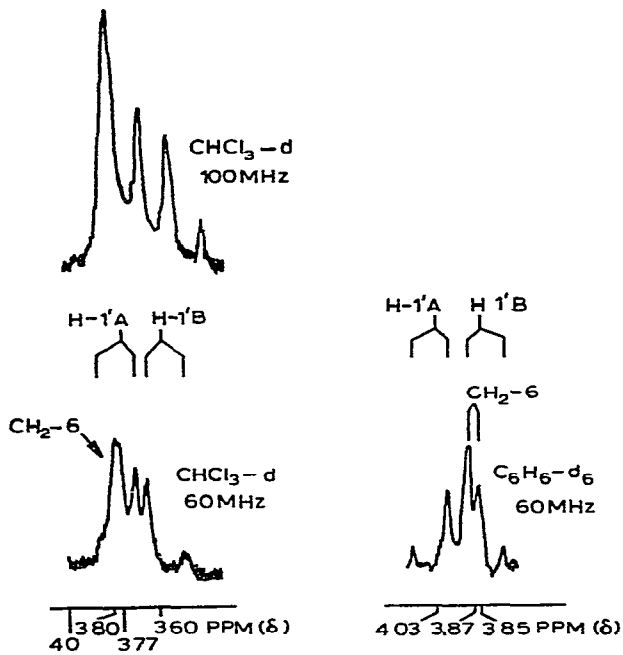


Fig. 1. Signals of the methylene-bridge protons in the n.m.r spectra of the trisaccharide hendecaacetate (5) in chloroform-*d* and in benzene-d_6.

In chloroform-*d*, the low-field side of this resonance appears as a broad absorption at δ 3.80 (half-band width, 5 Hz; integrated intensity, 2 H); three other sharp signals, showing the characteristic pattern of an AB system (the integrated intensity is a little less than 2 H, one of the outer peaks being hidden by the adjacent down-field resonance), are present: the resonance of *A* is at δ 3.77, and that of *B* at δ 3.60 (J_{gem} 9.5 Hz). In methanol-d_4, the spectrum is very similar to that observed in chloroform-*d*. In acetone-d_6, a better resolution of the peaks is observed, although the signals of the AB system show a less-pronounced nonequivalence; the signals are very close, and the outer one is very weak (J_{gem} 9 Hz). In benzene-d_6, the signals of the two methylene bridges overlap, but it is possible to distinguish a doublet at δ 3.87 (J 3 Hz) and a well-defined AB pattern (δ_A 4.03, δ_B 3.85, J_{gem} 10 Hz). The AB signal is assigned to CH_2-1′, as this methylene group has no vicinal proton. In fact, an AB system is expected for a methylene group with two nonequivalent protons, and such a pattern is usually observed when a methylene group is attached to an asymmetric carbon atom[6a]. On the other hand, the broad absorption at δ 3.80 in chloroform-*d* and the doublet in benzene-d_6 are assigned to the two almost equivalent protons at C-6, which are coupled with H-5. Evidently, for the C-6 methylene protons, the algebraic sum of the two factors determining the magnetic nonequivalence (different populations of the conformers and intrinsic asymmetry) is fortuitously zero[6b].

EXPERIMENTAL

General methods. — Infrared spectra were measured for potassium bromide discs. N.m.r. spectra were recorded with Varian A-60A and HA-100 spectrometers; chemical shifts were measured in p.p.m. (δ) from tetramethylsilane as the internal standard. Melting points were determined with a Kofler microscopic hot stage. Optical rotations were measured at 20 $\pm 3°$ with a Perkin–Elmer 141 Polarimeter.

Chromatographic methods. — Descending, paper chromatography was employed to establish identity and homogeneity of sugars and the corresponding alcohols, with (*a*) 6:4:3 butyl alcohol–pyridine–water[7]; (*b*) 2:1:5 ethyl acetate–pyridine–water[8]; (*c*) 7:1:2 propyl alcohol–ethyl acetate–water[9]. For the methylated and acetylated sugars, t.l.c. on Kieselgel G (Merck) was used with[10] (*d*) 15:1 chloroform–methanol; and (*e*) 1:1 benzene–acetone. For the detection of spots, benzidine[11], naphthoresorcinol–sulphuric acid[12], urea–hydrochloric acid[13], and aniline hydrogen phthalate[14] reagents were used. D-Glucitol was revealed with the sodium periodate–potassium permanganate–benzidine reagent[15]. For t.l.c., 50% sulphuric acid at 120° was used.

Electrophoresis. — Electrophoresis of methylated sugars was performed on Schleicher and Schull No. 2317 paper in 0.5M borate buffer (pH 9.2)[16] at 7–9° and at 50 volts/cm for 90 min. A high-voltage Pherograph apparatus was used. In these conditions, D-glucose moved 13 cm towards the anode.

Titration of aldose groups. — Aldose groups of the oligosaccharides were titrated with a modified[18] sodium hypoiodite method[17]. Found (aldose groups per mole of sugar): compound *A*, 1.04; *B*, 1.04; *C*, 1.17.

Borohydride reductions. — Borohydride reductions were performed following the method of Wolfrom and Thompson[19], on 200-mg samples, to give the following products: 6-*O*-β-D-fructofuranosyl-D-glucitol (from *A*), m.p. 70–72° (dec.), $[\alpha]_D$ −26° (*c* 1.0, water) (Found: C, 40.93; H, 6.89. $C_{12}H_{24}O_{11}$ calc.: C, 41.86; H, 7.03%); *O*-β-D-fructofuranosyl-(2→1)-*O*-β-D-fructofuranosyl-(2→6)-D-glucitol (from *B*), m.p. 74–76° (dec.), $[\alpha]_D$ −30° (*c* 1.0, water) (Found: C, 42.19; H, 6.95. $C_{18}H_{34}O_{16}$ calc.: C, 42.68; H, 6.77%); and *O*-β-D-fructofuranosyl-(2→1)-*O*-β-D-fructofuranosyl-(2→1)-*O*-β-D-fructofuranosyl-(2→6)-D-glucitol (from *C*), m.p. 114–116° (dec.), $[\alpha]_D$ −33° (*c* 1.0, water) (Found: C, 43.01; H, 6.83. $C_{24}H_{44}O_{21}$ calc.: C, 43.12; H, 6.63%).

Periodate-oxidation studies. — Periodate oxidations were performed at 5° in the dark, according to the procedure of Jeanloz and Forchielli[20]. The reaction was complete after 4 h.

Acid hydrolyses. — Alditols were hydrolyzed with N hydrochloric acid for 20 min at 100°. The *O*-β-D-fructofuranosyl linkages of methylated oligosaccharides were hydrolyzed selectively with 0.02N hydrochloric acid for 2 h at 100°; complete hydrolysis was performed with N acid for 4 h at 100°. Before analysis, the hydrolysates were neutralized with Dowex-1 x8 (HCO_3^- form).

Enzymic hydrolyses. — Yeast invertase (Nutritional Biochemical Corp.) was used. The reaction was carried out on 10-mg samples of the oligosaccharides in water

(1 ml) to which the enzymic preparation (10 mg) was added, and was complete in 4 h at 37°. Partial hydrolyses were performed at 20° with smaller amounts (10 to 100 times) of enzyme.

Methylations. — Each oligosaccharide (2g) was dissolved in water (10 ml) and treated[21] with methyl sulphate (7.5 ml) and sodium hydroxide (7.4 g in 14 ml of water), to give a partially methylated, syrupy product. For complete methylation[22], the partially methylated material was dissolved in anhydrous *N,N*-dimethylformamide (20 ml) and treated with barium oxide (7.5 g), barium hydroxide monohydrate (0.3 g), and methyl iodide (6 ml) for 24 h at room temperature with stirring. Processing of the mixture gave a syrup which was submitted to a second methylation. The final product was purified by chromatography on silicic acid, using chloroform containing 1% of ethanol as eluting agent. The fully methylated oligosaccharides were chromatographically homogeneous and showed no hydroxyl absorption in the infrared. Overall yield, 0.4–0.5 g.

Acetylation of the oligosaccharides. — The oligosaccharide (0.5 g) was suspended in pyridine (3.25 ml) and treated with acetic anhydride (2.5 ml) at 0°. After 18 h at 5°, and 18 h at room temperature, the solution was poured into cold water. The insoluble reaction product was recovered after several washings with water. The following acetates were obtained.

Octaacetate of A (225 mg, from aqueous methanol), m.p. 54–56° (dec.), $[\alpha]_D$ +6.4° (*c* 1, chloroform) (Found: C, 50.14; H, 5.79. $C_{28}H_{38}O_{19}$ calc.: C, 49.56; H, 5.64%).

Hendecaacetate of B (400 mg, crystals from 96% ethanol), m.p. 89–90° (dec.), $[\alpha]_D$ −14° (*c* 1, chloroform) (Found: C, 49.24; H, 5.65; O, 44.85. $C_{40}H_{54}O_{27}$ calc: C, 49.69; H, 5.63; O, 44.68%).

Tetradecaacetate of C (280 mg, amorphous), m.p. 68–70° (dec.), $[\alpha]_D$ −1.7° (*c* 1, chloroform) (Found: C, 50.24; H, 5.71. $C_{52}H_{70}O_{35}$ calc.: C, 49.76; H, 5.62%).

ACKNOWLEDGMENTS

The authors are indebted to Professor J. Edelman, Queen Elizabeth College, University of London, for a sample of 6-*O*-β-D-fructofuranosyl-D-glucose. Microanalyses were performed by Sig. A. Alemanni.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

SYNTHESIS AND PROPERTIES OF CELLULOSE FUROATES. NUCLEOPHILIC DISPLACEMENT REACTIONS OF BROMO-SUBSTITUTED CELLULOSE FUROATES WITH TERTIARY AMINES*

SUJAN SINGH** AND JETT C. ARTHUR, JR.

Southern Regional Research Laboratory, Southern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture, New Orleans, Louisiana 70119 (U. S. A.)

(Received November 3rd, 1969; in revised form, December 5th, 1969)

ABSTRACT

Cellulose furoates were prepared by the reaction of purified cotton cellulose with 2-furoyl chloride, 5-bromo-2-furoyl chloride, 5-methyl-2-furoyl chloride, and 2-furanacryloyl chloride in pyridine as the acid scavenger, with *N,N*-dimethylformamide as the diluent. The bromo-substituted cellulose furoates underwent nucleophilic displacement reactions with pyridine, *N,N*-dimethylcyclohexylamine, and triethylamine to give the corresponding quaternary salts. Infared spectra and physical properties of the fibrous cellulose furoates are reported. The mechanism of the nucleophilic displacement reaction is discussed.

INTRODUCTION

The properties of cotton cellulose have been modified by the preparation of chemical derivatives and graft copolymers of cellulose. For example, the reaction of 2-furoyl chloride with cellulose in the presence of pyridine was reported by Chatterjee and Stanonis[1] and Shaposhnikova *et al.*[2]. In the former article, no details were given regarding the identification or properties of the products. When the reactions were conducted in chloroform, dichloromethane, tetrachloroethane[3], and ethyl acetate, highly colored yarns were obtained. In dry *N,N*-dimethylformamide (DMF), the yarns obtained were less colored. The temperature during all of these reactions was 78–80°, at which, less colored yarns were obtained than at higher temperatures. Polyakov and Rogovin[4] found that, when cellulose was treated with thionyl chloride in dry DMF at 80° (without pyridine), reaction occurred and the hydrogen chloride evolved complexed with DMF. We have found that no reaction occurs when cellulose is treated with furan acid chlorides in dry DMF at 80°, unless pyridine is added. However, at 100–110°, reaction occurs without pyridine, and hydrogen chloride is evolved; at the end of the reaction, the treated yarns were found to be highly degraded by the acid.

*Presented at the 159th National Meeting of the American Chemical Society, Houston, Texas, February 22–27, 1970.

**NRC Resident Postdoctoral Research Associate

RESULTS AND DISCUSSION

The preparation of cellulose furoates is described in this report. In an attempt to prepare cotton cellulose yarns having modified physical properties, yarns were treated with 2-furoyl chloride, 5-methyl-2-furoyl chloride, 5-bromo-2-furoyl chloride, and 2-furanacryloyl chloride in pyridine as the acid scavenger, with dry DMF as the diluent.

The conditions for reaction of 2-furoyl chloride with purified cotton cellulose and the properties of the products are given in Table I. The degree of substitution (D. S.) of the products was dependent on the concentrations of the reactants and the

TABLE I

CONDITIONS FOR FORMATION, AND PROPERTIES, OF CELLULOSE FUROATES

Molar proportions of reactants[a]	*Reaction time, h*	*Degree of substitution*	*Breaking strength* $\times 10^{-3}$ (g)	*Color of product*
2-Furoyl chloride[b]				
(control)	—	0.00	4.77	white
1:4·8	6	1.26	4.84	very light-yellow
1:5:10	7	1.72	3.96	yellow
1:4.8	11	1.86	3 22	yellow
1:8:16	12	2.73	0.92	deep-yellow
5-Methyl-2-furoyl chloride[b]				
(control)	—	0.00	4.77	white
10.33:66	9	1.22	3.34	light-yellow
1:5:10	9	1.45	3.09	yellow
2-Furanacryloyl chloride[b]				
(control)	—	0.00	4.77	white
5:12·24	10	1.01	4.94	deep-yellow
5:38:76	10	1.89	1.44	brown-yellow
5-Bromo-2-furoyl chloride				
(control[b])	—	0.00	2 43	white
1:5:10[b]	6	79[c]	1.10	brown-black
1:4.8[b]	20	190[c]	0.64	black
1:4:8[d]	6	9.6[c]	1.59	white-yellow
1:4·8[e]	15	9.5[c]	1.71	yellow

[a]Molar ratios of D-glucose residue acid chloride·base; reaction temperature, 78–80°; DMF diluent. [b]Pyridine used. [c]Add-on, % [d]*N,N*-Dimethylcyclohexylamine used. [e]Triethylamine used.

reaction time. The i.r. spectrum of purified cotton cellulose is shown in Fig. 1A, and that of a product (D. S. 1.26), in Fig. 1B. The differential i.r. spectrum, obtained by instrumentally subtracting spectrum 1A from spectrum 1B, is shown in Fig. 1C; λ_{max}^{KBr} 5.80 (ester) and 6.35 μm (aromatic).

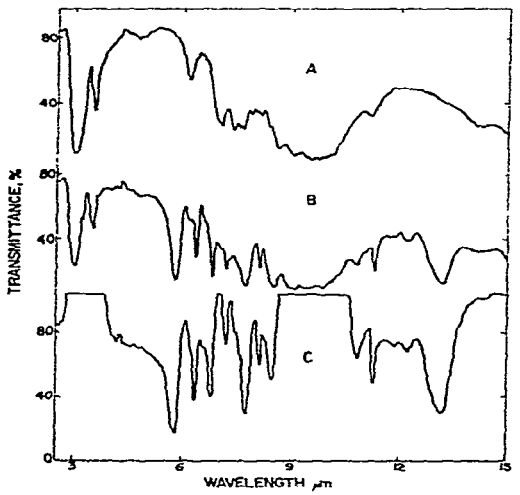


Fig 1. Infrared spectra of cotton cellulose (A) and of cotton cellulose treated with 2-furoyl chloride (B, product; C, differential spectrum).

The conditions for reaction of 5-methyl-2-furoyl chloride with cellulose, and the properties of the products, are given in Table I. The i.r. spectrum of a product (D. S. 1.45) is shown in Fig. 2A, and the differential i.r. spectrum in Fig. 2B; λ_{max}^{KBr} 5.80 (ester) and 6.28 μm (aromatic).

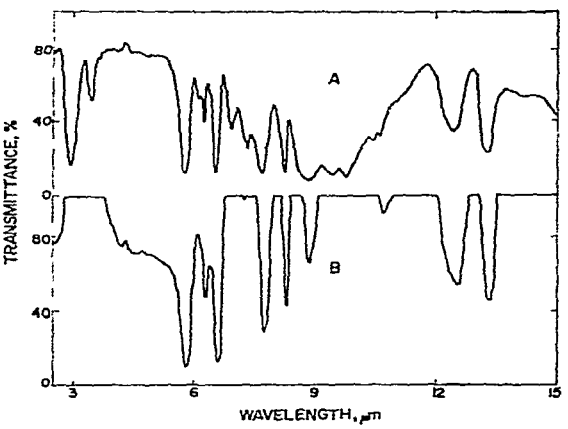


Fig. 2. Infrared spectrum of cotton cellulose treated with 5-methyl-2-furoyl chloride (A, product; B, differential spectrum)

The conditions for reaction of 2-furanacryloyl chloride with cellulose and the properties of the products are given in Table I. The i.r. spectrum of a product (D. S. 1.89) is shown in Fig. 3A, and the differential i.r. spectrum, in Fig. 3B; λ_{max}^{KBr} 5.81 (ester), 6.10 (C = C), and 6.41 μm (aromatic).

In dry DMF, the complex formed on reaction of pyridine with 2-furanacryloyl chloride was less soluble than the complexes formed on reaction with 2-furoyl chloride

or 5-methyl-2-furoyl chloride. Consequently, for the first complex, a larger proportion of dry DMF was needed in order to give a homogeneous solution than for the last two.

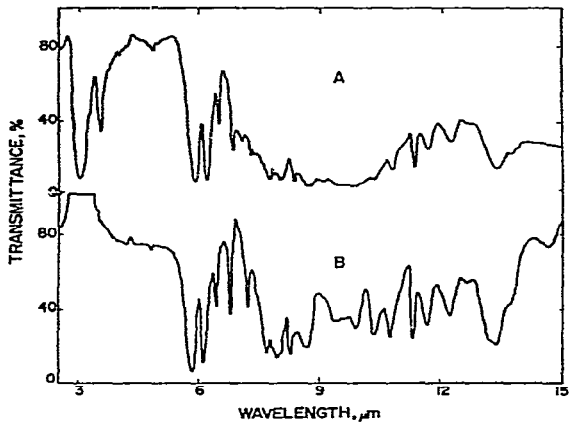
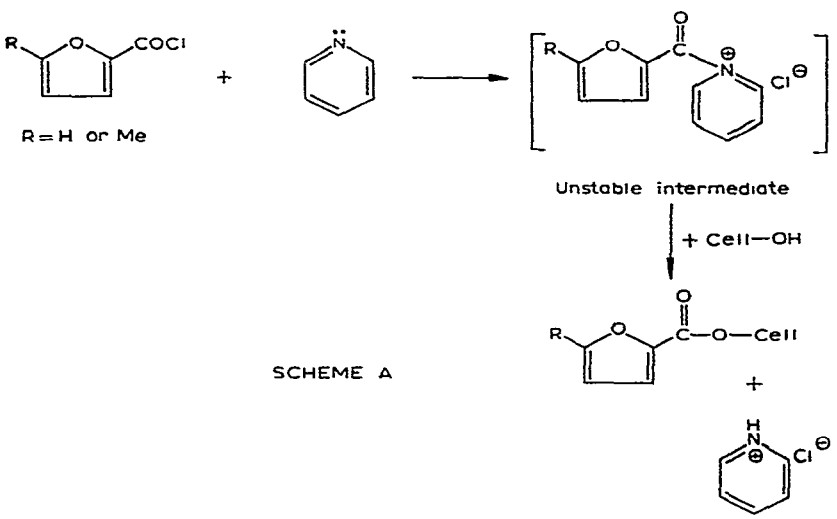


Fig. 3. Infrared spectrum of cotton cellulose treated with 2-furanacryloyl chloride (A, product; B, differential spectrum).

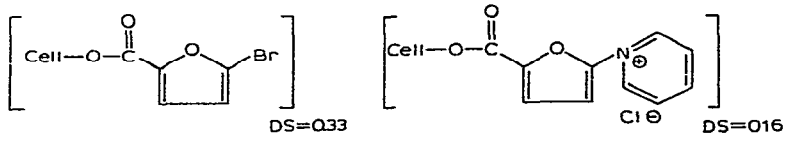
The i.r. spectra indicated that furoylation of cellulose occurred; a possible scheme is given in Scheme *A*. The ease[5] with which cinnamoyl chloride reacts with pyridine to give cinnamoylpyridinium chloride supports this Scheme.



The nucleophilic displacement reactions of vinyl halides have been described from both the mechanistic and the synthetic point of view[6,7]. The vinyl halides are usually unreactive in nucleophilic substitution reactions; they are reactive with such strong, electron-withdrawing groups as COR, COOR, SOR, SOOR, and CN, which, by inductive or mesomeric effects, or both, facilitate nucleophilic attack by the reagent

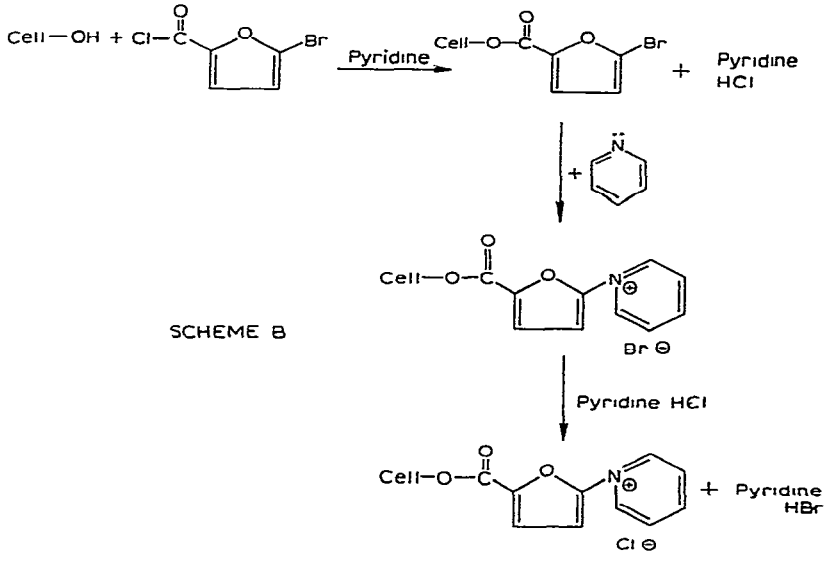
at the carbon atom bonded to the halogen atom[8–13]. The rates of reaction of halides of aliphatic and aromatic compounds with such nucleophilic reagents as hydroxides, alkoxides, ammonia, and amines have been reported[14]. Kinetic studies have been reported of the reaction of 2-bromofuran with piperidine at 199° in a sealed tube[15]. We have found[16] that, when 2-bromofuran is activated by electron-withdrawing groups, a nucleophilic displacement reaction with piperidine occurs at 80°. A prime reason for the present work was to compare the extent of reaction of halogen-substituted heterocyclic systems having electron-withdrawing groups with that of vinyl halides similarly substituted.

The conditions of reaction of 5-bromo-2-furoyl chloride with cellulose in pyridine, and the properties of the products, are given in Table I. The product obtained after reaction for 6 h had 79.4% "add-on" (increase in weight), and contained Br 10.09, Cl 2.15, N 0.81, and H_2O 3.10%. This composition corresponds to a mixed



(I)

derivative of cellulose, as in I. A possible reaction scheme is shown in Scheme *B*. The i.r. spectrum of the product is shown in Fig. 4A, and the differential i.r. spectrum,



SCHEME B

in Fig. 4B; λ_{max}^{KBr} 3.35 (aromatic CH, pyridine), 6.14, 6.25 (aromatic), and 5.80 μm (ester). These data further confirm that formation of the quaternary salt had occurred.

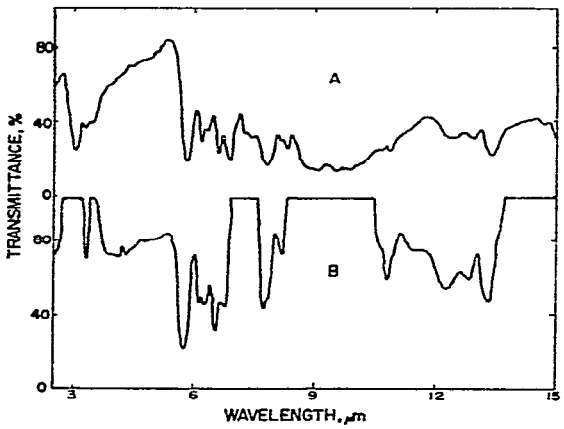
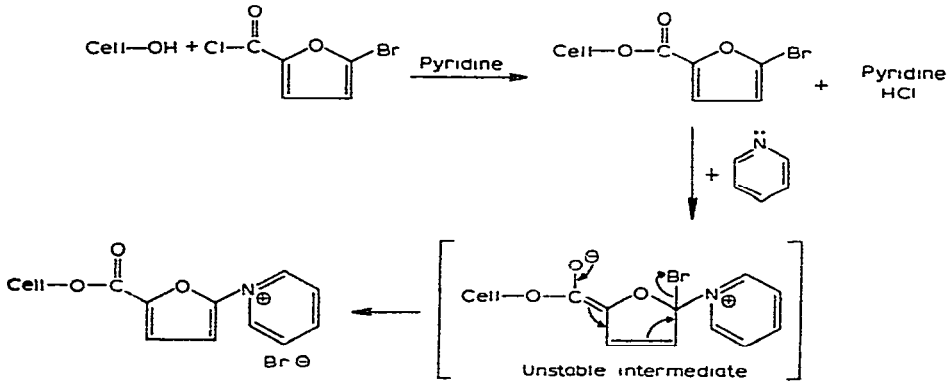


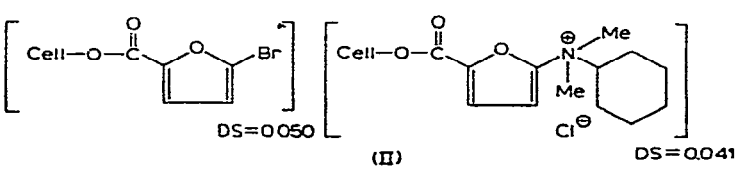
Fig. 4. Infrared spectrum of cotton cellulose treated with 5-bromo-2-furoyl chloride in pyridine (A, product; B, differential spectrum)

The most likely mechanism for the reaction of 5-bromo-2-furoyl chloride with cellulose in pyridine involves conjugate addition–elimination, as shown in Scheme *C*.



SCHEME C

Nucleophilic reactivity generally depends on the strength of the base used. Piperidine, a stronger base than pyridine, reacted with the acid chloride, and a quantitative yield of 5-bromo-*N*,*N*-pentamethylene-2-furoamide was obtained. In *N*,*N*-dimethylcyclohexylamine, a cellulosic product having 9.64% "add-on" was obtained; it contained Br 2.18, Cl 0.79, N 0.084, and H_2O 3.05%. This composition could correspond to a mixed derivative of cellulose, as in II.



(II)

Brady and Cropper[17] reported that amines having a number of alkyl groups attached to, or near, their nitrogen atoms are, in general, less reactive than amines having less branching near the nucleophilic center. They observed that, in some structures, alkyl groups interfere with the approach of the nitrogen atom to the aromatic carbon atom, whereas alkyl groups do not affect the basicity of the amine. Their argument, namely, that the intrinsic nucleophilic activity of the amines is modified by the steric effects of the alkyl groups, is convincing; it is applicable to *N,N*-dimethylcyclohexylamine and triethylamine. Cellulosic yarns treated with 5-bromo-2-furoyl chloride in *N,N*-dimethylcyclohexylamine and triethylamine are slightly yellow. When the reaction is conducted in pyridine, the yarns are dark-brown. The properties of the products obtained on reaction of 5-bromo-2-furoyl chloride with cellulose in *N,N*-dimethylcyclohexylamine are shown in Table I. The i.r. spectrum of the product is shown in Fig. 5A, and the differential i.r. spectrum, in Fig. 5B; λ_{max}^{KBr} 5.81 (ester) and 6.35 μm (aromatic).

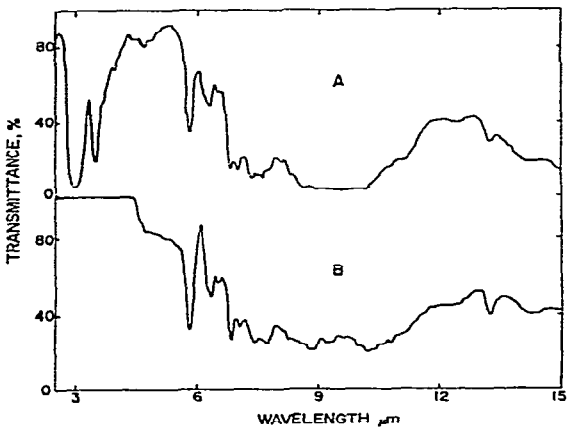


Fig. 5. Infrared spectrum of cotton cellulose treated with 5-bromo-2-furoyl chloride in *N,N*-dimethylcyclohexylamine (A, product; B, differential spectrum).

In triethylamine, a cellulosic product having 9.51% "add-on" was obtained; it contained Br 2.93, Cl 0.71, N 0.33, and H_2O 5.84%. This composition corresponds to a mixed derivative (III) of cellulose. The i.r. spectrum of the product is shown in

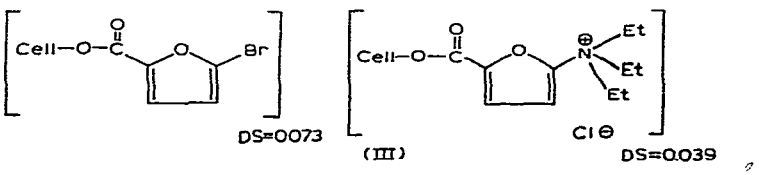


Fig. 6A, and the differential i.r. spectrum, in Fig. 6B; λ_{max}^{KBr} 5.82 (ester) and 6.35 μm (aromatic). The reaction conditions, and the properties of the products, are given in Table I.

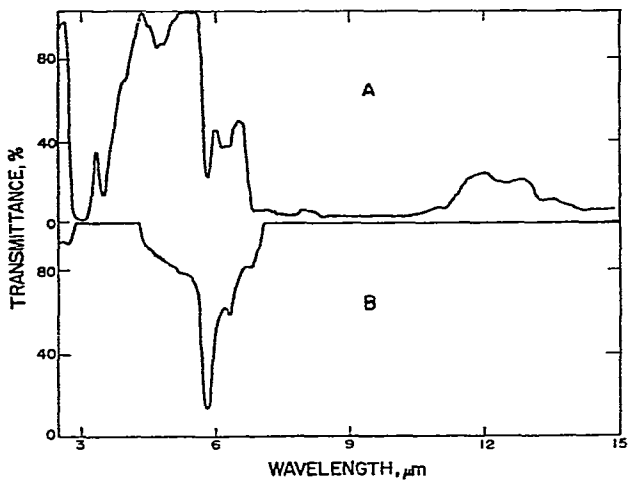


Fig. 6. Infrared spectrum of cotton cellulose treated with 5-bromo-2-furoyl chloride in triethylamine (A, product; B, differential spectrum).

EXPERIMENTAL

Materials. — Pyridine was dried over solid potassium hydroxide for 24 h, and distilled. *N,N*-Dimethylformamide (analytical grade) was dried over Drierite*, and purified by distillation. 2-Furoyl chloride was obtained from Eastman Kodak Co.; it was purified by distillation, b.p. 64–65°/10 torr.

5-Bromo-2-furoyl chloride was prepared by boiling a solution of 5-bromo-2-furoic acid (m.p. 186–187°; 1 mole) (prepared by the method of Whittaker[18]) and thionyl chloride (1.5 moles) in dry benzene for 20 h under reflux, and evaporating to dryness in a flash evaporator. The residue was vacuum-distilled, and a fraction of b.p. 78–81°/25 torr was collected; it solidified completely within 0.5 h; yield 90%, m.p. 59–60°; lit.[19] m.p. 58–60°.

5-Methyl-2-furoyl chloride was prepared by the method of Mudzhoyan *et al.*[20] by boiling 5-methyl-2-furoic acid (1 mole) and thionyl chloride (1.5 moles) in dry benzene under reflux, it was a colorless liquid, b.p. 136–138°/66 torr, yield 92%, that solidified completely on standing.

2-Furanacryloyl chloride was prepared by boiling a solution of 2-furanacrylic acid (*trans* acid, m.p. 141°; 1 mole) and thionyl chloride (1.5 moles) in dry benzene for 20 h under a reflux condenser (calcium chloride tube). The solution was evaporated to dryness under diminished pressure (25 torr), and the residue was distilled at 75–80°/0.45 torr; lit.[21] b.p. 90°/2 torr. The distillate, obtained in 90% yield, solidified on standing.

Deltapine cotton in the form of 7s/3 yarn was purified by the method of Arthur and Mares[22] to yield cellulose (mol. wt. 700,000).

*Trade names are given as part of the exact experimental conditions, and not as an endorsement of the products over those of other manufacturers.

Methods. — The reactions were conducted by placing weighed yarn in an all-glass, tube apparatus described by Hamalainen *et al.*[23]. Physical tests were performed by ASTM methods[24]. I.r. spectra of potassium bromide discs were recorded with a Perkin–Elmer double-beam spectrophotometer. Differential i.r. techniques were used for identifying the products. Elemental analyses were made by Galbraith Laboratories, Knoxville, Tennessee. The degree and uniformity of the esterification was determined by a dye test that uses mixed Acetate Yellow and Direct Cotton Blue[25]; the test was performed only for light-colored products.

Reaction conditions. — Dry DMF (100 to 300 ml, depending on the acid chloride used) was placed in the glass-tube apparatus[23], the desired amount of acid chloride (freshly distilled) was added, and dry pyridine (freshly distilled; 2 moles per mole of acid chloride) was added dropwise during 15 min. The mixture was then shaken by repeatedly inverting the tube; a colorless solution resulted. (A colored solution was formed if the acid chloride and pyridine were not freshly distilled.) Cellulose (~4–8g), in the form of yarn predried at 60° for 2 h, was added to the solution, and the tube was placed in an oil bath at 78–80°. The contents of the tube were shaken every 30 min. After completion of the reaction, the yarns were removed from the solution, washed twice with DMF, and immersed[26] in warm methanol (50–60°). The yarns were then kept in 10% potassium hydrogen carbonate solution for 0.5 h, and washed thoroughly with distilled water. Before being weighed or tested, all of the yarns were equilibrated for 24 h at about 50% relative humidity at 25°. The extent of reaction was ascertained by determining the increase in weight ("add-on") of these samples over that of the control yarn. Yarns esterified to various degrees of substitution (D.S.) with each acid were obtained by altering the ratio of molar concentration of acid chloride to cellulose, or the reaction time, or both.

A reaction did not occur between cellulose and 5-bromo-2-furoyl chloride when, instead of pyridine or *N,N*-dimethylcyclohexylamine, piperidine was used as the acid scavenger; 5-bromo-*N,N*-pentamethylene-2-furoamide was obtained in 89% yield. The crude amide was recrystallized twice from hexane (decolorizing carbon); m.p. 66–67°; $\lambda_{max}^{CHCl_3}$ 6.20 and 6.40 μm; n.m.r. data ($CDCl_3$): τ 3.10 (doublet, H-1), 3.60 (doublet, H-1), 6.30 (multiplet, H-4), and 8.35 (multiplet, H-6).

Anal. Calc. for $C_{10}H_{12}BrNO_2$: C, 46.51; H, 4.65; Br, 31.00; N, 5.42. Found: C, 46.32; H, 4.86; Br, 30.90; N, 5.52.

We thank Mr. Gordon J. Boudreaux for recording the n.m.r. spectra.

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Carbohyd. Res., 14 (1970) 73–82

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

CALCULATION OF MOLECULAR ROTATION BY SUMMATION OF PARTIAL CONFORMATIONAL CONTRIBUTIONS

PART II. ROTATIONS OF THE 1,6-ANHYDRO-DEOXY-β-D-HEXOPYRANOSES, 2,7-ANHYDRO-β-D-HEPTULOPYRANOSES, AND THEIR ACETATES*†

D. HORTON AND J. D. WANDER
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210 (U.S.A.)
(Received December 11th, 1969)

ABSTRACT

The optical rotations of two families of compounds, both having the 1,6-anhydro-β-D-hexopyranose structure as the fundamental skeletal unit, have been expressed as algebraic summations of empirical rotatory contributions from various conformational elements of asymmetry. The calculations have been made for 1,6-anhydro-monodeoxy-β-D-hexopyranoses and their diacetates, and for the 2,7-anhydro-β-D-heptulopyranoses and their tetraacetates, by using terms calculated[1] for the parent 1,6-anhydro-β-D-hexopyranoses and four new terms to describe interactions previously untreated. The calculated rotations are in satisfactory agreement with constants reported in the literature, and indicate conformational uniformity throughout the entire class of examples.

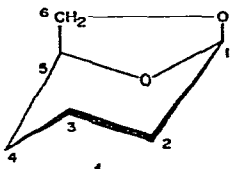
INTRODUCTION

Van't Hoff proposed that the net optical rotation of any molecule possessed of more than one center of asymmetry would be the algebraic sum of the rotatory powers of each of the asymmetric features[3], a hypothesis that was elegantly defended by Guye[4] and Walden[5], but was later discredited by Rosanoff[5]. Subsequent investigation showed that Van't Hoff's "principle" could be applied with systems having, between sets of asymmetric elements, separation sufficient to preclude significant vicinal interactions, as in the bis[(−)-menthylurethans] of the diethyl tetrarates[7], whereas, for flexible systems containing contiguous asymmetric centers, as in the hexonic acids[8], the "principle" was totally inapplicable.

N.m.r.[9–12] and i.r.[13] spectral data, and X-ray crystallographic[14,15] studies indicate that the favored conformation of derivatives of the 6,8-dioxabicyclo[3.2.1]octane skeleton is arrangement **1**, in which the 6-membered ring adopts[16] the *1C* conformation. Such an arrangement has considerable structural rigidity, and it is,

*Supported, in part, by a grant from the National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Maryland 20014, U.S.A.; Grant No. GM-11976-04 (The Ohio State University Research Foundation Project 1820).
† Part I of this series, see Ref. 1. For a preliminary report of the present work, see Ref. 2.

therefore, not surprising[17] that the specific rotations of the 1,6-anhydro-β-D-hexopyranoses[18], their 4-deoxy derivatives[19], and the 2,7-anhydro-β-D-heptulopyranoses[20] can, by the Van't Hoff approach, be interpreted in terms of a sum of contributions from the isolated *configurational* elements.



1

A more theoretically oriented approach, based on *conformational* elements of asymmetry, to the estimation of optical rotatory power in molecules containing contiguous asymmetric centers was initiated by Whiffen[21] in 1956. By this method, the molecule is dissected into contiguous, four-atom units of the form W–C–C–X, each of which is defined as a contributing rotational unit (±W/X), and a sum is made over all such possible units, to give a calculated rotation that is in reasonable agreement with the value observed. The complicated notation of Whiffen was simplified considerably by Brewster[22], and Lemieux[23] has recently shown that rotational values of a broad range of conformationally predictable, six-membered-ring monocycles can be estimated within reasonable limits by using only a few of the interactions originally considered by Whiffen.

In the previous paper[1] in this series, the rotations of the 1,6-anhydro-β-D-hexopyranoses were expressed as the algebraic sum of five conformational elements (see Fig. 1). The rotational contributions arising from vicinal, *gauche* interactions of

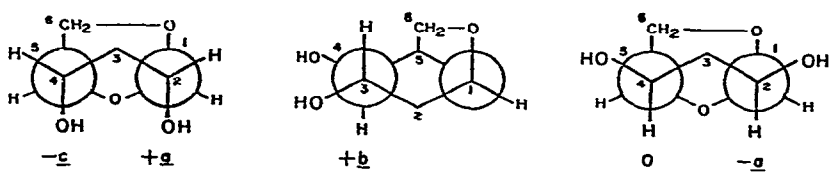


Fig. 1. Rotational contributions previously defined (Ref. 1) for the 1,6-anhydro-β-D-hexopyranoses.

oxygenated substituents along the C-1–C-2 bond were defined as *a*, and those along the C-2–C-3 or C-3–C-4 bonds as *b*. The signs of these interactions were defined by the chirality of the screw pattern relating them, so that one substituent advancing to the other as a right-hand screw gave a positive value, and the mirror-image relationship (left-hand screw) a negative one. Antiparallel pairs of oxygen atoms at these positions gave a contribution of zero. Additional rotatory contributions were also defined as follows: an axial substituent on C-2, C-3, or C-4 contributed *f* to the net rotation; the 6,8-dioxabicyclo[3.2.1]octane ring system itself gave a rotatory increment *d*; and an axial substituent at C-4 contributed $-c$. An equatorial group at C-4 (O-4 and O-5 antiparallel) was defined as contributing zero. It was noted[24] that the values of *c* and *d* are interrelated, and that, without data on the 1,6-anhydro-4-deoxy-β-D-

hexopyranoses, only relative values of these terms could be obtained. The choice of zero for the contribution corresponding to *c* in the examples having an equatorial 4-substituent was made in order to decrease the number of terms to be considered. With additional rotational data on the 4-deoxy compounds now available[19], the contribution from the bicyclic skeleton can be redefined to make it independent of the effect of interactions between the 4-substituent and the center at C-5. The resultant numerical values, in addition to permitting the calculation of accurate values for the rotation of any 1,6-anhydro-β-D-hexopyranose[1] (or its triacetate)[1], now permit calculation of the rotations of 1,6-anhydro-deoxy-β-D-hexopyranoses (and their acetates), and the results are in good agreement with literature values. The inclusion of only two additional parameters is sufficient to extend the applicability of this computation to the 2,7-anhydro-β-D-heptulopyranoses, their acetates, and their deoxy analogs.

DISCUSSION

1,6-Anhydro-monodeoxy-β-D-hexopyranoses. — Essentially the same conformational rotatory elements are employed for the deoxy compounds as were defined for the parent triols[1], namely:

(*1*) An algebraic value *a* is assigned to the *gauche* interaction along the C-1–C-2 bond between the substituent at C-2 and one of the oxygen atoms at C-1. The sign of this term is plus when the advance of O-2 into one oxygen atom at C-1 is as a right-hand screw, and minus for the enantiomorphic relationship (see Fig. 1). As O-2 is necessarily antiparallel to the other oxygen atom at C-1, there is no net rotational contribution from this second unit to be considered. The *a* term is unchanged from the original treatment[1], and is absent when the 1,6-anhydro-2-deoxy-β-D-hexopyranoses are considered.

(*2*) An algebraic value *b* is assigned to each vicinal diol group in a *gauche* relationship along the C-2–C-3 bond and the C-3–C-4 bond (see Fig. 1). It is immaterial whether the substituents are axially or equatorially attached. A vicinal diol group in the *trans* diaxial arrangement produces a rotatory increment of zero. The sign of *b* is determined by the criterion of chirality as defined for *a*, and is also unchanged from the previous treatment[1].

(*3*) An algebraic value c_{ax} is assigned to the *gauche* arrangement, along the C-4–C-5 bond, of the C-4 substituent (in the axial position) with the ring oxygen atom (O-5), and c_{eq} is the value in the 4-epimeric situation, in which O-4 is antiparallel to O-5, but in a *gauche* relationship to the C-6 methylene group (see Fig. 2). As each relationship is unique, the values are different for each term, and the signs of both are defined as positive in the D series and negative in the L, although the value of c_{ax} is negative. These definitions replace the term *c* in the previous treatment[1], and give new terms that are independent of the ring contribution. Establishment of these explicit C-4–C-5 contributions is made possible by use of data on the 1,6-anhydro-4-deoxy-β-D-hexopyranoses, in which both the c_{eq} and the c_{ax} contributions are absent.

(*4*) An algebraic value d_0 is assigned to the parent ring-structure **1**. The analogous contribution for the β-L enantiomorph is $-d_0$. Although this definition is little altered in principle from the earlier treatment, the actual numerical value is different

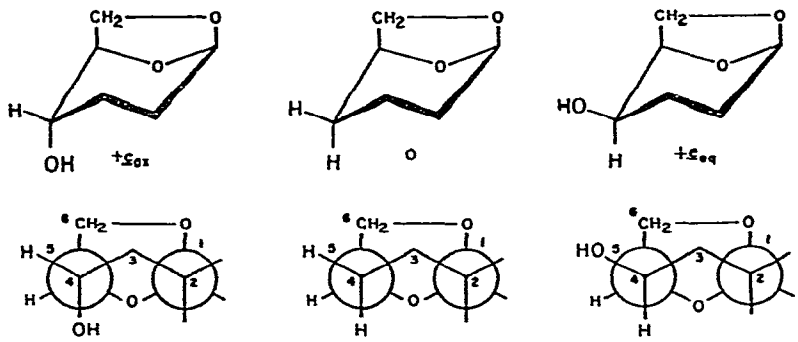


Fig 2. Interactions between C-4 and C-5.

from the value originally calculated for the contribution of the ring system[1] (because the original term *d* was not independent of the term *c*, whereas the new definitions for c_{ax} and c_{eq} are independent of the ring contribution), and so the subscript is added to indicate this distinction.

(*5*) An algebraic value *f* is assigned as a positive contribution for each substituent at C-2, C-3, or C-4 that is axial; this is identical in all respects with the previous definition[1]. A minus sign is prefixed to this term in the β-L series.

As the numerical values of *a*, *b*, and *f* remain as previously calculated[1] (see

TABLE I

PARTIAL OPTICAL ROTATORY CONTRIBUTIONS DETERMINED EMPIRICALLY FOR THE 1,6-ANHYDRO- AND 1,6-ANHYDRO-DEOXY-β-D-HEXOPYRANOSES AND THEIR PERACETATES

Structure	*Partial molecular rotatory contribution*[a, b] *(specific rotatory contributions given in parentheses)*[c]					
	a	b	c_{eq}[d]	c_{ax}[d]	d_0[d]	f
1,6-Anhydro-β-D-hexopyranoses	6,300 (39, 43)	4,400 (27,30)	+5,200[e] (+32, +36)	−8,000[e] (−49, −55)	−13,900[e] (−86, −95)	+1,600[e] (+10, +11)
1,6-Anhydro-β-D-hexopyranose peracetates	9,900 (34, 43)	3,500 (12, 15)	+4,800[e] (+17, +21)	−16,700[e] (−58, −73)	−16,800[e] (−58, −73)	+1,700[e] (+6, +7.5)

[a]The signs of *a* and *b* are given by the chirality of the screw pattern that they represent. [b]Molecular rotatory values are given as $[M]_D = [\alpha]_D \times$ mol. wt. [c]Obtained by dividing the corresponding partial molecular rotatory contributions by the molecular weight. The first value is derived for the parent compounds, and the second applies to the deoxy analogs. [d]These values replace the terms *c* and *d* previously used. The values −13,300 (−82.1) and −8,600 (−53.1), respectively, were given[1] for *c* and *d* for the parent triols, and −21,500 (−74.6) and −12,000 (−41.7), respectively, for the corresponding acetates [e]These signs are reversed when the corresponding 1,6-anhydro-β-L-hexopyranose is considered.

TABLE II

OBSERVED AND CALCULATED ROTATIONS OF SOME 1,6-ANHYDRO-MONODEOXY-β-D-HEXOPYRANOSES AND THEIR DIACETATES

Structure and configuration	*Sum of partial rotatory contributions*	1,6-*Anhydro*-β-D-*hexopyranoses* (*in water*)				*Refs*	1,6-*Anhydro*-β-D-*hexopyranose diacetates* (*in chloroform*)				*Refs.*
		$[\alpha]_D$, *degrees*[a]		$[M]_D$, *degrees*[a,b]			$[\alpha]_D$, *degrees*[a]		$[M]_D$, *degrees*[a,b]		
		obs.	*calc.*	*obs.*	*calc.*		*obs.*	*calc.*	*obs.*	*calc.*	
2-Deoxy											
ribo	$-b+c_{ax}+d_0+f$	−167	−169	−24,400	−24,600	26					
arabino	$c_{ax}+d_0+2f$	−118	−128	−17,200	−18,600	26					
xylo	$b+c_{eq}+d_0$	−21.4, −31	−29	−3,100	−4,200	27, 28					
lyxo	$-b+c_{eq}+d_0+f$	−70	−78	−10,200	−11,400	26					
3-Deoxy											
ribo	$a+c_{ax}+d_0+2f$	−80	−85	−11,700	−12,300	29	−74	−88	−17,000	−20,000	29
arabino	$-a+c_{ax}+d_0+f$	−156	−182	−22,800	−26,500	29	−139	−181	−32,000	−41,700	29
xylo	$a+c_{eq}+d_0+f$	[−8][d]	−5	[−1,200]	−700						
lyxo	$-a+c_{eq}+d_0$		−102		−14,900						
4-Deoxy											
ribo	$a+b+d_0+f$	−16	−11	−2,300	−1,600	19	−12	−8	−2,800	−1,700	19
arabino[e]	$-a-b+d_0$	−164	−168	−24,000	−24,600	30, 31[c]	−137	−131	−31,500	−30,200	32
xylo	$a+d_0+2f$	−39	−30	−5,700	−4,400	30					
lyxo	$-a+b+d_0+f$	−87	−97	−12,700	−14,700	19	−69	−93	−15,900	−21,500	19

[a] Determined in the temperature range of 15–24°. [b] $[M]_D = [\alpha]_D \times$ molecular weight. [c] Reported as the L enantiomorph, having the opposite sign of rotation. [d] M. MacLeod, L. Schroeder, and P. A. Seib have recently isolated an anhydride, m.p. 138–139°, in equilibrium with 3-deoxy-D-*xylo*-hexose. The pyranose structure is assigned on the basis of the agreement between predicted and observed rotation. (P. A. Seib, personal communication, Jan. 28, 1970). [e] Specific rotations in excellent agreement with the values here calculated have also been observed for 1,6-anhydro-4-deoxy-β-D-*arabino*-hexopyranose and its triacetate as isolated by degradation from a natural glycoside (Mme. J Polonsky, personal communication to D. H. at Gif-sur-Yvette, February 27th, 1970).

Table I), a narrow range of values for d_0 can be obtained directly by summing the terms for any one of the 1,6-anhydro-4-deoxy-β-D-hexopyranoses and equating the result with the known rotation. The optimum value for d_0 is presented in Table I. Because of the linear relationship of the terms c and d previously[1] used, $c_{ax} = -c+(d-d_0)$, and $c_{eq} = d-d_0$. Once d_0 has been evaluated, the values of c_{eq} and c_{ax} can also be obtained directly from summations of terms for any appropriately substituted 1,6-anhydro-β-D-hexopyranose (see Table I). Calculated rotations for the remaining isomers and homologs are then found in excellent agreement with the values determined experimentally (see Table II). A similarly consistent set of values was determined (see Table II) for the diacetates; the numerical magnitudes of the five terms (see Table I) differed somewhat from those determined for the parent alcohols.

The method is illustrated by means of the following examples.

In 1,6-anhydro-β-D-galactopyranose (**2**, see Fig. 3), the 2-hydroxyl group has a right-hand screw relationship to O-5 and gives a 1,2 contribution of $+a$; the anti-

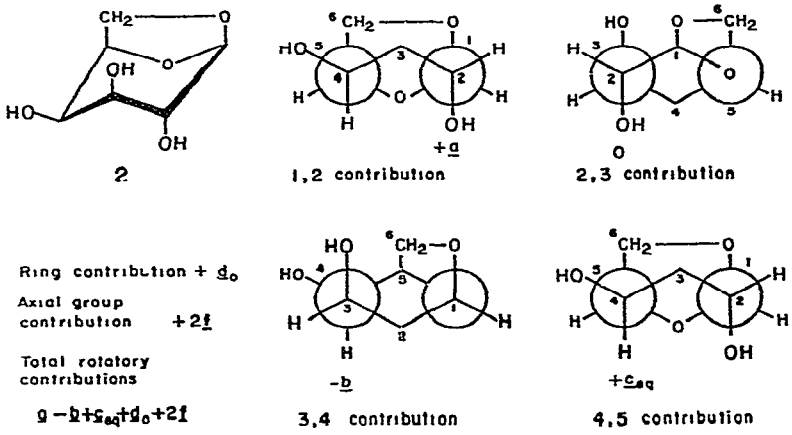


Fig. 3. Summation of rotatory contributions in 1,6-anhydro-β-D-galactopyranose (**2**).

parallel arrangement of O-2 and O-6 gives a contribution of zero. Along the C-2–C-3 bond, the two hydroxyl groups are *trans* diaxial, and so the 2,3 contribution is zero. Along the C-3–C-4 bond, there is a left-hand screw relationship of hydroxyl groups, giving a 3,4 contribution of $-b$. The interaction of the equatorial hydroxyl group at C-4 with the center at C-5 is accounted for by a 4,5 contribution of $+c_{eq}$. When the skeletal contribution ($+d_0$) and the contributions ($+2f$) for the axial substituents are included, summation of the terms gives the following equation:

$$\text{total rotation} = +a-b+c_{eq}+d_0+2f.$$

In 1,6-anhydro-β-D-mannopyranose (**2** in Ref. 1), the 4,5 contribution becomes $+c_{ax}$, and the summation of all of the partial rotatory contributions gives the following equation:

$$\text{total rotation} = -a+b+c_{ax}+d_0+2f.$$

In 1,6-anhydro-3-deoxy-β-D-*ribo*-hexopyranose (**3**, see Fig. 4), the hydroxyl group on C-2 advances into O-5 (the oxygen atom in the pyranose ring) as a right-hand

screw, giving a 1,2 contribution of $+a$. The absence of a hydroxyl group from C-3 removes any vicinal contributions from *gauche* interactions of hydroxyl groups along either the C-2–C-3 or the C-3–C-4 bond. The interaction of the axial hydroxyl group

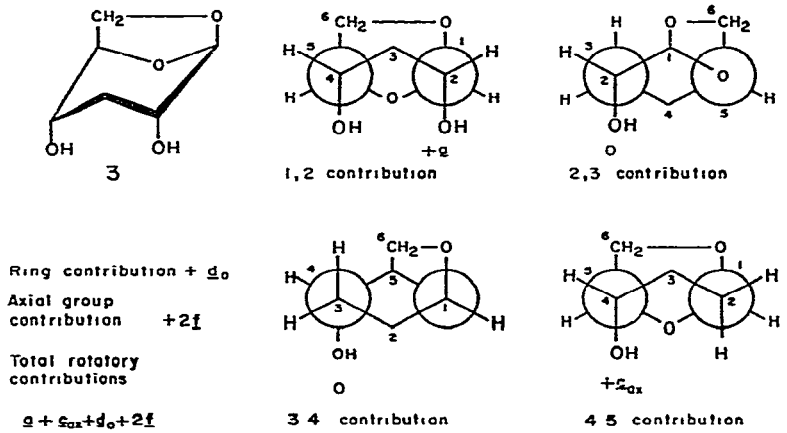


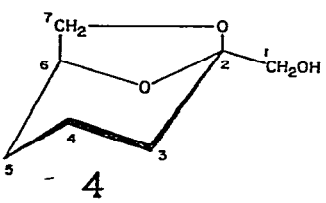
Fig. 4. Summation of rotatory contributions in 1,6-anhydro-3-deoxy-β-D-*ribo*-hexopyranose (3).

at C-4 with the center at C-5 is independent of chirality along C-4–C-5, contributing $+c_{ax}$ despite the left-hand screw relationship. Inclusion of the skeletal ($+d_0$) and axial group ($+2f$) contributions and summation of terms gives the following equation:

$$\text{total rotation} = +a+c_{ax}+d_0+2f$$

Corresponding summations are given in Table II for all twelve 1,6-anhydro-monodeoxy-β-D-hexopyranoses. The parameters calculated for the acetates are of like sign and order of magnitude, and there is good agreement between the experimental rotations and those calculated, indicating that the anisotropy of the acetoxyl substituents does not seriously complicate the calculation of rotation by summation of partial conformational contributions.

2,7-Anhydro-β-D-heptulopyranoses. — The members of this class are related to the corresponding 1,6-anhydro-β-D-hexopyranoses by replacement of the hydrogen atom at C-1 of the parent ring structure (**1**) by a hydroxymethyl group. It is thus



4

reasonable to expect that only those rotatory interactions directly related to C-2 in the new ring structure (**4**) will be altered, and so only two revised definitions are required.

(*1*) The *gauche* interaction, advancing as a right-hand screw along the C-2–C-3 bond, between a hydroxyl group at C-3 and one oxygen atom attached to the anomeric center, is assigned the algebraic value $+a'$ (see Fig. 5), and the corresponding inter-

action, in a left-hand screw arrangement, $-a'$. The antiparallel relation between the hydroxyl group at C-3 and the other oxygen atom attached to the anomeric carbon atom is zero by the arguments given previously, and the *gauche* interaction between the 3-hydroxyl group and the exocyclic hydroxymethyl group is included in the definition of a' because of the symmetrical disposition of the hydroxymethyl group with respect to the two orientations possible for the 3-substituent*.

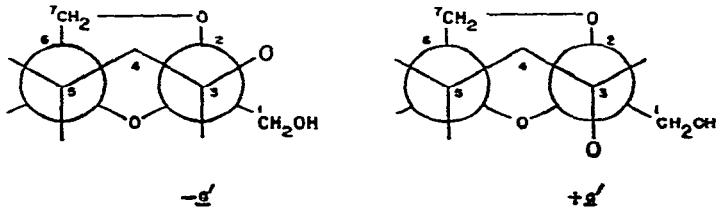


Fig. 5. Rotational contributions along the C-2–C-3 bond in 2,7-anhydro-β-D-heptulopyranoses.

(*2*) The algebraic value d_0' is assigned to the parent ring structure **4**. In **4**, the sign of d_0' is positive; in the β-L enantiomorph, the sign would be negative.

Solution of any two of the equations for the six known 2,7-anhydro-β-D-heptulopyranoses provides values (see Table III) for the two new terms, the others remaining constant.

This application is illustrated in Fig. 6 with 2,7-anhydro-β-D-*altro*-heptulopyranose (**5**) as the example. The 3-hydroxyl group advances along the C-2–C-3 bond into O-7 (the oxygen atom of the anhydro bridge) as a left-hand screw. The contribution $-a'$ thus accounts for the 2,3 interaction and takes into account the hydroxy-

TABLE III

PARTIAL OPTICAL ROTATORY CONTRIBUTIONS DETERMINED EMPIRICALLY FOR THE 2,7-ANHYDRO-β-D-HEPTULOPYRANOSES AND THEIR PERACETATES

Structure	*Partial molecular rotatory contributions*[a,b] *(specific rotatory contributions given in parentheses)*					
	a′	b	c_{eq}	c_{ax}	d′0	f
2,7-Anhydro-β-D-heptulopyranoses	2,900 (15.1)[d]	4,400 (22.9)[d]	+5,900[c] (+30.7)[d]	−7,300[c] (−37.3)[d]	−12,000[c] (−62.5)[d]	+1,600[c] (+8.3)[d]
2,7-Anhydro-β-D-heptulopyranose tetraacetates	5,000 (13.0)[d]	3,500 (9.7)[d]	+4,800[c] (+13.3)[d]	−16,700[c] (−46.4)[d]	−12,000[c] (−33.3)[d]	+1,700[c] (+4.7)[d]

[a,b]See Table I. [c]These signs are reversed in the β-L series. [d]Obtained by dividing the corresponding partial molecular rotatory contributions by the molecular weight.

*It would also be possible to define a term a_c as the interaction between the hydroxyl group at C-3 and the hydroxymethyl group, in which case a would retain its original definition and value. This procedure would, however, introduce one more contribution to be accounted for, and, as $+a'$ would equal $+a - a_c$, and $-a' = -a + a_c$ always, the decrease in the total number of operations involved on using a' justifies the introduction of this new term

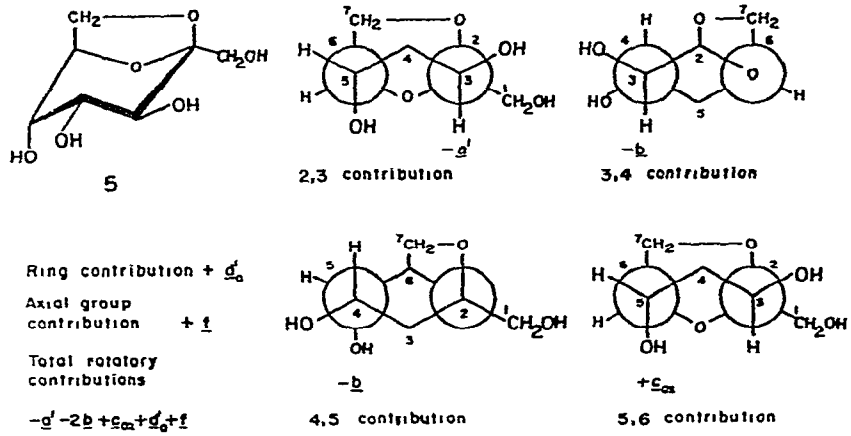


Fig. 6. Summation of rotatory contributions in 2,7-anhydro-β-D-*altro*-heptulopyranose (5).

methyl group; the necessarily antiparallel disposition of the 3-hydroxyl group and the other oxygen atom (O-5) at C-2 contributes zero. The hydroxyl group at C-3 also advances into the hydroxyl group at C-4 as a left-hand screw, giving a 3,4 contribution of $-b$, and the similar disposition of the hydroxyl groups at C-4 and C-5 gives a 4,5 contribution of $-b$. The interactions between the axial hydroxyl group at C-5 and the center at C-6 bring about a contribution of $+c_{ax}$. When the skeletal contribution ($+d'_0$) and the contribution ($+f$) of the single axial group are included, summation of the terms gives the following equation:

$$\text{total rotation} = -a'-2b+c_{ax}+d'_0+f.$$

Corresponding summations are given in Table IV for the eight parent configurations and for 2,7-anhydro-3-deoxy-β-D-*arabino*-heptulopyranose[25], together with calculated and experimental values for the rotations of these anhydroheptuloses and their peracetates.

The very satisfactory agreement between the rotations observed for the six known 2,7-anhydroheptuloses and the rotations calculated (see Table IV) by using these partial rotatory values testifies to the validity of the initial assumption. Experimentally determined rotations for only two of these anhydroheptuloses were needed in order to permit calculation of the two new values a' and d'_0. The paucity of data on the acetates precludes detailed comparison of calculated with observed values, but the similarity of sign and order of magnitude evident for the four examples for which comparisons can be made provides definite indication that the anisotropy of the acetoxyl groups does not hinder the calculation, by summation of partial rotatory contributions, of the rotatory power of these derivatives.

CONCLUSIONS

The approach based on additive conformational rotatory elements, developed by Whiffen[21], and the modifications thereof made by Brewster[22] and Lemieux[23], are capable of predicting the optical proclivities of a plethora of molecules, provided

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TABLE IV

OBSERVED AND CALCULATED ROTATIONS OF SOME 2,7-ANHYDRO-β-D-HEPTULOPYRANOSES AND THEIR PERACETATES

Configuration	*Sum of partial rotatory contributions*	*2,7-Anhydro-β-D-heptulopyranoses (in water)*				*Refs.*	*2,7-Anhydro-β-D-heptulopyranose acetates (in chloroform)*				*Refs.*
		$[\alpha]_D$, *degrees*[a]		$[M]_D$, *degrees*[a,b]			$[\alpha]_D$, *degrees*[a]		$[M]_D$, *degrees*[a,b]		
		obs.	*calc.*	*obs.*	*calc.*		*obs.*	*calc.*	*obs.*	*calc.*	
allo	$a'+c_{ax}+d'_0+2f$		−68.3		−13,200		−44.9, −46	−57	−16,600	−20,300	33, 34[c]
altro	$-a'+2b+c_{ax}+d'_0+f$	−146	−152.4	−28,000	−29,000	35	−110	−110	−39,600	−39,000	35
gluco	$a'+c_{ax}+d'_0+3f$	−63	−59.8	−12,100	−11,600	36, 37	−59	−52	−21,200	−18,600	36
manno	$-a'+b+c_{ax}+d'_0+2f$	−80	−75.4	−15,400	−14,600	37, 38					
gulo	$a'+2b+c_{eq}+d'_0+f$	+40	+37.4	+ 7,700	+ 7,200	39[c]					
ido	$-a'+c_{eq}+d'_0$	−41	−46.9	− 7,900	− 9,000	34					
galacto	$a'-b+c_{eq}+d'_0+2f$	−25.5	−22.2	− 4,900	− 4,400	40					
talo	$-a'+c_{eq}+d'_0+f$		−51.6		− 9,900						
3-deoxy *arabino*	$c_{ax}+d'_0+2f$	−90.2	−90.3	−15,900	−15,900	25	−98.3	−84	−29,700	−25,300	25

[a,b,c] See Table II.

that ideal bond-geometry can be assumed. The mild distortion of bond angles in the bicyclo[3.2.1]octane type of skeleton present in the 1,6-anhydro-β-D-hexopyranoses[14,15] excludes treatment of such derivatives by this approach. Furthermore, it is not clear how such derivatives as acetates can be treated in a simple way by these methods. The method of Černý, Pacák, and Staněk[18–20], based on Van't Hoff's idea of additive *configurational* elements, has demonstrated success with such compounds, but suffers from two general disadvantages: (*1*) the seven parameters derived for the 1,6-anhydro-β-D-hexopyranoses[18] are applicable only to that skeleton, and treatment of the 2,7-anhydro-β-D-heptulopyranoses requires a complete, new set of seven values[20], thereby limiting the predictive power of this approach to systems for which seven or more closely related examples are available; and (*2*) no provision is made for treatment of 1,6-anhydro-3-deoxy-β-D-hexopyranoses. The contributions of C-2 and C-4 depend on the configuration at C-3, and are undefined for the case where C-3 lacks a substituent.

The calculations described in this discussion represent a simple but general and accurate tool for predicting the optical rotatory power of molecules structurally related to the 1,6-anhydro-β-D-hexopyranoses, either by removal of oxygenated groups, or by addition of a hydroxymethyl group to C-1, or both. By introduction of sufficient new parameters to account for the various nitrogen–oxygen interactions, it is possible to extend this treatment to aminodeoxy and acetamidodeoxy 1,6-anhydro-β-D-hexopyranoses, although partially acetylated systems and amine salts are not suitable for this approach. Details of these calculations will be published in a separate report.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

1,2(*R*):5,6(*R*)-, 1,2(*R*):5,6(*S*)-, AND 1,2(*S*):5,6(*S*)-bis-*O*-(TRIFLUOROMETHYL)-ETHYLIDENE-D-MANNITOL

T. D. INCH AND R. V. LEY
Chemical Defence Establishment, Porton Down, Salisbury, Wiltshire (Great Britain)

(Received October 3rd, 1969, in revised form, December 8th, 1969)

ABSTRACT

The three possible 1,2:5,6-bis-*O*-(trifluoromethyl)ethylidene-D-mannitols have been isolated from the reaction between D-mannitol and 1,1,1-trifluoroacetone, and converted into 2,3-*O*-(trifluoromethyl)ethylidene derivatives of D-glycerol and D-glyceric acid. The assignment of configuration to the acetal carbon atom in the various products was based on n.m.r. studies. Chemical shift non-equivalence is exhibited by the acetal methyl group in 2,3-*O*-(*S*)-(trifluoromethyl)ethylidene-D-glycerol (*RS*)-atrolactate (*i.e.*, where the atrolactoyloxymethyl substituent is *cis* to the acetal methyl group), whereas the corresponding 2,3-*O*-(*R*)-(trifluoromethyl)-ethylidene-D-glycerol derivative shows only a single methyl signal.

INTRODUCTION

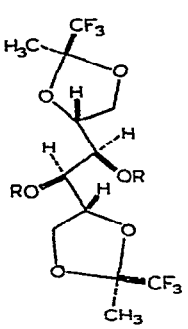
In 1960, Bourne and his co-workers[1] described the isolation of two of the three possible isomeric 1,2:5,6-bis-*O*-(trifluoromethyl)ethylidene-D-mannitols which differ in configuration at the carbon atoms carrying the trifluoromethyl groups, but the configuration of the isolated isomers was not established. We now report the isolation and structural assignments of the three possible isomeric 1,2:5,6-bis-*O*-(trifluoromethyl)ethylidene-D-mannitols (**1**, **2**, and **3**) and their subsequent conversion into the (*S*) (**4a** from **1**), (*S*) and (*R*) (**4a** and **5a** from **2**), and (*R*) (**5a** from **3**) isomers of 2,3-*O*-(trifluoromethyl)ethylidene-D-glyceric acid.

DISCUSSION

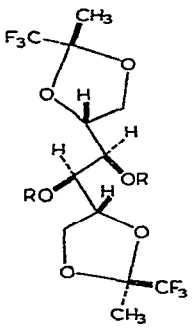
1,1,1-Trifluoroacetone was allowed to react with D-mannitol in sulphuric acid under essentially the conditions described by Bourne and his co-workers[1]. The three crystalline 1,2:5,6-bis-*O*-(trifluoromethyl)ethylidene-D mannitols (**1**, **2**, and **3**) were separated from each other and from other products* by chromatography over silica

*In addition to other (trifluoromethyl)ethylidene-D-mannitols, cyclic sulphates may also be formed in this kind of reaction. Two other crystalline products were also isolated, but their structures have not yet been established.

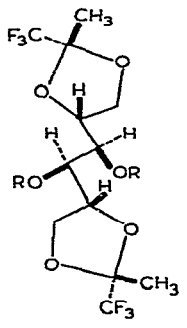
gel. The 1,2:5,6-substitution pattern was established by oxidation studies in which each diacetal rapidly consumed 1 mol. of sodium periodate. Sequential reduction with lithium aluminium hydride and oxidation with chromic acid of the crude periodate-oxidation products yielded, successively, 2,3-*O*-(trifluoromethyl)ethylidene-D-glycerol and 2,3-*O*-(trifluoromethyl)ethylidene-D-glyceric acid. From **1**, only one acid, identified as 2,3-*O*-(*S*)-(trifluoromethyl)ethylidene-D-glyceric acid (**4a**), was obtained, and **3** also yielded only one acid, identified as 2,3-*O*-(*R*)-(trifluoromethyl)-ethylidene-D-glyceric acid (**5a**). Compound **2** yielded both the (*S*) and (*R*) 2,3-*O*-(trifluoromethyl)ethylidene-D-glyceric acids which were separated by chromatography over silica gel. The oxidation studies established the unsymmetrical nature of **2** but did not permit **1** and **3** to be distinguished.



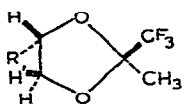
1 R = H
1a R = $COCH_3$



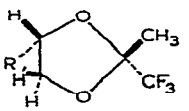
2 R = H
2a R = $COCH_3$



3 R = H
3a R = $COCH_3$

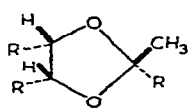


4 R = CH_2OH
4a R = CO_2H
4b R = CO_2Me

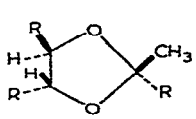


5 R = CH_2OH
5a R = CO_2H
5b R = CO_2Me

The n.m.r. method has been used previously for establishing the structure of 2,2,4-trisubstituted 1,3-dioxolanes[2], since, for example, methyl groups that are *β-cis* to two protons, as in **6**, resonate at higher field than when they are *β-cis* to an alkyl (or substituted alkyl) substituent, as in **7**. That is, alkyl groups deshield *β-cis* protons or groups of protons in 1,3-dioxolanes[3]. This approach has been used for structural assignments to two 1,2:5,6-di-*O*-bromoethylidene-D-mannitols[4] and in other cases[5].



6



7

The n.m.r. parameters of **1**, **2**, and **3** and their acetates are listed in Table I. In chloroform-*d*, all three compounds showed broad signals at 1.46 p.p.m. consistent with two equivalent methyl groups (some broadening of the signals was due to coupling with the CF_3 groups), but, on acetylation, isomer **2** showed two distinct acetoxyl methyl peaks, whereas isomers **1** and **3** each showed single acetoxyl methyl peaks corresponding to two equivalent acetoxyl methyl groups. This evidence showed that compound **2** was the unsymmetrical isomer, but permitted no distinction between **1** and **3**. However, in trifluoroacetic acid, isomer **2** showed two distinct CH_3 signals, and there was a clear difference between the CH_3 signals in **1** and **3**. Both compounds still showed two equivalent methyl signals, but the signal in **1** was at 0.04 p.p.m. to lower field than in **3**, thereby suggesting that **1** was 1,2(*S*):5,6(*S*)-bis-*O*-(trifluoromethyl)ethylidene-D-mannitol and that **3** was 1,2(*R*):5,6(*R*)-bis-*O*-(trifluoromethyl)-ethylidene-D-mannitol. Also, the CH_3 signal in the glycerol derivative **4** (from **1**) (Table II) was at lower field than the corresponding signal in **5** (from **3**), providing further evidence for the assigned structures, since there is unequivocal evidence that hydroxymethyl groups deshield *β-cis*-methyl groups in 1,3-dioxolanes[3,6]. Again, further evidence in support of the structures assigned to **1** and **3** was provided by the glyceric acid derivatives (**4a** and **5a**) and the methyl glycerate derivatives (**4b** and **5b**). In both **4a** and **4b**, the acetal methyl groups are at significantly lower field than in **5a** and **5b**, and, moreover, the difference in chemical shift is enhanced in benzene. This result is consistent with the findings of a detailed study of glyceric acid derivatives, in which it was shown that $-CO_2H$ and $-CO_2Me$ groups exhibit a stronger deshielding effect on *β-cis* protons than do $-CH_2OH$ or $-CH_3$ groups[3].

TABLE I

ASSIGNMENTS IN THE N.M R. SPECTRA OF 1,2:5,6-*bis*-*O*-(TRIFLUOROMETHYL)ETHYLIDENE-D-MANNITOLS

Compound	*Solvent*	*Chemical shifts*[a]		
		CH_3[b]	*$COCH_3$*	*CF_3*
1	CF_3CO_2H	1.60 (×2)	—	+7.08 (×2)
1	$CDCl_3$	1.46 (×2)	—	—
1a	$CDCl_3$	1.46 (×2)	2.10 (×2)	—
2	CF_3CO_2H	1.56,1.60	—	+7.08, +7.28
2	$CDCl_3$	1.46 (×2)	—	—
2a	$CDCl_3$	1.46 (×2)	2.08,2.12	—
3	CF_3CO_2H	1.56 (×2)	—	+7.26 (×2)
3	$CDCl_3$	1.46 (×2)	—	—
3a	$CDCl_3$	1.46 (×2)	2.10 (×2)	—

[a]In p.p.m. from tetramethylsilane as internal standard for proton shifts, and upfield (+) from TFA (trifluoroacetic acid) for ^{19}F shifts; [b](×2) indicates that the intensity of the signal corresponded to two methyl groups.

Since CF_3 groups are known to be "repulsively deshielded" by neighbouring bulky groups[7], it was expected that the CF_3 groups in **1** and **4** would be at higher field than in **3** and **5**. This was not found to be the case. The CF_3 groups in **1** were

TABLE II

ASSIGNMENTS IN THE N.M R. SPECTRA OF SOME 4-SUBSTITUTED-2-METHYL-2-TRIFLUOROMETHYL-1,3-DIOXOLANES

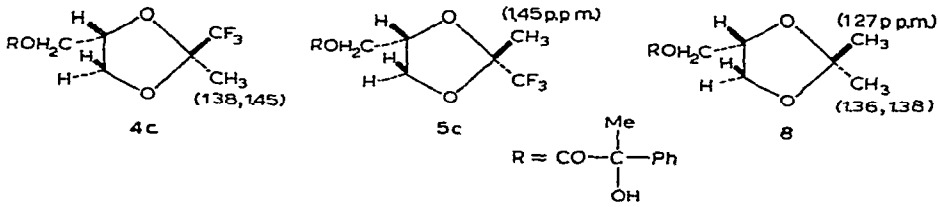
Compound	*Chemical shifts*[a]					*Coupling constants*[a]		
	H-4	*H-5*	*H-5'*	*CH_3*	*CF_3*	$J_{4,5}$	$J_{4,5'}$	$J_{5,5'}$
4				1.51	+7.7			
5				1.50	+8.15			
4a	4.87	4.45	4.35	1.65	+7.82	4.5	7.3	8.5
				(1.55)[b]		[$J_{CF_3,H-5}$ 1.1][c]		
5a	4.83	4.48	4.22	1.55	+7.12	7.5	7.5	8.2
				(1.30)[b]		[$J_{CF_3,H-5}$ 1.6, $J_{CF_3,H-5'}$ 1.1]		
4b	4.80	4.38	4.28	1.63				
				(1.55)[b]				
5b	4.77	4.43	4.23	1.55				
				(1.30)[b]				

[a]Proton chemical shifts in p.p.m. from tetramethylsilane in $CDCl_3$ ^{19}F chemical shifts in p.p.m. from trifluoroacetic acid in $CDCl_3$. Coupling constants in Hz. [b]Figures in parentheses are shift values in benzene [c]No accurate value for the $J_{CF_3,H-5}$ coupling constant was measurable, but it was not greater than 2 Hz.

equivalent and at appreciably lower field than in **3**, and similarly the CF_3 group in **4** was at lower field than in **5**. However, the $-CO_2R$ groups exhibited the expected deshielding effect, and the CF_3 signals in **5a** and **5b** were at lower field than in **4a** and **4b**. No explanation is at present offered to account for these effects.

Any doubt cast on the validity of the above structural assignments by the ambiguity of the ^{19}F n.m.r. studies was dispelled by the following investigations. The 2,3-*O*-(trifluoromethyl)ethylidene-D-glycerols were envisaged as potential agents for assessing the optical purity, by n.m.r. techniques[8], of stereoselectively synthesised 2-alkyl-2-hydroxy-2-phenylacetic acids. However, the general utility of the method for measuring enantiomeric ratios, which involves conversion of enantiomers into diastereoisomers by reaction with optically pure, derivatising agents, not only necessitates the availability of such pure agents but also requires that the agents should preferably give rise to strong single or double peaks which do not overlap with signals due to other portions of the molecule. It is not easy to satisfy these requirements, and the observation that the diastereoisomeric esters (**8**) from racemic atrolactic acid and the readily available, optically pure 2,3-*O*-isopropylidene-D-glycerol showed a chemical-shift nonequivalence of 2 Hz for the low-field isopropylidene methyl signals (at 1.36 and 1.38 p.p.m.) therefore prompted the investigation of 2,3-*O*-(trifluoromethyl)-ethylidene-D-glycerols as optically pure, derivatising agents for chiral acids. It was found that in 2.3-*O*-(*S*)-(trifluoromethyl)ethylidene-D-glycerol (*R.S*)-atrolactate (**4c**, formed from **4** and racemic atrolactic acid), the acetal methyl group showed two signals at 1.38 and 1.45 p.p.m., whereas 2,3-*O*-(*R*)-(trifluoromethyl)ethylidene-D-glycerol (*R*, *S*)-atrolactate (**5c**, formed from **5** and racemic atrolactic acid) showed only one acetal methyl signal. This evidence was a strong indication of the validity

of the structural assignments to **4** and **5**, and hence to **1** and **3**. Unfortunately, the CF_3 group in **5c** did not show the hoped for non-equivalence, and so **5** has no real utility as a derivatising agent.



The tentative chemical-shift assignments to the ring protons in **4a** and **5a** (Table II) were made in the following manner. The low-field signal is reasonably assigned to H-4 because of the deshielding effect of the carboxy substituent, and because the higher-field protons show the common, geminal, coupling constants (provided the geminal couplings are larger than vicinal couplings in 1,3-dioxolanes, and there is no evidence to the contrary[3]). The high-field, ring-proton signal was assigned to H-5′, since it has been established that 4-alkyl or 4-carboxy substituents shield *cis*-vicinal protons[3]. On the basis of these assignments, in both **4a** and **5a**, H-5 and H-5′ are coupled similarly to CF_3, but no coupling between CF_3 and H-4 was detected. The approximately equal coupling of CF_3–H-5 and CF_3–H-5′ is not surprising, since it has been shown recently that if the proximity requirements for long-range coupling for one proton of a geminal pair are satisfied, then the apotropic geminal proton will also show long-range coupling[9]. However, it is surprising that no CF_3–H-4 coupling was detected in either isomer, but whether this is a direct consequence of the 4-substituent[10] or reflects some other aspect of the coupling mechanism is uncertain.

Attempts to provide chemical evidence for the structure of the *O*-(trifluoromethyl)ethylideneglycerols were unsuccessful. For certain 4-hydroxymethyl-1,3-dioxolanes, it is possible to promote intramolecular alkylation of the 4-hydroxymethyl group by a *cis*-oriented bromomethyl group on C-2, but no synthesis of 2-bromomethyl-4-hydroxymethyl-2-trifluoromethyl-1,3-dioxolanes was possible. A variety of conditions[11] were investigated for the acid-catalysed condensation of glycerol and 1-bromo-3,3,3-trifluoropropan-2-one, but no evidence for dioxolane formation was obtained. It was also not possible to confirm a report that 2-bromomethyl-4-hydroxymethyl-2-methyl-1,3-dioxolane was obtained by bromination of the isopropylidene acetal of glycerol with bromine or *N*-bromosuccinimide in carbon tetrachloride or pyridine[12]. In our hands, bromination of isopropylidene or (trifluoromethyl)ethylidene acetals of glycerol gave a multiplicity of products, but in no case was there any n.m.r. evidence for substitution of the 2-methyl substituent.

It is of interest to compare the physical constants of isomers[1] *A* and *B* with those for isomers **1**, **2**, and **3**. Isomer *A* appears to correspond closely with **2**, and this similarity is supported by the fact that we were also able to obtain **2** by fractional

crystallisation (after repeated crystallisation, traces of **1** and **3** were still chromatographically detectable, and homogeneous **2** was only obtained by chromatography). Isomer *B* appears to correspond most closely with isomer **1**. However, the conclusion that isomers *A* and **2** are the same and *B* and **1** are the same is not supported by the previous evidence that in *A* both acetal carbon atoms had the same configuration, and in *B* the acetal carbon atoms differed in configuration.

EXPERIMENTAL

Thin-layer chromatography was performed on microscope slides coated with Merck Silica Gel G, and column chromatography was performed with Merck Silica Gel of particle size 0.05–0.2 mm. The chromatoplates were developed with 50% sulphuric acid and/or iodine vapour. P.m.r. spectra were measured with a JEOL, JNM-4-H-100 n.m.r. spectrometer at 100 MHz, and ^{19}F spectra were obtained at 94 MHz. Acetylations were carried out with acetic anhydride in pyridine at room temperature.

Reaction of D-*mannitol with* 1,1,1-*trifluoroacetone.* — 1,1,1-Trifluoroacetone (3.2 ml) was added to a cooled solution of D-mannitol (3 g) in conc. sulphuric acid (11 ml). The solution was stirred for 48 h at room temperature, poured into water (200 ml), and extracted with ether (5 × 50 ml). The extract was washed with aqueous sodium hydrogen carbonate, dried ($MgSO_4$), and concentrated. The crude product was chromatographed over silica gel with 1:9 acetone–light petroleum (b.p. 60–80°). and the eluate was examined by t.l.c. in 3:7 acetone–light petroleum (b.p. 60–80°); R_F values were measured in this solvent. The components with R_F values higher than 0.75 [presumably tris-*O*-(trifluoromethyl)ethylidene-D-mannitols] were not separated or further investigated. Three crystalline 1,2:5,6-bis-*O*-(trifluoromethyl)ethylidene-D-mannitols were isolated:

Isomer **1** (R_F 0.72) was 1,2(*S*):5,6(*S*)-bis-*O*-(trifluoromethyl)ethylidene-D-mannitol (0.45 g, 7%), m.p. 82–83° (from ethanol or light petroleum), $[\alpha]_D$ +1° (*c* 3, ethanol). Isomer **1** gave a *diacetate*, m.p. 131–132° (from ethanol), $[\alpha]_D$ +18° (*c* 1, chloroform). Isomer **1** appears to correspond with isomer B^1, m.p. 82–83°, $[\alpha]_D$ +2.2° (*c* 0.92, chloroform), which gave a diacetate, m.p. 131–132°, $[\alpha]_D$ +19.7° (*c* 6.9, chloroform).

Isomer **2** (R_F 0.68) was 1,2(*R*):5,6(*S*)-bis-*O*-(trifluoromethyl)ethylidene-D-mannitol (0.45 g, 7%), m.p. 124–125° [from light petroleum (b.p. 60–80°)], $[\alpha]_D$ +10° (*c* 2.5, ethanol). Isomer **2** gave a diacetate, m.p. 95° (from ethanol), $[\alpha]_D$ +26° (*c* 1.5, chloroform). Isomer **2** appears to correspond with isomer A^1, m.p. 128–129°, $[\alpha]_D$ +12° (*c* 2.5, ethanol), which gave a diacetate, m.p. 91–93°, $[\alpha]_D^{13}$ +28° (*c* 2.65, chloroform).

Isomer **3** (R_F 0.64) was 1,2(*R*):5,6(*R*)-bis-*O*-(trifluoromethyl)ethylidene-D-mannitol (0.35 g, 5%), m.p. 121–122° (from ethanol), $[\alpha]_D$ +10° (*c* 2.0, ethanol) (Found: C, 39.72; H, 4.09. $C_{12}H_{16}F_6O_6$ calc.: C, 38.93; H, 4.36%). Acetylation of isomer **3** yielded a diacetate, m.p. 94° (from ethanol), $[\alpha]_D$ +36° (*c* 2.5. chloroform) (Found: C, 42.43; H, 4.29. $C_{16}H_{20}F_6O_8$ calc.: C, 42.30; H, 4.44%).

Oxidation of cis,trans-*1,2-O-(trifluoromethyl)ethylidene-*DL-*glycerol.* — A mixture of conc. sulphuric acid (10 ml) and water (10 ml) was added dropwise to a cooled solution of *O*-(trifluoromethyl)ethylidene-DL-glycerol (10 g, prepared by reaction between glycerol and 1,1,1-trifluoroacetone in sulphuric acid) and sodium dichromate (10 g) in water. The reaction was monitored by t.l.c. (ether–light petroleum, 1:1), and stirring was continued until no *O*-(trifluoromethyl)ethylidene-DL-glycerol remained. The reaction mixture was diluted with water and extracted with ether. The extract, which contained *cis,trans-O*-(trifluoromethyl)ethylidene-DL-glycerol *cis, trans-O*-(trifluoromethyl)ethylidene-DL-glycerate, and *cis,trans-O*-(trifluoromethyl)-ethylidene-DL-glyceric acid, was concentrated, and hydrolysed with aqueous, methanolic potassium hydroxide. The hydrolysate was diluted with water, and extracted with ether. The aqueous solution was acidified and extracted with ether, and the extract was dried and concentrated. Chromatographic resolution of the residue over silica gel with ether–light petroleum (9:1, containing 1% of trifluoroacetic acid) yielded the *cis* and *trans* acids which were crystallised from light petroleum to give *trans-O*-(trifluoromethyl)ethylidene-DL-glyceric acid, m.p. 75° (R_F 0.4) (Found: C, 36.4, H, 3.8. $C_6H_7F_3O_4$ calc.: C, 36.0; H, 3.5%), and *cis-O*-(trifluoromethyl)ethylidene-DL-glyceric acid, m.p. 58° (R_F 0.33).

*Preparation of 2,3-*O-(S)-*(trifluoromethyl)ethylidene-*D-*glycerol and* -D-*glyceric acid* (**4a**) *from* **1**. — A solution of sodium periodate (3 g) in water was added to a solution of **1** (2.56 g) in 80% aqueous ethanol, and the mixture was stored for 10 min. The solution was diluted with water and thoroughly extracted with ether, and the extract was dried, concentrated, and cautiously added to an excess of lithium aluminium hydride in ether. Excess of reductant was destroyed with ethyl acetate and the resulting alkoxides with water, and the solution was filtered, dried, and concentrated. The residue was distilled to yield 2,3-*O*-(*S*)-(trifluoromethyl)ethylidene-D-glycerol, b.p. 68°/3mmHg, $[\alpha]_D$ −2° (*c* 9, chloroform), which was oxidised by the procedure described for the *cis,trans* mixture to yield 2,3-*O*-(*S*)-(trifluoromethyl)ethylidene-D-glyceric acid (**4a**; 1.09 g, 39%), m.p. 54° (from light petroleum), $[\alpha]_D$ ~0° (*c* 2, chloroform), which was spectroscopically indistinguishable from its racemic analogue.

*Preparation of 2,3-*O-(R)- *and* -(S)-*(trifluoromethyl)ethylidene-*D-*glyceric acid from* **2**. — Sequential application of periodate oxidation, lithium aluminium hydride reduction, and chromic acid oxidation to **2** (6.9 g), as described for **1**, produced a mixture of D-glyceric acid derivatives which was resolved chromatographically over silica gel (9:1 ether–light petroleum, containing 1% of trifluoroacetic acid) to yield 2,3-*O*-(*S*)-(trifluoromethyl)ethylidene-D-glyceric acid (1.4 g, 19%), m.p. 54°, and the (*R*)-acid (1.85 g, 25%), which was crystalline but difficult to free from trifluoroacetic acid; variable melting points were obtained.

*Preparation of 2,3-*O-(R)-*(trifluoromethyl)ethylidene-*D-*glycerol and* -D-*glyceric acid from* **3**. — Successive treatment of **3** with periodate and lithium aluminium hydride yielded 2,3-*O*-(*R*)-(trifluoromethyl)ethylidene-D-glycerol (**5**), $[\alpha]_D$ +21° (*c* 7, chloroform). Chromic acid oxidation of **5** yielded 2,3-*O*-(*R*)-(trifluoromethyl)ethylidene-D-glyceric acid (**5a**), $[\alpha]_D$ +31° (*c* 4, chloroform).

Formation of esters **4c**, **5c**, *and* **8**. — 2,3-*O*-Isopropylidene-D-glycerol (*R*,*S*)-atrolactate (**8**) was formed by the sodium methoxide-catalysed transesterification of methyl (*R*,*S*)-atrolactate with 2,3-*O*-isopropylidene-D-glycerol in benzene. Similar transesterification of methyl (*R*,*S*)-atrolactate with **4** or **5** was unsuccessful, and consequently the following procedure was adopted. Racemic 2-benzyloxy-2-phenylpropionic acid was converted into its acid chloride by treatment with $SOCl_2$ in dry carbon tetrachloride containing a small proportion of *N*,*N*-dimethylformamide. The glycerol derivatives **4** and **5** were treated with this acid chloride in carbon tetrachloride containing pyridine. The products (R_F 0.5) were purified by chromatography in 1:3 ether–light petroleum and hydrogenolysed in methanol over palladium-on-charcoal to yield **4c** and **5c** as chromatographically homogeneous syrups (R_F 0.2, ether–light petroleum, 1:3).

ACKNOWLEDGMENTS

The authors thank Miss C. Stanley for measuring the ^{19}F n.m.r spectra and some of the p.m.r. spectra.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

ANOMALOUS REACTION OF D-GLUCONAMIDE WITH BENZOYL CHLORIDE IN ANHYDROUS PYRIDINE

JORGE O. DEFERRARI, ROSA M. DE LEDERKREMER*, BETTY MATSUHIRO, AND MARTA I. LITTER

Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Perú 222, Buenos Aires (Argentina)

(Received January 6th, 1970)

ABSTRACT

The reaction of D-gluconamide with benzoyl chloride and pyridine gave a complex mixture from which ethyl or methyl 2,3,5,6-tetra-*O*-benzoyl-D-gluconate (**5** and **6**) crystallized after treatment with ethanol or methanol, respectively. These esters were formed by alcoholysis of 2,3,5,6-tetra-*O*-benzoyl-D-glucono-1,4-lactone (**4**), which was isolated by column chromatography, together with penta-*O*-benzoyl-D-gluconononitrile (**7**), penta-*O*-benzoyl-D-gluconamide (**8**), *N*-benzoyl-penta-*O*-benzoyl-D-gluconamide (**9**), and benzamide when an excess of benzoyl chloride was used. The isolation of **4** and benzamide was explained by the formation and hydrolysis of a benzoylated imino-lactone intermediate (**3**).

INTRODUCTION

Benzoylation of the four-carbon amides erythronamide[1,2] and threonamide[3,4] with benzoyl chloride and pyridine afforded the corresponding tri-*O*-benzoylaldonamides in high yield. However, treatment of the six- and seven-carbon aldonamides with the same reagents did not lead to the expected per-*O*-benzoylaldonamides, but gave a complex mixture of products[5,6]. The benzoylation of D-gluconamide has now been studied in detail.

RESULTS AND DISCUSSION

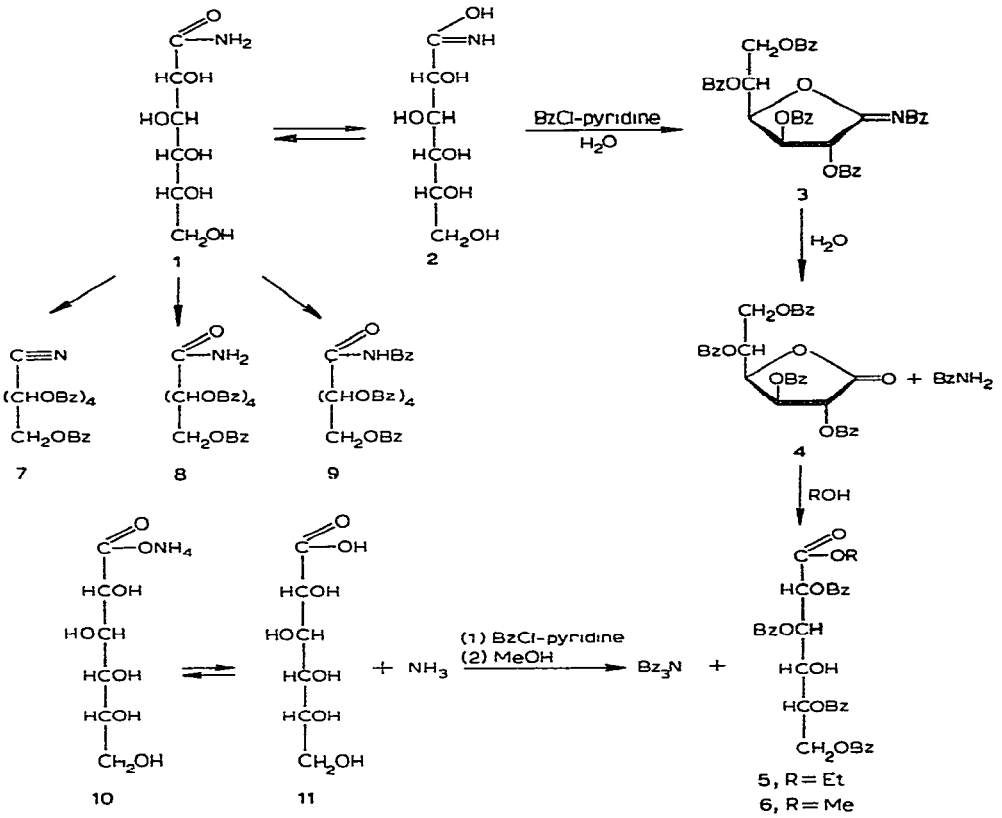
D-Gluconamide (**1**) was benzoylated in anhydrous pyridine with a slight excess of benzoyl chloride, as described by Jelinek and Upson[1]. When the benzoylation product was dissolved in warm ethanol and the solution was kept for a few days, ethyl 2,3,5,6-tetra-*O*-benzoyl-D-gluconate (**5**) crystallized. Treatment with methanol instead of ethanol afforded the methyl ester (**6**). These partially benzoylated esters were identical with the products obtained by (ethyl or methyl) alcoholysis of tetra-

*Research Member of the Consejo Nacional de Investigaciones Científicas y Técnicas

O-benzoyl-D-glucono-1,4-lactone, and their structure was confirmed by n.m.r. spectroscopy[7].

Benzoylation of **1** in chloroform with an excess of benzoyl chloride and pyridine afforded penta-*O*-benzoyl-D-gluconononitrile (**7**) as the main product, together with penta-*O*-benzoyl-D-gluconamide (**8**), *N*-benzoyl-penta-*O*-benzoyl-D-gluconamide (**9**), tetra-*O*-benzoyl-D-glucono-1,4-lactone (**4**), and benzamide. This mixture was separated by column chromatography on silica gel.

It is important to recrystallize amide **1** from an anhydrous solvent. D-Gluconamide was purified by recrystallization from 2-methoxyethanol as recommended by Wolfrom and co-workers[8]. Recrystallization from 90% ethanol, as recommended by Weerman[9], gave a mixture (of D-gluconamide and ammonium D-gluconate) having the same melting point as the pure amide. In one experiment, D-gluconamide recrystallized from aqueous ethanol was benzoylated, and, after treatment of the reaction mixture with methanol, tribenzamide was isolated, together with **6**. We proved that these products were formed from ammonium D-gluconate (**10**) by benzoylation of the pure salt under the same conditions.



A course of reaction that could explain the formation of esters **5** and **6** is: D-gluconamide (**1**), in pyridine, would be in equilibrium with the imino form (**2**)

which, under the anhydrous conditions of benzoylation, would cyclize with loss of the elements of water to give the benzoylated iminolactone **3**. The *N*-benzoyl-2,3,5,6-tetra-*O*-benzoyl-D-glucono-1,4-iminolactone (**3**) would be hydrolyzed on pouring the mixture into water, to give lactone **4** and benzamide. Alcoholysis of **4** would yield **5** or **6**.

It is interesting that there is a correlation between the hydrolytic stability of the aldonamides and the results of the reaction with benzoyl chloride and pyridine. Wolfrom and co-workers[8,10] found that D-gluconamide and other aldonamides containing six (or five) carbon atoms undergo "mutarotation" in aqueous solution, but that the amides of the aldotetronic acids[4] are stable to hydrolysis, and they postulated the formation of a 1,5-lactone intermediate to explain the hydrolysis of five- and six-carbon aldonamides in contrast to the stability of the four-carbon aldonamides in solution. In our case, the experimental facts indicate the formation of a 1,4-cyclic intermediate. The fact that benzoylation of tetronamides[1–4] affords the *O*-benzoylated amides in high yield could be explained by a fast benzoylation of the primary hydroxyl group that would prevent interaction with the 1-hydroxyl groups of the iminohydrin form to give the 1,4-iminolactone intermediate.

EXPERIMENTAL

General. — Melting points were determined with a Thomas–Hoover apparatus (Arthur H. Thomas Co., Philadelphia, Pennsylvania) and are uncorrected. Specific rotations were measured with a 1-dm tube. Infrared spectra were recorded with a Perkin–Elmer Infracord Spectrophotometer. Thin-layer chromatography (t.l.c.) was performed on Silica Gel G (E. Merck, Darmstadt, Germany), with detection with iodine vapor or by spraying with 5% ethanolic sulfuric acid followed by heating for 10 min at 110–120°. Column chromatography was performed with Silica Gel Davison; grade 923, 100–200 mesh.

Preparation of D-*gluconamide* (**1**). — D-Gluconamide (**1**) was prepared by treating D-glucono-1,5-lactone with concentrated ammonium hydroxide (sp. gr. 0.880), with subsequent precipitation by ethanol[11]. It was purified by recrystallization from 2-methoxyethanol, m.p. 142–143°, $[\alpha]_D^{25}$ +30.9° (*c* 1.8, water), in agreement with the reported values[8].

Reaction of D-*gluconamide with benzoyl chloride in anhydrous pyridine.* — *A. Isolation of penta-*O*-benzoyl-*D*-glucononitrile* (**7**), *tetra-*O*-benzoyl-*D*-glucono-1,4-lactone* (**4**), N-*benzoyl-penta-*O*-benzoyl-*D*-gluconamide* (**9**), *penta-*O*-benzoyl-*D*-gluconamide* (**8**), *and benzamide.* D-Gluconamide (2 g) was added slowly, with stirring, to a solution of benzoyl chloride (13 ml) and pyridine (20 ml) in chloroform (24 ml), cooled to 0°. After being kept overnight at room temperature, the mixture was diluted with an equal volume of chloroform; the solution was successively washed with 0.5 M sulfuric acid, water, half-saturated sodium hydrogen carbonate, and water, dried (sodium sulfate), and evaporated under diminished pressure. The resulting syrup

(6.6 g) showed several spots on t.l.c. with 1:4 (v/v) ethyl acetate–benzene. Part (2 g) of the product was dissolved in benzene and chromatographed on a column (700 × 35 mm) of silica gel, and 15-ml fractions were collected. Elution was effected with benzene (2 liters) and then with 1:49 (v/v) ethyl acetate–benzene (15 liters). From fractions 180–200, **7** crystallized upon evaporation; yield 0.520 g (24.4%), m.p. and mixed m.p. 116–117°, $[\alpha]_D^{20}$ +16.4° (*c* 1, chloroform); lit.[12] m.p. 118°, $[\alpha]_D$ +15.1°. Fractions 201–240 gave 0.200 g of a mixture. Fractions 300–400 afforded 0.370 g (21%) of **4** as an amorphous solid, $[\alpha]_D^{20}$ +44.5° (*c* 1, chloroform). The optical rotation, i.r. spectrum, and R_F value (silica gel) were identical with the reported values[7]. Fractions 430–750 afforded 0.200 g (7.6%) of **9** which, after purification from ether–petroleum ether, had m.p. 85–87°, $[\alpha]_D^{20}$ +11.8° (*c* 0.9, chloroform); ν_{max}^{Nujol} 3250 (secondary amide), 1720 (benzoyl carbonyl), and 1690 cm^{-1} (amide carbonyl).

Anal. Calc. for $C_{48}H_{37}NO_{12}$: C, 70.33; H, 4.51; N, 1.71. Found: C, 70.63; H, 4.50; N, 2.09.

Elution with 1:19 (v/v) ethyl acetate–benzene afforded, from fractions 1550–1770, 0.30 g (14%) of **8**. Recrystallization from ethanol gave the pure product, m.p. and mixed m.p. 101–102°, $[\alpha]_D^{20}$ +30.2° (*c* 1, chloroform) in agreement with the literature values[13]. Fractions 1790–2200 gave 0.090 g (25%) of benzamide that was purified by sublimation; m.p. and mixed m.p. 126–127°.

*B. Formation of ethyl 2,3,4,6-tetra-*O*-benzoyl-*D*-gluconate* (**5**). To a suspension of D-gluconamide (2 g) in pyridine (15 ml) at 0° was added benzoyl chloride (6.5 ml). After being kept overnight, the mixture was poured into ice–water. The oil formed was washed several times with water, and extracted with chloroform. The extract was washed with water, dried (sodium sulfate), and evaporated under diminished pressure. The residue was dissolved in warm ethanol, and after the solution had been kept for a few days, crystalline product was formed. The crystals were filtered off, and, on being kept, the mother liquor afforded more product. Total yield 0.500 g (13.3%) in eight crops. Recrystallization from 3:2 (v/v) acetone–ethanol gave pure **5**, m.p. and mixed m.p. 174–175°, $[\alpha]_D^{20}$ +30.5° (*c* 1, chloroform), in agreement with the reported values[7]. From the mother liquors, 0.100 g of **7** was obtained. Recrystallization from ethanol afforded the pure product; m.p. and mixed m.p. 116–117°. T.l.c. of the mother liquors, with 1:4 (v/v) ethyl acetate–benzene as developer, showed the presence of **5**, R_F 0.56; **8**, R_F 0.29, and material at the starting line. Benzamide, R_F 0.27, was detected by using 1:1 (v/v) ethyl acetate–benzene as the developer.

*C. Formation of methyl 2,3,4,6 tetra-*O*-benzoyl-*D*-gluconate* (**6**). D-Gluconamide (1 g) was treated with pyridine and benzoyl chloride as described under *B*. The resulting product was dissolved in warm methanol and, after a few days, the crystals that formed were filtered off. From the mother liquors, more product was obtained; total yield 0.550 g (17.2%). Recrystallization from benzene afforded pure **6**, m.p. and mixed m.p. 182–183°, $[\alpha]_D^{20}$ +35.5° (*c* 1, chloroform), in agreement with the reported values[7]. T.l.c. of the mother liquors showed the presence of **7**, **8**, and benzamide.

Reaction of ammonium D*-gluconate with benzoyl chloride and pyridine.* — To a solution of benzoyl chloride (8 ml) and pyridine (12 ml) in chloroform (15 ml), cooled

to 0°, ammonium D-gluconate (1 g) was slowly added, with stirring. After being kept overnight, the mixture was diluted with chloroform, and the solution was successively washed with 0.5 M sulfuric acid, water, half-saturated sodium hydrogen carbonate solution, and water, dried (sodium sulfate), and evaporated under diminished pressure. Tribenzamide was obtained by treating the residue with methanol; yield 0.950 g (64%), m.p. and mixed m.p. 203–204°. After several days, **6** was obtained from the mother liquors; yield 1.300 g (44%).

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

KETO GROUPS IN CELLULOSE AND MANNAN OXIDIZED BY DINITROGEN TETROXIDE

C. MERCER AND H. I. BOLKER*

Organic Chemistry Section, Pulp and Paper Research Institute of Canada, and Department of Chemistry, McGill University, Montreal (Canada)

(Received April 14th, 1969; in revised form, January 16th, 1970)

ABSTRACT

Mannan B and cellulose were oxidized with nitrogen tetroxide to transform the C-6 hydroxyls into carboxyl groups. In both products keto groups had formed on secondary carbons in side-reactions. After reduction of the oxidized polysaccharides with sodium borohydride-t_4, determination of the radioactivity indicated that the ratio of keto groups in oxycellulose to those in oxymannan was 2:1. Comparison of the count of radioactivity with that of diphenylmethanol-t_2 prepared from a similarly reduced sample of diphenyl ketone showed that the oxymannan contained one keto group for every 10 anhydro-D-mannose units.

INTRODUCTION

The oxidation of mannan with nitrogen tetroxide has not hitherto been reported. By analogy with the oxidation of cellulose[1], the main reaction of mannan should be the nearly complete conversion of primary hydroxyls at C-6 into carboxyl groups, with simultaneous oxidation of some secondary hydroxyls to keto groups. The precise degree and course of the latter reaction in cellulose is not known, hence the effect of configurational differences in mannan cannot be predicted.

The purpose of this paper is to describe the oxidation by nitrogen tetroxide of a suspension of mannan B (from ivory nuts) in carbon tetrachloride, and to report the results of measuring the keto content of the oxidized mannan, as well as that of oxidized celluloses, by a novel technique. The method was based on reduction of the oxidized polysaccharides by sodium borohydride-t_4.

DISCUSSION

In one experiment, samples of oxidized mannan B and commercial oxycellulose ("Oxycel", Parke, Davis and Co.) were reduced with equal aliquots of the same

*To whom enquiries should be addressed.

sample of sodium borohydride-t_4. After precipitation, washing, and drying of the polyuronic acids, they were burned in Schöniger flasks and the activity of the water collected was determined. The ratio of activities of reduced oxycellulose to reduced oxymannan was almost exactly 2:1.

In a second experiment, the sodium borohydride-t_4 had a specific activity different from that of the sample used in the first, and equal aliquots of it were employed to reduce four different materials. The first two materials were the same oxycellulose and oxymannan that had been used in the first experiment, and the ratios of activities found confirmed the previous results. The third material was an independently prepared sample of oxycellulose (from cellulose regenerated from its acetate), and the specific activity after reduction agreed with that of the reduced commercial oxycellulose. Thus, it appears that oxidation of cellulose with nitrogen tetroxide results in the formation of twice as many keto groups (per monomer unit) as the oxidation of mannan B.

The possibility was considered that if the aldehyde end-groups had not been oxidized during isolation of the polysaccharides, or later by nitrogen tetroxide, they might interfere in the measurement, and their presence might make the results dependent upon the molecular weights of the cellulose and mannan, respectively. In model experiments with 2,3,4,6-tetra-*O*-methyl-D-mannose and 2,3,4,6-tetra-*O*-acetyl-D-glucose it was found that nitrogen tetroxide oxidized both compounds completely to carboxylic acids. Although the final products were not positively identified, it seems a reasonable presumption that they were the corresponding derivatives of D-mannonic and D-gluconic acids. Similar oxidation of end-groups probably occurred in the polymers, and rendered them unreducible by sodium borohydride.

In the second reduction experiment, the fourth substance reduced by an aliquot of sodium borohydride-t_4 was diphenyl ketone. The reduction was not as complete as might be desired, thus rendering the product difficult to separate from the starting material. However, separation was achieved, and the purified diphenylmethanol-t_2 having a tritium content proportional to a known weight of carbonyl group, provided a standard from which the absolute content of the original polyuronic acids could be calculated. The oxidized mannan contained one keto group per ten D-mannuronic residues, whereas the oxidized cellulose had one keto group per five D-glucuronic acid residues.

The low yield of diphenylmethanol-t_2 apparently did not affect the results, since the ratio found for oxycellulose agreed with the value of 18:100 reported by Kaverzneva *et al.*[2,3], who had determined it by the Benedict's reagent and hydroxylamine methods.

There are at least two possible reasons for the degree of oxidation of secondary alcohol groups by nitrogen tetroxide in cellulose and mannan to differ. One is that the reaction being heterogeneous, its course may be affected by the secondary structures of these polymers. The second possibility is that oxidation of alcohols by nitrogen tetroxide may occur through the formation of a nitrite ester intermediate[4], and may

be controlled by steric requirements so that the oxidizing species can only enter at an unhindered site; consequently, equatorial hydroxyl groups at both C-2 and C-3 (as in cellulose) must represent less hindrance than a combination of an axial hydroxyl group at C-2 and an equatorial hydroxyl at C-3 (as in mannan). This second possibility will be investigated, in future experiments, by the oxidation of suitable model compounds.

EXPERIMENTAL

Extraction of ivory nut mannan. — The mannans of ivory nuts were extracted according to the method of Aspinall *et al.*[5]. Only mannan B was used for our experiments.

Regeneration of cellulose from cellulose acetate. — Commercial cellulose acetate (300 g) was suspended in a solution of sodium hydroxide (320 g) and sodium chloride (50 g) in methanol (8 l) and water (8 l) and stirred for 12 h. The saponified cellulose was separated by filtration and thoroughly washed with water, and then stirred at room temperature in hydrochloric acid (10 l) at pH 3.0 for 4 h. After being filtered off and washed with water, the cellulose was air dried (168 g). The average D.P. (obtained by viscosity measurements in cupriethylenediamine) was 117. A sample of commercial oxycellulose was obtained from Parke, Davis and Co.

Oxidation with dinitrogen tetroxide. — Samples of the mannan B and the regenerated cellulose were dried in a vacuum desiccator over phosphorus pentoxide for several days before use.

Commercial nitrogen tetroxide was dried by passing it through two U-tubes both packed with a mixture of calcium chloride lumps and phosphorus pentoxide, and then was condensed in a preweighed glass pressure bottle cooled in dry ice–acetone. The polysaccharide was added to a solution of the nitrogen tetroxide in dry carbon tetrachloride, and the bottle was removed from the cold bath and stoppered with a P.T.F.E. plug. The bottle was shaken on a wrist-action shaker, and after 24 h the reaction was stopped by flushing out the excess of nitrogen tetroxide with nitrogen. After the solids had been filtered off on preweighed, sintered-glass crucibles, any remaining nitrogen oxides were washed out first with carbon tetrachloride, and then with diethyl ether, both in copious amounts. The ratio of nitrogen tetroxide to carbon tetrachloride to polysaccharide was 15:8:0.57 in all cases.

The carboxyl content of each oxidized product was measured by treating a known weight of the material with calcium acetate (0.1M) for 18 h and titrating the liberated acetic acid with sodium hydroxide (0.01M) in presence of an indicator made up of 1 vol. of Cresol Red and 2 vol. of Thymol Blue.

The carboxyl content of the commercial oxycellulose was not measured, but the mannan B and the regenerated cellulose gave values of 24.1% (by weight) and 25.4% respectively (theoretical, 25.6%) after oxidation with nitrogen tetroxide.

Reduction of oxidized polysaccharides with sodium borohydride-t_4. — In the first experiment, the oxidized mannan B (158.5 mg) and commercial oxycellulose (191.5 mg)

were reduced with sodium borohydride-t_4 (82 mCi/mmole). Sodium borohydride (1.0 g) was dissolved in water (50.0 ml) and allowed to stabilize for a few min. This was done so that a minimum of tritium would be lost when the sodium borohydride-t_4 was dissolved in the solution. (It was found convenient to break the ampoule containing the tritide and to add the glass and its content to the sodium borohydride solution.) In the second experiment the oxidized mannan B (90.8 mg), the commercial oxycellulose (70.8 mg), the oxidized regenerated cellulose (99.0 mg) and diphenyl ketone (49.9 mg) were reduced with sodium borohydride-t_4 (722 mCi/mmol). In both experiments each sample to be reduced was placed in a three-necked pear-shaped Quickfit flask (25 ml), fitted with a dropping funnel, an air-driven stirrer, and a glass stopper. The polysaccharide samples were suspended in distilled water (5 ml) and the diphenyl ketone was dissolved in methanol (5 ml). An aliquot (12.5 ml) of the reducing solution was placed in each dropping funnel and the reactions were started by adding several drops of the sodium borohydride-t_4 solution, followed by a dropwise addition over the course of 1 h. The walls of the funnels were carefully rinsed with water (2.5 ml) and the washings were added to the flasks. The reaction was stopped after a total of 6 h by slowly adding dilute acetic acid (25%) to destroy any excess of sodium borohydride-t_4. Each solution was allowed to equilibrate overnight and then was poured into cold methanol (0–5°). The reduced polysaccharides precipitated and coagulated after about 2 h. The products were isolated by filtration on preweighed sintered-glass crucibles, and were washed thoroughly with dry methanol, and then diethyl ether.

Since diphenylmethanol is soluble in methanol, it could not be precipitated in the same way. Instead, very dilute hydrochloric acid (at about 5°) was added to the solution after the sodium borohydride-t_4 had been destroyed. The diphenylmethanol separated out, was filtered off on a sintered-glass crucible, and dried over phosphorus pentoxide. The product, m.p. 55°, was deposited on a thin-layer plate (Silica Gel G) and eluted with 2:1:2 benzene–ethyl acetate–petroleum ether (65–110°). Two spots having the same mobilities as authentic diphenyl ketone and diphenylmethanol confirmed that the product contained some starting material. The diphenylmethanol was separated on a column of silica gel (0.05–0.2 mm) by elution with the same eluent, and was obtained in poor yield as delicate needles, m.p. 66°.

Combustion of reduced samples and determination of their activities. — The combustion of the samples was based on the method of Kelly *et al.*[6] using Schöniger flasks. The activities of the tritiated water samples were measured with a scintillation counter (Packard Instrument Co., Inc.), using a scintillation solution made by dissolving 1,4-bis-2-(5-phenyloxazolyl)-benzene (250 mg), 2,5-diphenyloxazole (10 g) in *p*-dioxane (Eastman 2144, 770 ml) and adding ethanol (230 ml) and naphthalene (200 g). An internal standard was made by diluting tritiated toluene (5 ml) (2.39×10^6 c.p.m./ml) to 100 ml with scintillation-grade toluene. The counts, after correction for quenching effects, gave ratios of 2:1 for cellulose and mannan B; 1.97:1 for regenerated cellulose and mannan B; and 1:1.2 for regenerated cellulose and cellulose, respectively.

Reaction of model sugars with dinitrogen tetroxide. — For oxidation, the 2,3,4,6-tetra-*O*-methyl-D-mannopyranose, obtained by saponification of 1-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-α-D-mannopyranose, and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose were dissolved separately in carbon tetrachloride (10 ml) in small glass-pressure bottles fitted with ground-glass stoppers. Dry nitrogen tetroxide (4 g) was added to each bottle which was then securely stoppered and shaken overnight. The remaining nitrogen oxides were flushed out by a stream of nitrogen; then the carbon tetrachloride was evaporated to give straw coloured syrups. A little of each syrup was dissolved in chloroform and applied to thin-layer silica gel plates and eluted with ethyl acetate. The spots had different mobilities than their precursors. Visualization of the chromatograms was obtained with an ammonium sulfate–sulfuric acid spray, followed by heating for 10–15 min at 110°.

ACKNOWLEDGMENT

We thank Dr. A. S. Perlin for valuable discussion and for the gift of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose and 1-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-α-D-mannopyranose.

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Elsevier Publishing Company, Amsterdam
Printed in Belgium

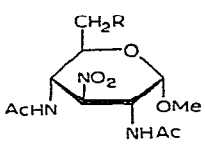
Note

Synthesis of methyl 2,3,4,6-tetraamino-2,3,4,6-tetradeoxy-α-D-glucopyranoside tetrahydrochloride

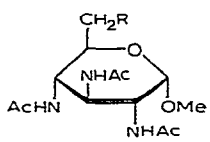
HANS H. BAER AND MONIKA BAYER
Department of Chemistry, University of Ottawa, Ottawa 2 (Canada)

(Received December 2nd, 1969)

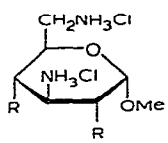
Tetraamino sugars were unknown until quite recently when the first compounds in this class were synthesized by Ali and Richardson[1]. Starting from suitably blocked monoamino sugars, the authors employed the sequence[2] of sulfonylation, displacement by azide, and hydrogenation, for attaching amino groups to C-3, C-4, and C-6 of the sugar chain; configurational inversions were effected at various stages, and derivatives of tetraamino hexoses having the D-*galacto*[1a], D-*ido*[1a], and D-*gluco*[1b] configurations were obtained. Methyl 2,3,4,6-tetraacetamido-2,3,4,6-tetradeoxy-α-D-glucopyranoside (**8**) was the final product of one of these syntheses, although in this particular instance the route was stated [1b] to be tedious. Having recently described[3] a convenient synthesis of methyl 2,4-diacetamido-2,3,4-trideoxy-3-nitro-α-D-glucopyranoside (**1**) by nucleophilic amination of a 3-deoxy-3-nitro sugar, we now record the conversion of **1** and the 2,3,4,-triacetamido analog[3] (**2**) into the tetraacetamido derivative **8** and the title compound **9**.



1 R = OH
3 R = OMs
5 R = N_3



2 R = OH
4 R = OMs
6 R = N_3
8 R = NHAc



7 R = NHAc
9 R = NH_3Cl

Both the nitro sugar **1** and the triacetamido derivative **2** were readily mesylated. The 6-methanesulfonates **3** and **4** were converted by sodium azide into the 6-azido-6-deoxy glycosides **5** and **6**, respectively. Such displacements of primary sulfonyloxy groups with azide ion have been performed[2] by long boiling in aqueous acetone solution, although dipolar, aprotic solvents (methyl sulfoxide, *N*,*N*-dimethylformamide, and recently[1] hexamethylphosphoric triamide) are needed when secondary as well as primary sulfonic esters are the substrates[4]. The present displacements proceeded readily in *water* (3 h on a steam bath), and the azido sugars crystallized spontaneously from the reaction mixtures in good to excellent yields. In the azidolysis of **4** a minor by-product was isolated from the mother liquor and identified as **2**,

indicating that hydrolysis was a side-reaction. Inasmuch as no **1** was observed to arise from **3** under identical conditions, the inference might be made that the acetamido group at C-3 in **4** is capable of transannular assistance in the solvolysis at C-6, although no other evidence to support this assumption is available.

The azidonitro derivative **5** was hydrogenated over platinum in the presence of hydrochloric acid to produce methyl 2,4-diacetamido-3,6-diamino-2,3,4,6-tetradeoxy-α-D-glucopyranoside dihydrochloride (**7**), which was subsequently acetylated to the tetra-*N*-acetyl derivative **8**. The latter was also obtained by similar hydrogenation of **6**, followed by acetylation. The over-all yields of **8** were 47% (from **1**) and 19% (from **2**).

All attempts to hydrolyze the glycosidic bonds in **7** and **8** were unsuccessful. With 6M hydrochloric acid (24 h at 100°), compound **7** was largely hydrolyzed to methyl 2,3,4,6-tetraamino-2,3,4,6-tetradeoxy-α-D-glucopyranoside tetrahydrochloride (**9**), which was isolated as crystals in 88% yield, but no reducing sugar could be detected by means of Fehling and Tollens tests. Similar hydrolysis of **8** resulted in extensive charring, and **9** was obtained in 21% yield only. It is well known that certain amino glycosides are difficult to hydrolyze (see Ref. 4, pp. 92–94), although methyl 2-acetamido-3-amino-2,3-dideoxy-α-D-glucopyranoside hydrochloride was hydrolyzed[3] to give the reducing diamino sugar dihydrochloride in 30% yield.

EXPERIMENTAL*

Methyl 2,4-diacetamido-2,3,4-trideoxy-6-O-(methylsulfonyl)-3-nitro-α-D-glucopyranoside (**3**). — Methyl 2,4-diacetamido-2,3,4-trideoxy-3-nitro-α-D-glucopyranoside[3] (**1**, 2.5 g) in pyridine (35 ml, dried over calcium hydride) was treated with methanesulfonyl chloride (1.25 ml) for 15 h at 5°. The mixture was poured into a large amount of ice–water, which was evaporated to give a residue that crystallized upon trituration with some fresh water. Recrystallization from ethanol gave **3** (2.6 g, 82.5%) as colorless needles, m.p. 221–222° (dec.), $[\alpha]_D^{25}$ +104° (*c* 0.8, water), +90° (*c* 0.8, methanol); i.r. data, 3345 and 3315 (NH), 1666 (amide I), 1553 (NO_2), 1528 (amide II), 1335 and 1165 cm^{-1} (sulfonate).

Anal. Calc. for $C_{12}H_{21}N_3O_9S$ (383.4): C, 37.59; H, 5.52; S, 8.36. Found: C, 37.79; H, 5.64; S, 8.49.

Methyl 2,3,4-triacetamido-2,3,4-trideoxy-6-O-(methylsulfonyl)-α-D-glucopyranoside (**4**). — Methyl 2,3,4-triacetamido-2,3,4-trideoxy-α-D-glucopyranoside[3] (**2**, 1 g) was dissolved (if incompletely) in dry pyridine (60 ml) by gentle warming, and, after cooling of the solution to 0°, methanesulfonyl chloride (1.2 ml) was added, with stirring. The reaction was allowed to proceed for 20 h at 5° and for 5 h at 25°. Work-up as described for **3** gave a first crop (600 mg) of **4** as slightly brownish crystals (from water). A second crop was obtained from the mother liquor, after deionization with Dowex-1 X2 (CO_3^{2-}), treatment with decolorizing carbon, and crystallization from

*For general methods see Ref 3

methanol. The two crops, which gave a single, and identical, spot on t.l.c., were jointly recrystallized from methanol to give **4** (705 mg, 56%) as needles, m.p. 241° (dec.), $[\alpha]_D^{25}$ +105° (*c* 0.6, methanol); i.r. data, 3270 with shoulder at 3310 (NH), 1633 and 1525 (amide I and II), 1330 and 1150 cm^{-1} (sulfonate).

Anal. Calc. for $C_{14}H_{25}N_3O_8S$ (395.4): C, 42.53; H, 6.37; S, 8.11. Found: C, 42.66; H, 6.56; S, 7.99.

Methyl 2,4-diacetamido-6-azido-2,3,4,6-tetradeoxy-3-nitro-α-D-glucopyranoside (**5**). — The mesyl derivative **3** (1.5 g) and sodium azide (1.5 g) were heated in water (12 ml) for 3 h on a steam bath. The colorless solution began to deposit cm-long needles during that period, after which it was concentrated to about 4 ml, and cooled. The product (980 mg, 75%) was collected and washed with cold water; it gave a single spot on t.l.c., and the mother liquor appeared to contain only traces of other organic material (except for NaOMs). Compound **5** decomposed at 234–235° (236° after recrystallization from ethanol) and showed $[\alpha]_D^{25}$ +122° (*c* 0.9, methanol); i.r. data, 3325 and 3300 (NH), 2100 (N_3), 1668 (amide I), 1550–1525 cm^{-1} (NO_2 and amide II).

Anal. Calc. for $C_{11}H_{18}N_6O_6$ (330.3): C, 40.00; H, 5.49; N, 25.45. Found: C, 40.15; H, 5.61; N, 25.37.

Methyl 2,3,4-triacetamido-6-azido-2,3,4,6-tetradeoxy-α-D-glucopyranoside (**6**). — The mesyl derivative **4** (540 mg) and sodium azide (540 mg) were heated in water (6 ml) on a steam bath. Dissolution of **4** was complete within 10 min, and long needles of **6** began to separate within 1 h. T.l.c. with 9:1 chloroform–methanol revealed complete consumption of **4** after 3 h and indicated **6** (R_F 0.3) to be the major product, together with a minor product (R_F 0.1). The cooled reaction mixture was then filtered, and the crystals were washed with cold water and recrystallized from methanol to give **6** (240 mg, 51%), $[\alpha]_D^{25}$ +97° (*c* 0.8, methanol), decomposing above 270°. I.r. data, 3270 (NH), 2085 (N_3), 1640 and 1525 cm^{-1} (amide I and II).

Anal. Calc. for $C_{13}H_{22}N_6O_5$ (342.4): C, 45.61; H, 6.48; N, 24.55. Found: C, 45.76; H, 6.60; N, 24.78.

The aqueous mother liquor of the above reaction was evaporated to dryness. A part (100 mg) of the residue (which was estimated on the basis of t.l.c. to contain **6** and the by-product in a ratio of about 3:1 was chromatographed on two thin-layer plates (20 × 20 cm) of Silica Gel G with 9:1 chloroform–methanol. The slower-moving band was extracted with methanol, and the extract furnished 9 mg of crystalline **2** that darkened above 305° and largely decomposed from 350° (as reported[3]) alone and in admixture with authentic **2**. The i.r. spectrum was superposable on that of **2**.

Methyl 2,4-diacetamido-3,6-diamino-2,3,4,6-tetradeoxy-α-D-glucopyranoside dihydrochloride (**7**). — A suspension of the azidonitro derivative **5** (600 mg) in water (40 ml) was added to a suspension of platinum catalyst (600 mg of PtO_2, prehydrogenated) in water (36 ml) and M hydrochloric acid (4.4 ml), and the mixture was shaken under hydrogen at ordinary temperature and pressure. The reaction was monitored by paper chromatography, which indicated very rapid formation of a product (R_{GN} 2.0, doubtless the 6-amino-3-nitro intermediate) that was slowly converted into the diamine **7** (R_{GN} 0.9) (compare the slow hydrogenation[3] of **1**). After 3 days, the

filtered solution was evaporated with several additions of water and finally with added ethanol and benzene. The resultant, colorless residue crystallized on treatment with boiling ethanol to give fine, slightly hygroscopic needles (580 mg, 93%) of **7**, dec. >285°, $[\alpha]_D^{25}$ +84° (*c* 1, water); i.r. data, 3500–3300 (weak, NH), 2700–2500 and 1900 (broad, weak, NH_3^+), 1660 (amide I), 1550–1500 cm^{-1} (NH_3^+ and amide II).

Anal. Calc. for $C_{11}H_{24}Cl_2N_4O_4$ (347.3): C, 38.04; H, 6.97; Cl, 20.42. Found: C, 37.88; H, 7.11; Cl, 20.64.

Methyl 2,3,4,6-tetraacetamido-2,3,4,6-tetradeoxy-α-D-glucopyranoside (**8**). — *A. From* **7**. The dihydrochloride **7** (330 mg) was *N*-acetylated with acetic anhydride (5 ml) in water (10 ml) and methanol (2 ml) in the presence of Dowex-1X2 (CO_3^{2-}), for 5 h at 0°. The filtered solution was treated with a small amount of Dowex-50 (H^+), filtered, and evaporated with several successive additions of methanol. The product (**8**) was thus obtained as a white powder (275 mg, 81%) that was nearly pure according to t.l.c.; R_F 0.5 in 4:1 chloroform–methanol. Crystallization was difficult because of the unfavorable solubility characteristics and a tendency of the product to appear as a gel from alcohols and alcohol–water mixtures. The analytical sample was obtained as a microcrystalline material (that could be filtered off readily) when **8** was dissolved in a large volume of hot methanol and the solution was concentrated and the inside of the vessel was scratched. The substance did not melt below 360°, and it showed $[\alpha]_D^{25}$ +104° (*c* 0.6, water) [lit.[1b], m.p. >350°, $[\alpha]_D$ +100° (*c* 0.2, water)]; i.r. data, 3270 with shoulder at 3200 (NH), 1640 (amide I), and 1550–1525 cm^{-1} (amide II). An n.m.r. spectrum in D_2O showed a single OCH_3 peak and 4 separate peaks of equal intensity in the N–$COCH_3$ region.

Anal. Calc. for $C_{15}H_{26}N_4O_6$ (358.4): C, 50.28; H, 7.31; N, 15.64. Found: C, 50.15; H, 7.15; N, 15.53.

B. From **6**. The azido glucoside **6** (200 mg) was hydrogenated as described for the hydrogenation of **5**. Work-up after 24 h afforded a chromatographically homogeneous, white solid presumed to be the corresponding 6-amino glycoside hydrochloride (180 mg). This was *N*-acetylated as described for **7**, and the product (140 mg, 67% based on **6**) was identical with **8** by t.l.c., i.r. spectrum, and optical rotation.

Methyl 2,3,4,6-tetraamino-2,3,4,6-tetradeoxy-α-D-glucopyranoside tetrahydrochloride (**9**). — The dihydrochloride **7** (200 mg) was hydrolyzed in half-concentrated hydrochloric acid (12 ml) for 24 h on a steam bath. Most of the acid was then removed by numerous co-evaporations with water. The residue was triturated with about 4 ml of methanol to give a thin gel, which suddenly crystallized on brief heating. After partial concentration of the mother liquor the colorless needles (170 mg, 88%) were isolated and washed with methanol and ether. The compound darkened and partially sublimed above 270°; R_{GN} 0.53; $[\alpha]_D^{25}$ +87° (*c* 0.9, water); i.r. data, 3560 and 3430 (weak, NH), 2700–2200, 1565 and 1485 cm^{-1} (NH_3^+). When recrystallized from acetic acid containing a small amount of water, the compound showed small differences in the i.r. pattern. A 60-MHz n.m.r. spectrum in D_2O showed a sharp 3-proton singlet (OCH_3), 1.28 p.p.m. upfield from the HOD signal, and no signals were present in the acetyl region.

Anal. Calc. for $C_7H_{22}Cl_4N_4O_2$ (336.1): C, 25.01; H, 6.60; Cl, 42.18. Found: C, 24.86; H, 6.90; Cl, 40.65.

Similar hydrolysis (20 h) of **8** (100 mg) gave a dark brown solution and some tar. After removal of excess acid, an aqueous solution of the residue was treated with activated charcoal (acid-washed), and 20 mg of **9** was then obtained, as slightly brownish needles, by crystallization as described above.

ACKNOWLEDGMENT

Financial assistance from the Life Insurance Medical Research Fund, Rosemont, Pennsylvania, is gratefully acknowledged.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

Note

Preparation of sulfate esters. *N,N'*-Dicyclohexylcarbodiimide-mediated sulfation of carbohydrate derivatives

RALPH O. MUMMA, CHARLES P. HOIBERG, AND RENNY SIMPSON

The Pesticide Research Laboratory and Graduate Study Center, The Department of Entomology, and The Department of Biochemistry, The Pennsylvania State University, University Park, Pa 16802 (U. S. A.)

(Received December 22nd, 1969)

INTRODUCTION

Many naturally occurring sugar sulfates are found in plants and animals[1]. Recently, considerable attention has been focused on the physiological role of sulfated polysaccharides[2,3] and on the genetic disease metachromatic leucodystrophy, which is caused by the accumulation of cerebroside sulfate[4]. The synthetic methods most commonly used in preparing sugar sulfates involve the sulfation of a sugar or derivative with chlorosulfonic acid[5] or with sulfur trioxide adducts such as pyridine–sulfur trioxide[6–8] or *N,N*,-dimethylformamide–sulfur trioxide[9].

Recently, in radiochemical investigations, Hoiberg and Mumma[10–12] have shown that various nucleophiles, sulfuric acid and *N,N'*-dicyclohexylcarbodiimide (DCC) react in *N,N*-dimethylformamide ($HCONMe_2$) to produce ^{35}S-labeled products. In these studies only one ^{35}S-labeled product was formed under dilute reaction conditions with isopropylidene acetals of carbohydrates; it was proposed that these products were monosulfate esters. To confirm the proposed structures and to demonstrate the usefulness of this reaction to carbohydrate chemistry, four isopropylidene acetals of sugar sulfates were synthesized. The synthetic procedure is particularly well suited for the preparation of ^{35}S-labeled compounds because of the readily available $^{35}SO_4^{2-}$. The isopropylidene groups can be readily removed by acid to produce the sugar sulfates[6]. Such labeled compounds should prove useful in investigations of metabolism of sugar sulfates in living systems.

RESULTS AND DISCUSSION

The respective isopropylidene acetal, DCC, and sulfuric acid react in *N,N*-dimethylformamide to produce 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose 6-sulfate, 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose 3-sulfate, and 2',3'-*O*-isopropylideneadenosine 5'-sulfate. The reaction is very rapid and goes to completion within

a few min. The yield of sulfated product is dependent upon the order of addition of the reactants (DCC plus alcohol plus sulfuric acid), the solvent, and to some extent upon the water content of the solvent[12]. If sulfuric acid was used in greater than 1.5-molar excess, or if DCC were used in less than a 5-molar excess, the yield of product was decreased. The reaction produces a voluminous, relatively insoluble by-product (*N,N'*-dicyclohexylurea), which can be directly filtered from the solvent; however, under these conditions some of the sulfated product is occluded in the urea precipitate.

The sulfated products were isolated by column chromatography on *O*-(2-diethylamino)ethylcellulose (DEAE-cellulose) and were subsequently converted into the potassium salts on Dowex-50 columns. Potassium salts were prepared because they are generally not as hygroscopic as sodium salts; interpretation of the elemental analyses is thus simplified. In the preparation of ^{35}S-labeled products, the conversion into the potassium salt is not necessary; the sulfated product can be isolated directly by t.l.c. The labile 5,6-*O*-isopropylidene group is partially hydrolyzed from the 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose 3-sulfate during the isolation procedure.

The amount of acetone needed to dissolve the *O*-isopropylidene sugar sulfate is dependent upon the particular compound and the water content of the acetone. Excess acetone should be avoided so that appreciable amounts of ammonium chloride and ammonium sulfate, also eluted from the DEAE-cellulose column, are not dissolved. In the preparation of 2',3'-*O*-isopropylideneadenosine 5'-sulfate it was found necessary to make the reaction mixture basic (pH 9.5–10) so that the 6-amino group is unprotonated, allowing the product to be bound to the ion-exchange column.

The products contained variable proportions of acetone of crystallization and had i.r. spectra consistent with sugar sulfates[1]. The potassium salt of 2',3'-*O*-isopropylideneadenosine 5'-sulfate absorbed maximally at 260 nm in the u.v., indicating that the 6-amino group of the purine moiety is not sulfated. Formation of this product demonstrates the selective sulfation of the hydroxyl group *versus* the amino group.

The yields of sugar sulfates prepared by the DCC-mediated sulfation are somewhat lower than those typically obtained by more classical means[1]. However, this new sulfation procedure is an alternative method, rapid, general in scope, and is particularly suited for the synthesis of ^{35}S-labeled sulfates of sugars.

EXPERIMENTAL

Materials and methods. — All solvents used were anhydrous. *N,N*-Dimethylformamide (certified) was purchased from Fisher Scientific Co. (water content less than 0.005%). The DCC, 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose, 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose, and 2',3'-*O*-isopropylideneadenosine were purchased from Eastman Organic Chemicals, Rochester, N.Y. The purity of all reagents used was checked by t.l.c. Whatman *O*-(2-diethylamino)ethylcellulose (DE-11) and Dowex-50W X8 columns were used as ion-exchangers. I.r. spectra were obtained with a Perkin–Elmer Model 521 spectrophotometer (KBr pellet) and u.v.

spectra with a Bausch and Lomb Model 600 spectrophotometer. Elemental analyses were performed by Midwest Microlab, Inc., Indianapolis, Ind.

T.l.c. was performed with 0.5 mm and 1.5 mm thick Silica Gel-G (Supelcosil-12B, Sulpelco Inc., Bellefonte, Pa.) and with Eastman Kodak precoated plates. The t.l.c. plates were developed with 65:25:4 (v/v) chloroform–methanol–water and the spots were visualized with 20% perchloric acid, with a solution of *p*-toluenesulfonic acid, or by fluorescence.

*Potassium 1,2:3,4-di-*O*-isopropylidene-α-*D*-galactopyranose 6-sulfate* (**1**). — 1,2:3,4-Di-*O*-isopropylidene-α-D-galactopyranose (1 mmole) dissolved in 8 ml of $HCONMe_2$ was added to DCC (5 mmoles) dissolved in 12 ml of $HCONMe_2$ in a 100-ml, ice-cooled, Erlenmeyer flask. To this ice-cooled mixture was added dropwise 6 ml of $HCONMe_2$ (containing 1.5 mmoles of conc. H_2SO_4). After 15 min the entire mixture was transferred to a DEAE-cellulose column (1.5 × 20 cm, chloride form). The column was washed with 800 ml of methanol to elute the unreacted DCC and isopropylidene acetal, the $HCONMe_2$, and the relatively insoluble *N,N'*-dicyclohexylurea. The sugar sulfate was then eluted with 300 ml of M methanolic ammonium hydroxide (65 ml of conc. NH_4OH diluted to one liter with methanol). This fraction was immediately flash-evaporated, the residue suspended in 300 ml of dry acetone, and the mixture filtered. The acetone solution was then passed through a Dowex-50W X8 column (1 × 30 cm, K^+ form) and after flash-evaporation, 160 mg (42% yield) of **1** was obtained, m.p. 172–177°; i.r. data: 1240, (S–O) and 800 cm^{-1} (C–O–S).

Anal. Calc. for $C_{12}H_{19}O_9KS$: C, 38.06; H, 5.06; S, 8.48. Found: C, 37.98; H, 5.32; S, 7.82.

The reaction and experimental procedure were followed in each step by t.l.c. analyses.

*Potassium 1,2:5,6-di-*O*-isopropylidene-α-*D*-glucofuranose 3-sulfate* (**2**) and *potassium 1,2-*O*-isopropylidene-α-*D*-glucofuranose 3-sulfate* (**3**). — 1,2:5,6-Di-*O*-isopropylidene-α-D-glucofuranose (1 mmole), DCC (5 mmoles), and H_2SO_4 (1.5 mmoles) were mixed and the products were worked up as described in the preparation of **1**, to give 150 mg of sulfated product. The t.l.c. analysis of this substance indicated the presence of two products, a mono- and a di-isopropylidene acetal. The 5,6-isopropylidene group was apparently partially hydrolyzed during flash-evaporation of the methanolic ammonium hydroxide.

The mono- and the di-isopropylidene acetals were separated by preparative t.l.c. and eluted from the adsorbent with acetone, to give 90 mg of **2** (23% yield), m.p. 159–163°; i.r. data: 1240 (S–O), and 840 cm^{-1} (C–S–O), and 60 mg of **3** (18% yield), m.p. 139–145°; i.r. data: 1240, (S–O) and 840 cm^{-1} (C–O–S).

Anal. Calc. for $C_9H_{15}O_9KS \cdot 1/2\ C_3H_6O$: C, 34.14; H, 4.90; S. 8.79. Found: C, 34.08; H, 4.97; S, 8.94.

Anal. Calc. for $C_{12}H_{19}O_9KS \cdot 1/2\ C_3H_6O$: C, 39.65; H, 5.42; S, 7.92. Found: C, 39.41; H, 5.73; S, 7.80.

*Potassium 2',3'-*O*-isopropylidene adenosine 5'-sulfate* (**4**). — 2',3'-O-isopropylideneadenosine (1 mmole), DCC (5 mmoles), and H_2SO_4 (1,5 mmoles) were mixed

as described in the preparation of **1**. After 15 min 3 drops of water was added to the reaction mixture to decompose the excess DCC, and dilute, methanolic ammonium hydroxide was added to bring the pH to 9.5–10. The basic reaction mixture was immediately placed on a DEAE-cellulose column (chloride form, 1.5 × 16 cm) and the column was washed with 900 ml of methanol. The sulfated nucleoside derivative was eluted with 450 ml of M methanolic ammonium hydroxide and the effluent was flash-evaporated. The residue was suspended in 400 ml of acetone and filtered. The acetone filtrate was passed through a Dowex-50 column (K^+ form), as previously described, and the effluent was evaporated to give 106 mg of **4** (25% yield), m.p. 205–210°; i.r. data: 1240 (S–O), and 800 cm^{-1} (C–O–S); u.v. data: λ_{max} 260 nm.

Anal. Calc. for $C_{13}H_{16}N_5O_7KS \cdot C_3H_6O$: C, 39.72; H, 4.59; N, 14.49; S, 6.63. Found: C, 39.23; H, 4.47; N, 14.32; S, 6.30.

ACKNOWLEDGMENTS

This work was supported in part by the U.S. Public Health Service (Grant AMO8481) and by the Pennsylvania Agricultural Experiment Station.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

Note

The reaction of resonance-stabilized ylids with D-ribose and D-glucose derivatives[1]

ROBERT E. HARMON, GEORGE WELLMAN, AND S. K. GUPTA
Department of Chemistry, Western Michigan University, Kalamazoo, Michigan 49001 (U. S. A.)
(Received December 11th, 1969; in revised form, January 10th, 1970)

INTRODUCTION

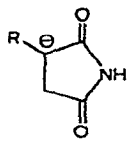
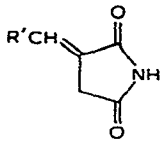
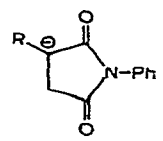
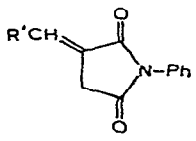
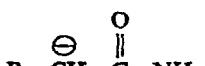
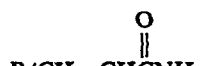
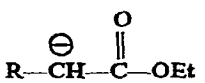
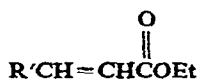
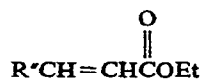
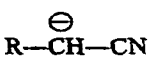
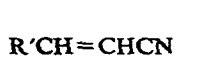
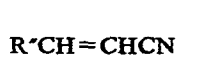
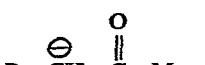
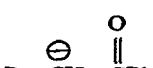
Recently, we reported[2] the preparation of intermediates for the total synthesis of the antibiotic Showdomycin by the reaction of ylids of maleimide with derivatives of D-ribose. As an extension of this work, we prepared a number of ylids that are resonance-stabilized by a variety of electron-withdrawing groups (such as ester, amide, imide, and nitrile) and studied their reaction with D-glucose and D-ribose derivatives. In the past, Wittig reactions involving resonance-stabilized ylids have been used for preparing useful intermediates for the synthesis of several unsaturated and long-chain carbohydrates[3]. Both protected and unprotected monosaccharides have been used in these reactions[4]. The reaction of resonance-stabilized ylids with carbohydrates is generally quite simple and affords high yields of the Wittig reaction-products. The failure to isolate any products in certain of the reactions is, perhaps, attributable to competing reactions, such as the aldol reaction (catalyzed by the basic ylids) or the reaction of the ylid with the protecting groups on the carbohydrate[5].

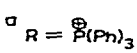
RESULTS AND DISCUSSION

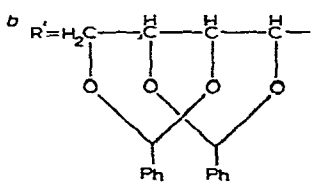
In order to determine the relative reactivity of various ylids with carbohydrates containing different protecting groups, seven stabilized ylids were prepared. (Triphenylphosphoranylidene)succinimide (**1**) and its *N*-phenyl derivative **2** were prepared by treating triphenylphosphine with maleimide and *N*-phenylmaleimide, respectively, by the procedure of Heyda and Theodoropulos[6]. The ylids **3**–**7** were prepared by the dehydrohalogenation of the corresponding phosphonium salts[7]. 2,4:3,5-Di-*O*-benzylidene-*aldehydo*-D-ribose (**8**) was prepared by the procedure of Potgieter and MacDonald[8], and penta-*O*-acetyl-*aldehydo*-D-glucose (**9**) was obtained as described by Wolfrom and Thompson[9]. The *aldehydo* sugars **8** and **9** were separately treated with each of the ylids **1**–**7** and the results are summarized in Table I. The D-ribose derivative **8** reacted with all of the ylids and gave the expected Wittig reaction-products

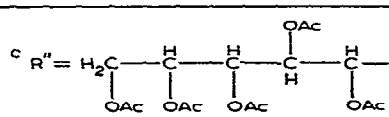
TABLE I

PRODUCTS FROM THE REACTION OF RESONANCE STABILIZED YLIDS WITH DERIVATIVES OF (A) D-RIBOSE AND (B) D-GLUCOSE

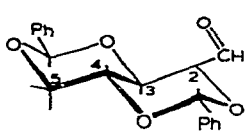
Ylid[a]	*Product*[b] (A)	*Yield* (%)	*Product*[c] (B)	*Yield* (%)
 1	 10	86	intractable mixture	
 2	 11	85	intractable mixture	
 3	 12	64	 17	55
 4	 13	75	 18	80
 5	 14	60	 19	48
 6	 15	82	no reaction	
 7	 16	63	 20	57

[a] 

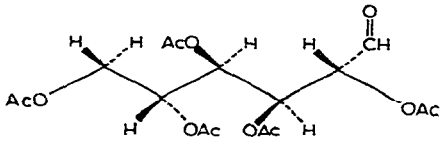
[b] 

[c] 

10–16. The reaction between the respective ylids **3**, **4**, **5**, and **7** and the D-glucose derivative **9** afforded products **17–20**, respectively. However, compound **9** did not react with ylid **6**, and, with ylids **1** and **2**, it afforded intractable, complex mixtures.

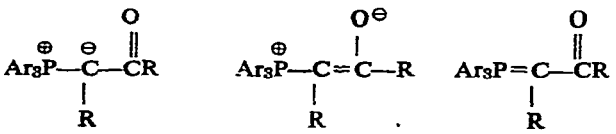


8



9

The resonance structures **21–23** can be considered to represent the stabilized phosphonium ylids. The greater the charge localization on the carbon atom (as shown in structure **21**), the more basic (and, presumably, more nucleophilic) should the ylid



21 **22** **23**

be[10]. Thus, an increase in the pK_a of the ylid should increase its reactivity. Likewise, the carbonyl band in the infrared spectrum of the ylid should show a marked shift to longer wavelengths as such structures as **22** become more important. Hence, it would be expected that the relative basicities and the shifts in the carbonyl stretching frequency of the ylids would be good measures of the relative reactivities of a particular series of ylids.

In 1963, Speziale and Ratts[10] attempted, for a series of resonance-stabilized halo-ylids, to correlate the reactivity to their basicity and to the carbonyl stretching frequency in their infrared spectra. However, their results were insufficiently conclusive to permit any structure–reactivity correlations for the halo-ylids. In an attempt to explain the observed differences in the reactivity of the ylids **1–7** with the D-ribose and D-glucose derivatives **8** and **9**, respectively, we determined their relative pK_a values and recorded their infrared spectra.

The relative basicities and infrared spectral data of the ylids are given in Table II. In this Table, the ylids are arranged in order of decreasing basicity. There is, apparently, no definite correlation between the pK_a values and the shift in the carbonyl stretching vibration of the various phosphorus ylids. Similarly, comparison of the data given in Tables I and II does not reveal any significant trend. It therefore appears that the reaction between the ylids **1–7** and the *aldehydo* sugars **8** and **9** is a rather complex reaction that is partially dependent on factors other than the basicity and nucleophilicity of the ylid; steric hindrance (by substituents on the carbohydrate and on the ylid) probably plays a significant role in the overall rate of the Wittig reaction.

TABLE II

RELATIVE BASICITIES AND INFRARED SPECTRA (STRETCHING FREQUENCY OF C=O) OF YLIDS 1–7

Ylid	pK_a	ν_{max}^{Nujol} (cm^{-1}) *for C=O*
3	10.6	1626
4	8.8	1615
5	7.7	——
1	7.1	1620
2	6.5	1640
6	6.4	1525[a]
7	6.0	1590

[a]Probably the C–O stretch of an enolate ion.

EXPERIMENTAL

Melting points were determined with a Thomas–Hoover melting-point apparatus and are corrected. The elemental analyses were performed by Midwest Microlab Inc., Indianapolis, Indiana. A Beckman IR-8 spectrophotometer was used for recording the i.r. spectra of Nujol mulls of the compounds.

Determination of relative pK_a*'s* (Table II). — The method of Speziale and Ratts[10] was used for determining the relative pK_a's by potentiometric titration (Beckman pH meter and a mini-electrode) of methanolic solutions of the ylids with hydrochloric acid.

General procedure for Wittig reactions. — A solution of equimolar amounts (~6 mmoles) of the appropriate ylid and the *aldehydo* sugar in tetrahydrofuran (100 ml) was boiled for 2 h under reflux, and evaporated to dryness under diminished pressure. The residue was dissolved in the minimal volume of absolute ethanol or butyl ether, and on cooling the solution to about 10°, the product crystallized, leaving the triphenylphosphine oxide (by-product) in solution. Filtration of the suspension, followed by recrystallization from the same solvent afforded the analytical samples.

2,4:3,5-Di-*O*-benzylidene-1-deoxy-1-(2,5-dioxo-3-pyrrolidinylidene)-D-ribitol (**10**), m.p. 194–195°; lit.[2] m.p. 194–195°.

2,4:3,5-Di-*O*-benzylidene-1-deoxy-1-(2,5-dioxo-1-phenyl-3-pyrrolidinylidene)-D-ribitol (**11**), m.p. 212–213°; lit.[2] m.p. 212–213°.

4,6:5,7-Di-*O*-benzylidene-2,3-dideoxy-D-*ribo*-hept-2-enonamide (**12**), m.p. 260°.

Anal. Calc. for $C_{21}H_{21}NO_5$: C, 68.65; H, 5.76; N, 3.81. Found: C, 68.80; H, 5.63; N, 3.79.

Ethyl 4,6:5,7-di-*O*-benzylidene-2,3-dideoxy-D-*ribo*-hept-2-enonate (**13**), m.p. 160–161°.

Anal. Calc. for $C_{23}H_{24}O_6$: C, 69.68; H, 6.10. Found: C, 69.54; H, 6.13.

4,6:5,7-Di-*O*-benzylidene-2,3-dideoxy-D-*ribo*-hept-2-enononitrile (**14**), m.p. 169–171°.

Anal. Calc. for $C_{21}H_{19}NO_4$: C, 72.19; H, 5.48; N, 4.00. Found: C, 71.80; H, 5.55; N, 3.92.

5,7:6,8-Di-*O*-benzylidene-1,3,4-trideoxy-D-*ribo*-oct-3-enulose (**15**), m.p. 196–197°.

Anal. Calc. for $C_{22}H_{22}O_5$: C, 72.11; H, 6.05. Found: C, 71.89; H, 6.04.

4,6:5,7-Di-*O*-benzylidene-2,3-dideoxy-1-*C*-phenyl-D-*ribo*-hept-2-enose (**16**), m.p. 186–187°.

Anal. Calc. for $C_{27}H_{24}O_5$: C, 75. 68; H, 5.64. Found: C, 75.84; H, 5.69.

2,3-Dideoxy-D-*gluco*-oct-2-enonamide 4,5,6,7.8-pentaacetate (**17**), m.p. 130–131°.

Anal. Calc. for $C_{18}H_{25}NO_{11}$: C, 50.11; H, 5.84; N, 3.24. Found: C, 50.07; H, 5.60; N, 3.26.

Ethyl 2,3-dideoxy-D-*gluco*-oct-2-enonate 4,5,6,7.8-pentaacetate (**18**), m.p. 133–135°; lit.[11] m.p. 133–134.5°.

2,3-Dideoxy-D-*gluco*-oct-2-enononitrile 4,5,6,7,8-pentaacetate (**19**), m.p. 125–127°.

Anal. Calc. for $C_{18}H_{23}NO_{10}$: C, 52.42; H, 5.62; N, 3.39. Found: C, 52.28; H, 5.48; N, 3.32.

2,3-Dideoxy-1-*C*-phenyl-D-*gluco*-oct-2-enose 4,5,6,7,8-pentaacetate (**20**), m.p. 93–94°.

Anal. Calc. for $C_{24}H_{28}O_{11}$: C, 58.53; H, 5.73. Found: C, 58.27; H, 5.69.

ACKNOWLEDGMENT

This work was supported by Grant No. CA-06140 from the National Cancer Institute.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
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Note

Correlation of conformational stability of pentose dialkyl dithioacetals and their conversion into 2,5-anhydro derivatives upon attempted *p*-toluenesulfonylation*†

J. DEFAYE

Institut de Chimie des Substances Naturelles, Centre National de la Recherche Scientifique, 91-Gif-sur-Yvette (France)

AND D. HORTON

Department of Chemistry, The Ohio State University, Columbus, Ohio 43210 (U. S. A.)

(Received January 24th, 1970)

By use of n.m.r. spectroscopy, it has been shown that acyclic sugar derivatives in solution favor a conformation having an extended, planar, zigzag arrangement of carbon atoms, corresponding to maximum staggering of small–medium–large sets of groups along each carbon–carbon bond[1], unless such an arrangement would generate a parallel, 1,3-interaction of a pair of substituents along the chain[2]. It was pointed out[2] in 1964, by J. C. P. Schwarz to one of the authors, that in the latter situation, steric destabilization corresponding to a 1,3-*syn*-diaxial interaction in a cyclohexane system should exist. In 1968, El Khadem, Horton, and Page[2] showed experimentally that, when such a situation would exist in the extended-chain form, the molecule favors an alternative conformation having the carbon chain in a bent or "sickle" conformation[3] formed by rotation about one carbon–carbon bond to remove the 1,3-interaction. Further experimental evidence in support of these generalizations has been presented with acetylated aldose dialkyl dithioacetals[3–7], acetylated *aldehydo* sugars[5], and other systems[5].

For the acetylated pentose dialkyl dithioacetals, the conformations established by n.m.r. spectroscopy have been shown[3,4] to accord with predictions; the D-arabinose isomer appears entirely stable in the extended, planar, zigzag conformation, whereas the D-ribose and D-xylose derivatives adopt sickle conformations formed from the extended conformation by rotation about the C-3–C-4 and the C-2–C-3 bond,

*Part 5 in the series "Conformation of Acyclic Derivatives of Sugars" (for parts 1–4, see Refs. 1–4) and part 11 in the series "2,5-Anhydro Derivatives of Sugars" [for part 10, see J. Defaye and T. Reyners, *Bull. Soc. Chim. Biol.*, 50 (1968) 1625].

†Supported, in part, by the National Science Foundation, Grant No. GP-9646 (The Ohio State University Research Foundation Project 2861) and, in part, by the Délégation Générale à la recherche scientifique auprès du Premier Ministre, Comité de Biologie Moléculaire, research grant No. 6600308.

respectively; the D-lyxose isomer also experiences some conformational destabilization in the fully extended form.

There exists a striking and hitherto unexplained difference between dialkyl dithioacetals of the four pentoses in their behavior on being treated at −10° with one molar equivalent of *p*-toluenesulfonyl chloride in pyridine, the mixture then being kept at room temperature. Various dialkyl dithioacetals of D-arabinose all undergo reaction under these conditions to give the 5-*p*-toluenesulfonates in high yield[6], with no indication of further transformation. In contrast, the diisobutyl dithioacetals of D-lyxose[7], D-ribose[8], and D-xylose[8] do not give the 5-*p*-toluenesulfonates under these conditions, and the only products isolable are the 2,5-anhydropentose diisobutyl dithioacetals, which are obtained in good yield (see Chart I and Table I). With the information now at hand on the conformations of the acetylated pentose dithioacetals[3,4], the direct correlation between conformational stability of these derivatives and the mode of reaction of pentose dithioacetals with *p*-toluenesulfonyl chloride is immediately evident.

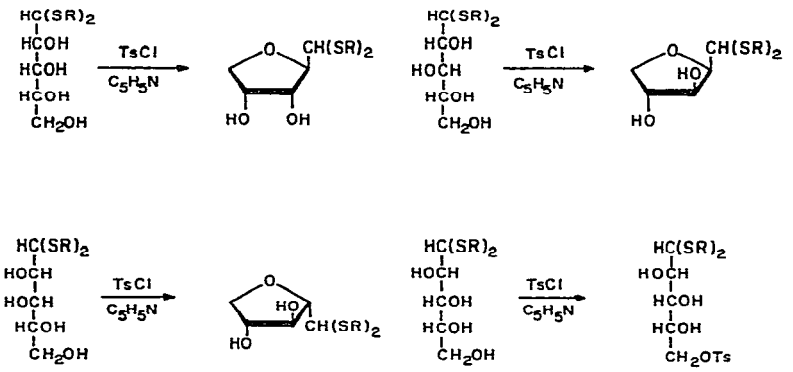


CHART I

TABLE I

YIELDS OF 2,5-ANHYDROPENTOSE DITHIOACETALS OBSERVED AFTER TREATMENT OF PENTOSE DITHIOACETALS WITH *p*-TOLUENESULFONYL CHLORIDE IN PYRIDINE[a]

Dialkyl dithioacetal of	*Yield (%) of 2,5-anhydride for given alkyl group*							*References*
	Methyl	*Ethyl*	*Propyl*	*Isopropyl*	*Butyl*	*Isobutyl*	*Benzyl*	
D-Ribose			47			59		8
D-Xylose						84[b]	29	8
D-Lyxose						73		7
D-Arabinose	0	0		0	0	0[b]	0	6
	[66][c]	[75][c]		[64][c]	[69][c]	[78][b,c,d]	[75][c]	6

[a]With 1.1 moles/mole at −10° and then at room temperature [b]See Experimental section. [c]Yield of 5-*p*-toluenesulfonate [d]For the L enantiomorph.

The mechanism by which the pentose dithioacetals are converted into 2,5-anhydrides in this reaction has not been established, but it is reasonable to suppose that *p*-toluenesulfonylation at O-5 occurs first and that a subsequent, rapid displacement of the 5-*p*-tolylsulfonyloxy group by O-2 takes place in each of the examples, except for the *arabino* isomer. Assuming that the first step in each case involves formation of the 5-*p*-toluenesulfonate, the rate at which this derivative is converted into the 2,5-anhydride will be controlled by the difference in energy between the ground state and the transition state for forming the anhydro ring, and the statistical frequency with which O-2 can be located in a position suitable for attack on C-5.

The favored conformations of the acetylated dithioacetals of D-ribose and D-xylose have been shown[3–5] to be sickle arrangements (**1** and **2**, respectively) in which the chain of carbon atoms is not planar; and, for the corresponding D-lyxose derivative, a form (**3**) having O-2 bisecting the angle of the two sulfur atoms at C-1 has been shown to be the favored conformation (Chart II). Similar sickle arrangements

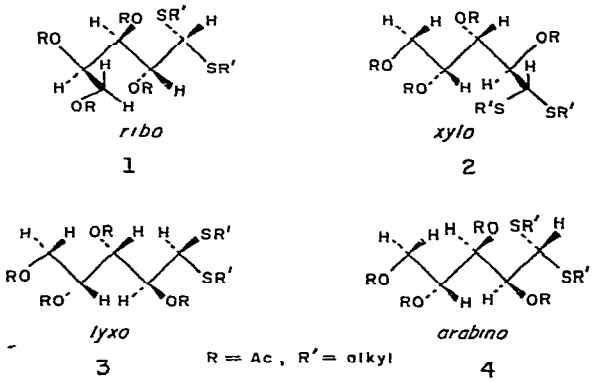


CHART II

may be anticipated for the non-acetylated analogs (compare Ref. 2). These non-extended forms represent the most frequently populated conformational states in a time-average of various conformations, and it is to be expected that, with these two configurations, the energy requirements will be relatively low for the chain to adopt a conformation in which O-2 could effect an internal SN2 type of attack on C-5 to displace a *p*-tolylsulfonyloxy group. Furthermore, the progress of such an attack toward a transition state should not be hindered appreciably by generation of unfavorable steric interactions; the substituent groups on C-2, C-3, and C-4 are staggered mutually *trans* in the D-xylose derivative; and, in the two other examples (D-*ribo* and D-*lyxo*), two contiguous substituent groups are on the same side of the developing ring, but the third is on the opposite side (see Fig. 1).

For the D-arabinose derivative, the planar, extended, zigzag arrangement (**4**) shown for the acetate can be expected to be the conformation of maximum stability, and a substantial amount of potential energy will need to be expended to bring O-2 into a position suitable for internal SN2 displacement of a *p*-tolylsulfonyloxy group from C-5. In addition, as a transition state for formation of an anhydro ring is approached,

a situation would arise where serious steric repulsions would exist between the substituent groups on C-2, C-3, and C-4, because all of these would be on the same side of the ring[9] (see Fig. 1).

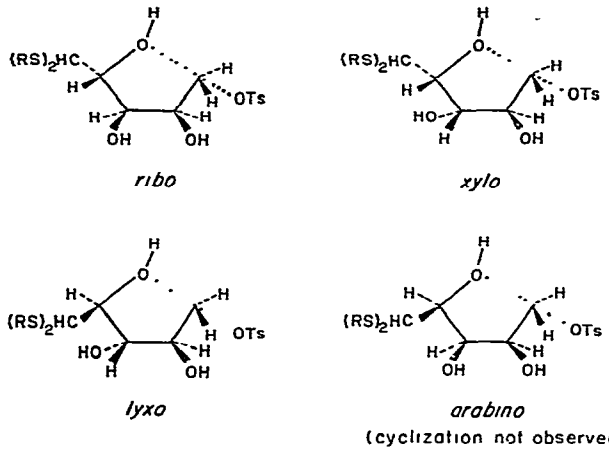


Fig 1. Steric disposition of substituents during ring-closure in the formation of 2,5-anhydropentose dialkyl dithioacetals

These conformational rationalizations accommodate the observed, striking differences in reactivity between the four configurational series in the reaction studied. Similar considerations of conformational stability in acyclic sugar derivatives may be of use in predicting the outcome of other kinetically controlled, ring-forming reactions of the sugars, and related studies along this line are in progress.

EXPERIMENTAL

5-O-p-Tolylsulfonyl-L-arabinose diisobutyl dithioacetal. — Carefully dried L-arabinose diisobutyldithioacetal[10] (5 g, 16 mmoles) was dissolved in pyridine (30 ml), and to the magnetically stirred solution at −10° was slowly added *p*-toluenesulfonyl chloride (3.35 g, 17.6 mmoles) in pyridine (30 ml), with continuous stirring. After 2 h at −10°, the solution was kept for 24 h at room temperature and then poured onto ice. After 10 min, the mixture was extracted with three 200-ml portions of chloroform; the extracts were combined, successively washed at 0° with 5% potassium hydrogen sulfate solution, saturated sodium hydrogen carbonate solution, and water, dried (sodium sulfate), and evaporated under diminished pressure to an oil that crystallized spontaneously. The product was recrystallized from benzene–petroleum ether (b.p. 50–75°); yield 5.8 g (78%), m.p. 71–73°, $[\alpha]_D^{25}$ +57.5° (*c* 0.5, chloroform).

Anal. Calc. for $C_{20}H_{36}O_6S_3$: C, 51.49; H, 7.35; S. 20.57. Found: C, 51.54; H, 7.25; S, 20.23.

2,5-Anhydro-D-xylose diisobutyl dithioacetal. — D-Xylose diisobutyl dithiocetal[11] (28 g, 90 mmoles), predried for 2 h at 70° at 0.01 torr, was dissolved in pyridine

(70 ml). To this solution, kept at −10°, was added slowly, with stirring, a solution of *p*-toluenesulfonyl chloride (19 g, 100 mmoles) in pyridine (70 ml). After 2 h at −10°, the solution was kept for 24 h at room temperature and then poured onto ice. The product was extracted as in the preceding experiment. The crude product crystallized spontaneously, and was recrystallized from chloroform–petroleum ether (b.p. 50–75°); yield 22.1 g (84%), m.p. 93–94°.

For this product, prepared by a similar procedure, Zinner and coworkers[8] recorded m.p. 94°, but a yield of only 21%.

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CORRIGENDUM

Carbohyd. Res., 13 (1970) page 208, line 6 from bottom should read:

5.5 (broad band, OH); 4.8 and 4.5 (2 multiplets, 2 protons); and 2.7 (broad band, NH).

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Elsevier Publishing Company Amsterdam
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MASS SPECTROMETRY OF SULFUR-CONTAINING DERIVATIVES OF SUGARS

PART I FRAGMENTATION OF SULFONIC ESTERS OF PENTOSE DERIVATIVES*

V KOVAČIK AND P KOVÁČ
Institute of Chemistry, Slovak Academy of Sciences, Bratislava (Czechoslovakia)

AND R L WHISTLER
Department of Biochemistry, Purdue University, Lafayette, Indiana 47907 (U S A)

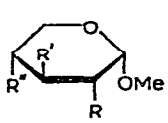
(Received November 24th, 1969, in revised form, January 9th, 1970)

ABSTRACT

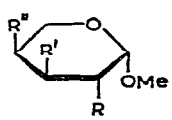
The mass spectra of some methanesulfonic and *p*-toluenesulfonic esters of methyl pentopyranosides have been studied. The fragmentation of the molecular ions is demonstrated in four fragmentation series Methanesulfonyl (Ms) components are removed principally as Ms or MsO radicals or as MsOMs, MsOH, and CH_2SO_2. On the other hand, *p*-toluenesulfonyl (Ts) groups are split off only in the form of TsOH and, perhaps, as TsO radicals Respective fragmentation mechanisms are proposed and discussed

INTRODUCTION

Sulfur-containing derivatives of sugars have often been intermediates in the synthesis of new derivatives of carbohydrates So far, only diethyl dithioacetals[1] and ethylene dithioacetals[2] have attracted appreciable attention We have now examined the fragmentation of methanesulfonic esters (**1–4**) of methyl α-D-xylopyranoside and *p*-toluenesulfonic esters (**5–9**) of methyl β-L-arabinopyranoside, some of which contained acetyl or benzoyl substituents



1–4



5–9

	R	*R'*	*R"*
1	OMs	OMs	OMs
2	OMs	OAc	OMs
3	OMs	OH	OMs
4	OMs	OD	OMs

	R	*R'*	*R"*
5	OH	OH	OTs
6	OAc	OAc	OTs
7	OBz	OBz	OTs
8	OBz	OBz	OH**
9	OBz	OBz	OD**

*Journal paper number 3908 of the Purdue Agricultural Experiment Station
**The spectra of compounds 8 and 9 were recorded in order to clarify the fragmentation of compound 7.

The mass spectra of the methanesulfonic esters **1–4** (see Figs. 1–3) exhibit slight peaks of molecular ions. The most intense peak is given by $MeSO_2^-$ at *m/e* 79. The methanesulfonyl group is split off in the form of the following radicals and neutral molecules: $MeSO_2O$ (95), $MeSO_2$· (79), $MeSO_2OH$, $CH_2{=}SO_2$, and $MeSO_2$–O–SO_2Me.

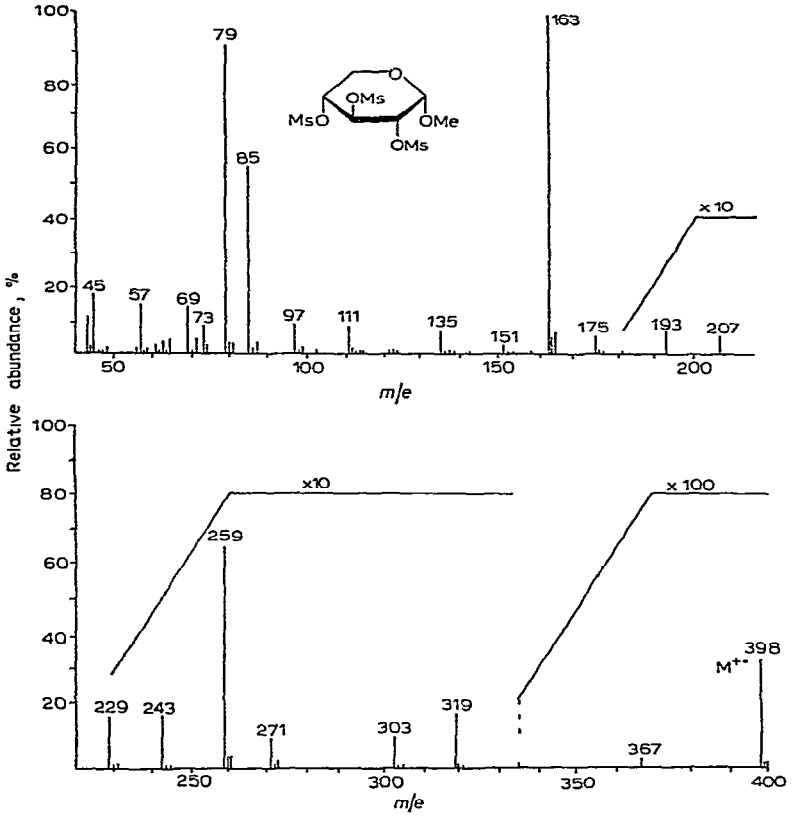
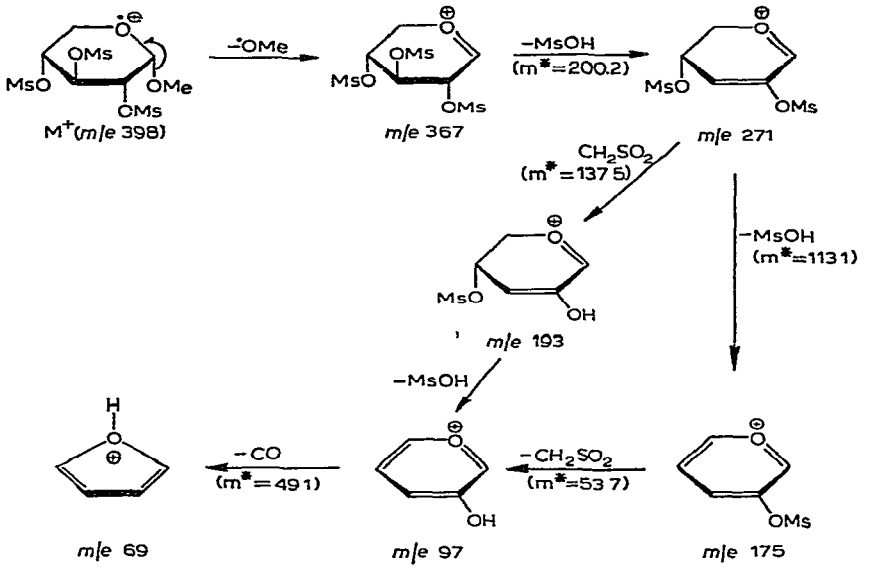


Fig. 1. Mass spectrum of methyl 2,3,4-tri-*O*-(methylsulfonyl)-α-D-xylopyranoside (**1**)

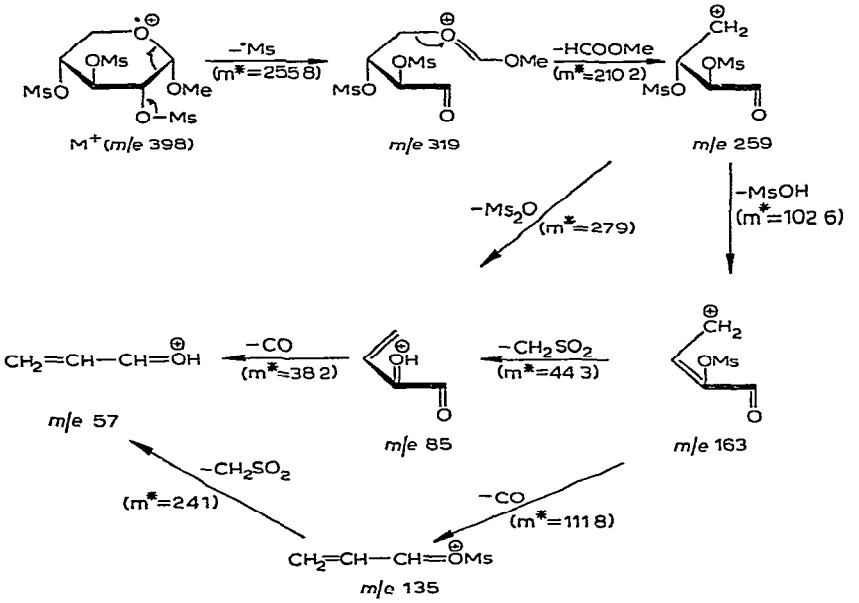
As a result, the mass spectra of methanesulfonic esters exhibit a number of peaks. Fragmentation of the molecular ions proceeds in four series (A_s, B_s, C_s, and D_s)*, illustrated in Schemes 1–4 for methyl 2,3,4-tri-*O*-(methylsulfonyl)-α-D-xylopyranoside (**1**). Series A_s (see Scheme 1) shows cleavage of the methoxyl group in the form of a radical, this is followed by the elimination of MsOH and CH_2SO_2, and the series is terminated by fission of a molecule of CO. This fragmentation mechanism resembles the fragmentation of pentose acetates[3].

*To distinguish the symbols A, B, C, and D from the ones commonly used for discussion of the fragmentation mechanism of acetylated[3–5] or methylated[4,5] monosaccharides, the symbols A_S, B_S, C_S, and D_S are introduced.



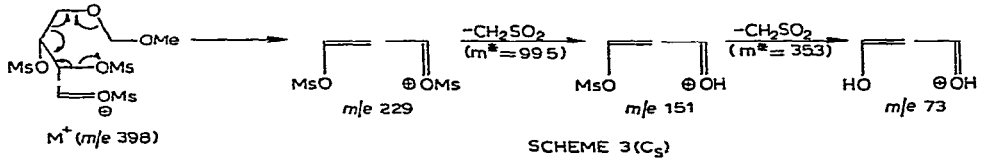
SCHEME 1 (A_S)

Series B_s begins with the elimination of a Ms radical from the O-2, and is followed by elimination of HCOOMe, MsOH, CH_2SO_2, and CO or Ms_2O (see Scheme 2)



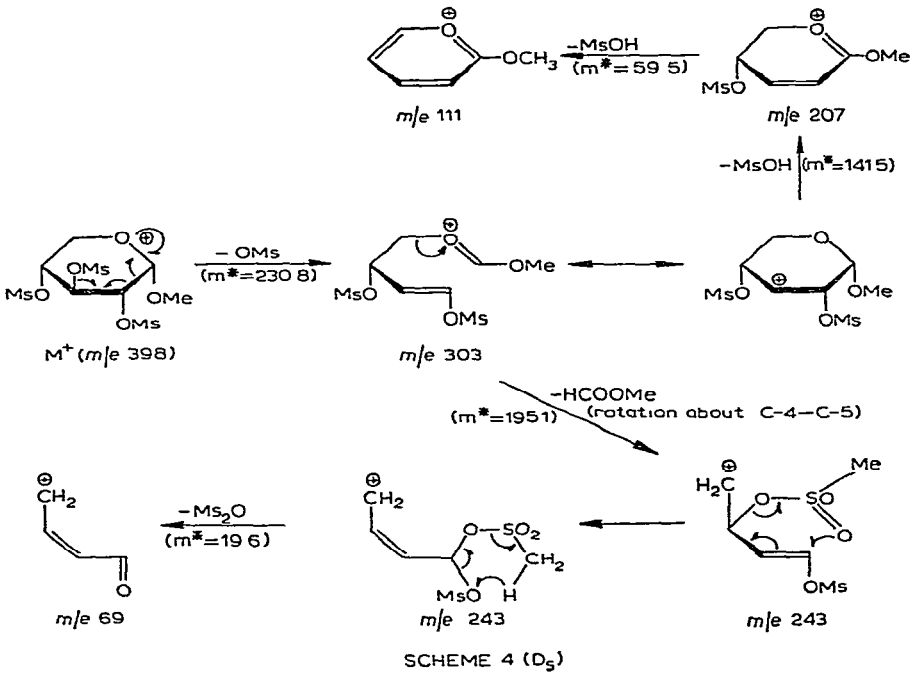
SCHEME 2 (B_S)

Series C_s anticipates a transfer of the substituent from C-3 to C-1 by the mechanism commonly occurring when methylated sugars are fragmented[4,5], and is followed by eliminations of CH_2SO_2. The pathway C_s is given in Scheme 3.



SCHEME 3 (C_S)

Series D_s begins with elimination of the OMs (OAc with compound **2**) radical from C-3 and is followed by the elimination of MsOH or HCOOMe and Ms_2O (see Scheme 4)



SCHEME 4 (D_S)

The ions of the series A_s at *m/e* 271 (see Scheme 1), visible in the spectra of methyl 3-*O*-acetyl-2,4-di-*O*-(methylsulfonyl)-α-D-xylopyranoside (**2**) (see Fig 2), methyl 2,4-di-*O*-(methylsulfonyl)-α-D-xylopyranoside (**3**) (see Fig 3), and its deuterated analog **4**, are formed from the ions $(M\text{-}MeO)^+$ at *m/e* 331, 289, and 290, after elimination of AcOH (m* = 222 0, 331→271), H_2O (m* = 254 0, 289→271) or HOD.

The disintegration of the ions $(M\text{-}Ms\text{-}HCO_2Me)^+$ at *m/e* 223 (series B_s, Scheme 2) during the fragmentation of **2** (see Fig 2) becomes more complicated, owing to the presence of two different substituents at C-3 and C-4, as shown in Scheme 5

The ions at *m/e* 85 (86) in compounds **3** and **4** are formed after elimination of MsOH (m* = 39.8,181→85) from the ions $(M\text{-}Ms\text{-}HCOOMe)^+$ at *m/e* 181 (182)

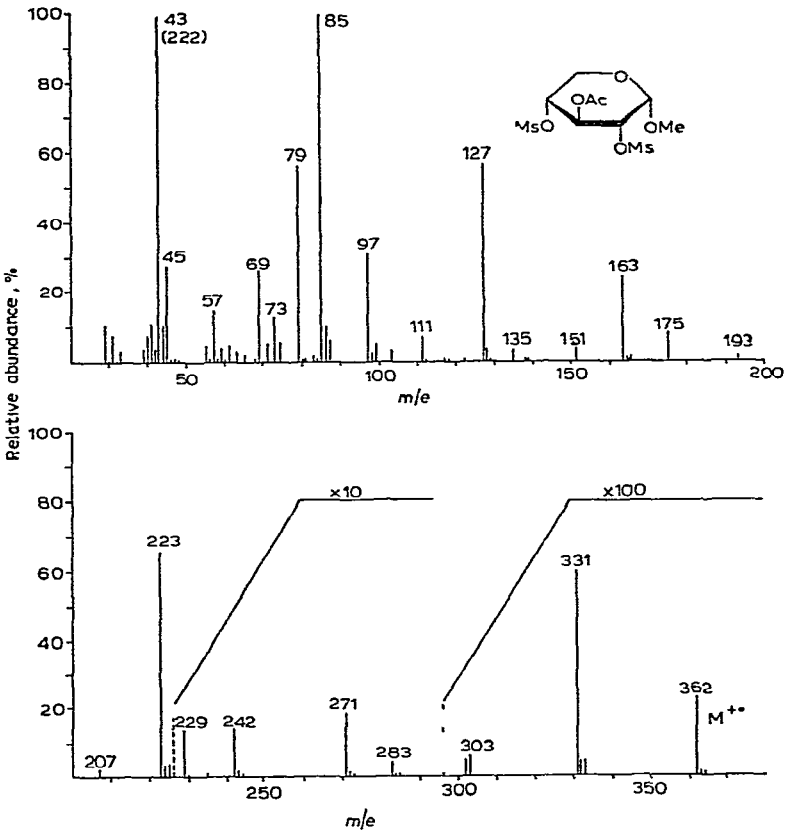
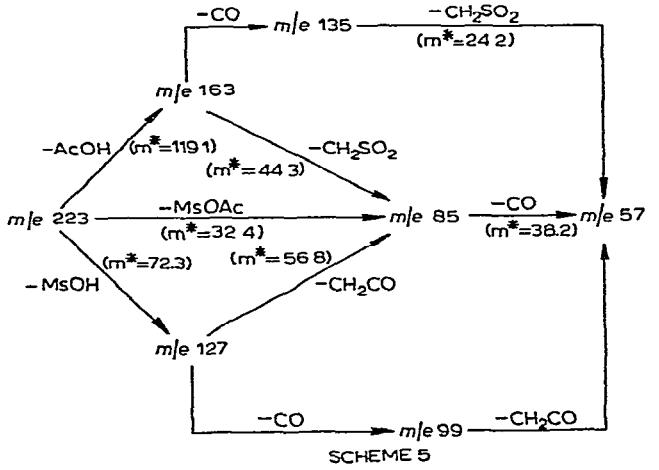
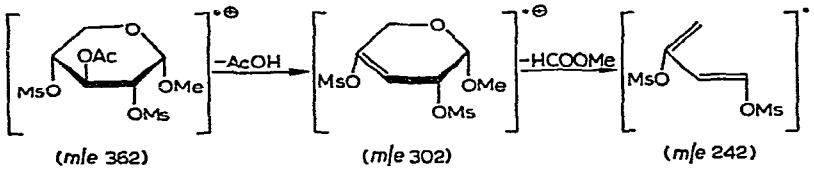


Fig 2 Mass spectrum of methyl 3-*O*-acetyl-2,3-di-*O*-(methylsulfonyl)-α-D-xylopyranoside (2) Numbers in parentheses indicate relative ion-abundances greater than 100%



SCHEME 5

Owing to the absence of a substituent on C-3, the ions of series C_s and D_s appear at the same *m/e* in the spectrum of **2** (see Fig. 2) In addition to these, in the spectrum of **2**, the most pronounced peaks are at *m/e* 43, representing the ions CH_3CO^+, as well as peaks at *m/e* 302 and 242 The last two are produced through deacetylation of molecular ions[5], followed by retro-Diels–Alder fragmentation (see Scheme 6).



SCHEME 6

The absence of peaks of the series C_s and D_s from the spectrum (see Fig 3) of compound **3** (and **4**) could be explained by resistance of the hydroxyl group on C-3 to fission as a radical

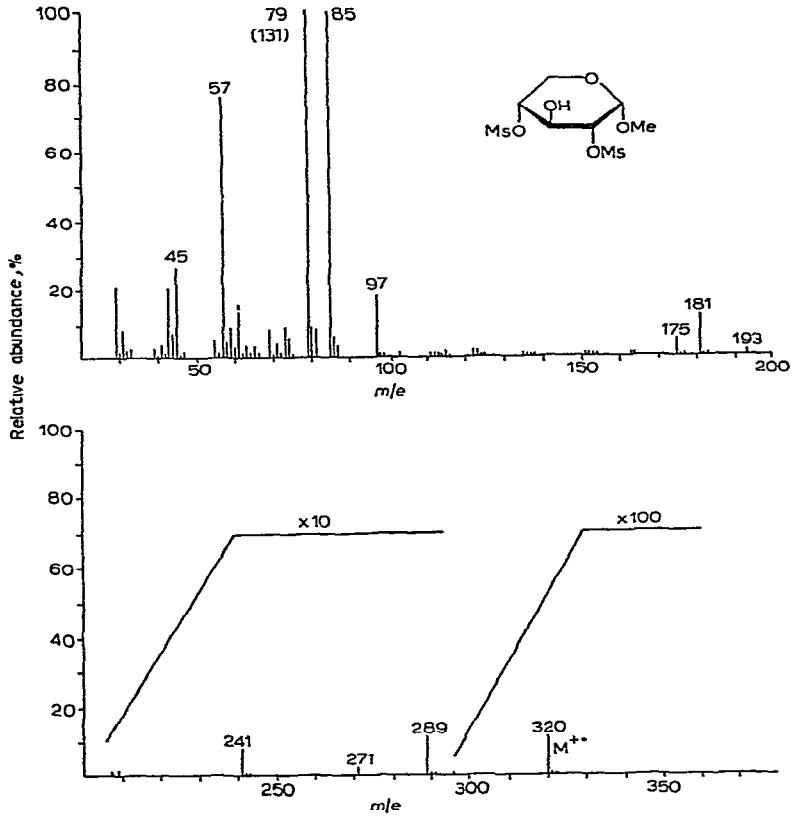
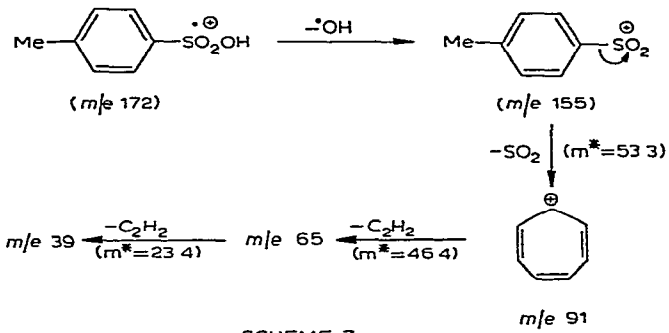


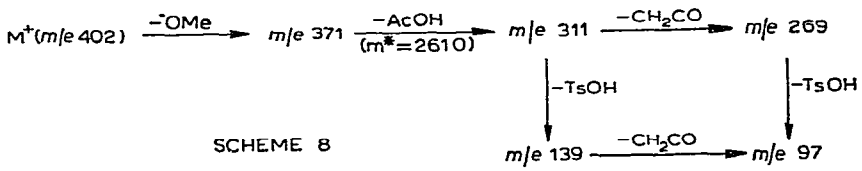
Fig 3 Mass spectrum of methyl 2,4-di-*O*-(methylsufonyl)-α-D-xylopyranoside (**3**) Numbers in parentheses indicate relative ion-abundances greater than 100%

The *p*-toluenesulfonyl group is eliminated from carbohydrate derivatives in the form of *p*-toluenesulfonic acid (TsOH) and, perhaps, as the radical OTs As the elimination of a *p*-toluenesulfonyl group as a ketene is impossible, because of the aromatic character of the substituent, the fragmentation of a *p*-toluenesulfonic ester is less complicated than for a methanesulfonic ester. When the spectrum of methyl 4-*O*-*p*-tolylsulfonyl-β-L-arabinopyranoside (**5**) was determined, complete detosylation occurred, even though the fragmentation was conducted by evaporation at 70°, and at 120°, in the ionizing chambers The spectrum actually represents the spectrum of *p*-toluenesulfonic acid, the fragmentation of which proceeds as shown in Scheme 7.



SCHEME 7

p-Toluenesulfonyl ions at *m/e* 155 and tropylium ions at *m/e* 91 are well pronounced in all of the spectra of the *p*-tolylsulfonyl derivatives, and are typical of the presence of a *p*-tolylsulfonyl group in a carbohydrate derivative In the spectrum of methyl 2,3-di-*O*-acetyl-4-*O*-*p*-tolylsulfonyl-β-L-arabinopyranoside (**6**) (see Fig 4), a minor peak for the molecular ions is found The fragmentation according to series A_s is illustrated in Scheme 8



SCHEME 8

The peaks at *m/e* 269 and 227 represent the ions of the series C_s

$$AcO{-}CH{=}CH{-}CH{=}O^{\oplus}Ts \xrightarrow[(m^* = 191.7)]{-CH_2CO} HO{-}CH{=}CH{-}CH{=}O^{\oplus}Ts$$

The ions at *m/e* 342 originate after deacetylation of the molecular ions, and, after retro-Diels–Alder fragmentation, give rise to ions $(M\text{-}AcOH\text{-}HCOOMe)^+$ at *m/e* 282 The series proceeds through eliminations of CH_2CO and TsOH, or, perhaps, of a TsO· radical

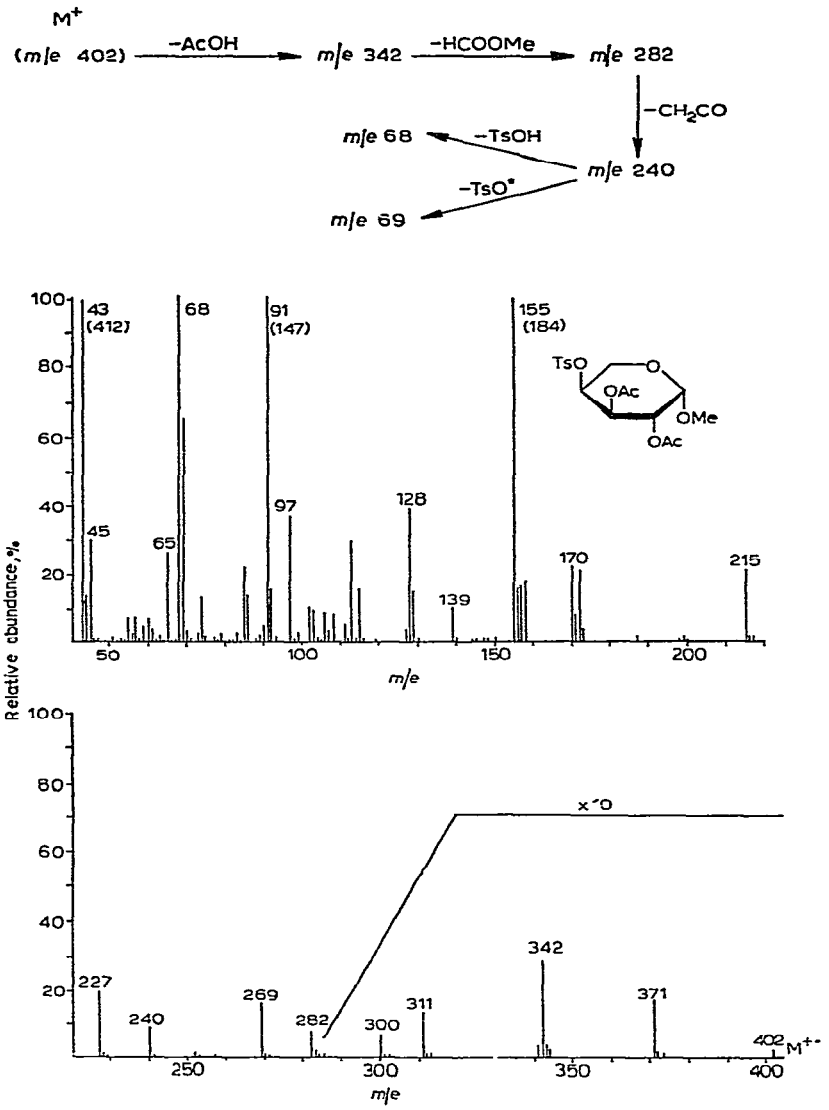
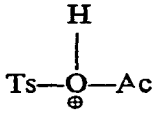
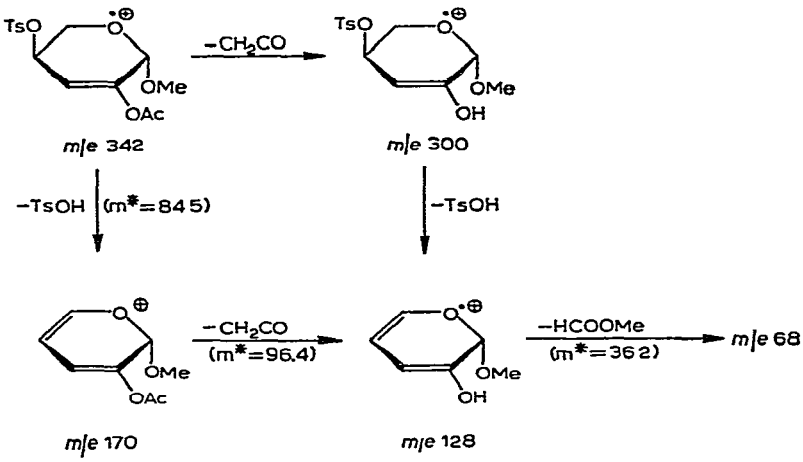


Fig 4 Mass spectrum of methyl 2,3-di-*O*-acetyl-4-*O*-*p*-tolylsulfonyl-β-L-arabinopyranoside (6) Numbers in parentheses indicate relative ion-abundances greater than 100%

A different course of fragmentation of the ions $(M\text{-}AcOH)^+$ of *m/e* 342 affords the ions at *m/e* 300, 170, 128, and 68, and is shown in Scheme 9.

The ions at *m/e* 215 probably belong to the ions of a protonated, mixed anhydride.





SCHEME 9

The peaks at *m/e* 105 and 77 (m* = 56.5; 105→77) are the most pronounced ones in the spectra of benzoic esters. In the spectrum of methyl 2,3-di-*O*-benzoyl-4-

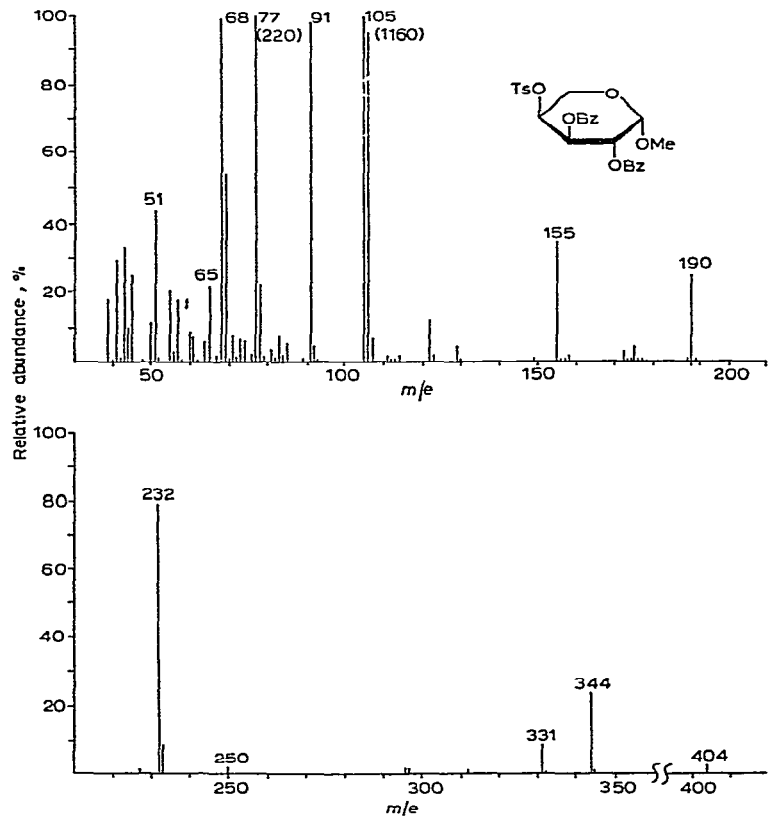
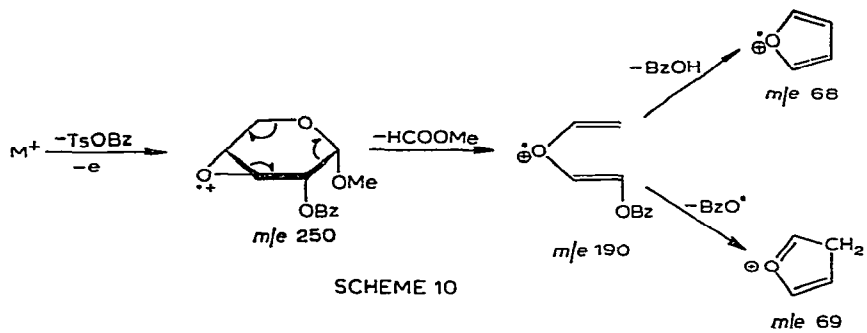


Fig 5 Mass spectrum of methyl 2,3-di-*O*-benzoyl-4-*O*-*p*-tolylsulfonyl-β-L-arabinopyranoside (7) Numbers in parentheses indicate relative ion-abundances greater than 100%

O-*p*-tolylsulfonyl-β-L-arabinopyranoside (**7**) (see Fig 5), neither the peaks of the molecular ions nor the peaks of series A_s are to be found Series C_s is represented by the peak at *m/e* 331, which corresponds to the TsO–CH=CH–CH=OBz ions The molecular ions are debenzoylated, giving rise to the ions $(M\text{-}BzOH)^+$ at *m/e* 404, from which, after elimination of HCOOMe, or a molecule of *p*-toluenesulfonic acid, the ions at *m/e* 344 or 232 are formed

Rather unexpected is the formation of the ions $(M\text{-}276)^+$ at *m/e* 250, these may be formed through the elimination of a molecule of the mixed anhydride TsOBz from the molecular ions. This peculiar fragmentation is probably occasioned by the bulky substituents on C-3 and C-4. The ions $(M\text{–}TsOBz)^+$ eliminate HCOOMe, BzOH, or BzO·, and then disintegrate to the ions at *m/e* 68 and 69 (see Scheme 10)



SCHEME 10

To confirm the pathway postulated in Scheme 10, we recorded the mass spectra of methyl 2,3-di-*O*-benzoyl-β-L-arabinopyranoside (**8**) and its deuterated analog (**9**) The fragmentation of **8** proceeds mainly through the formation of the ions $(M\text{-}BzOH)^+$ at *m/e* 250, which can be found unaltered in the spectrum of **9** Peaks at *m/e* 190, 69, and 68 remain.

EXPERIMENTAL

The mass spectra were obtained with a MCh 1306 mass spectrometer (U S S R) modified for direct introduction of the sample, at an ionizing potential of 70 eV and an intensity of 0 5 mA The temperature at the site of evaporation was, according to the volatility of the compounds, 40–70°, and the temperature in the ionizing chamber was 120–130°

O-Deuterated compounds **4** and **9** were prepared by evaporation of the solvent (EtOD) from solutions of **3** and **4**, directly in the mass spectrometer The degree of deuteration was 66 and 64%, respectively

ACKNOWLEDGMENT

The authors are grateful to Mr. S Markovič for skilful technical assistance.

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Carbohydrate Research
Elsevier Publishing Company Amsterdam
Printed in Belgium

SULPHATED POLYSACCHARIDES OF THE GRATELOUPIACEAE FAMILY

PART IV[1]. METHYLATION ANALYSIS OF PHYLLYMENAN AND DESULPHATED PHYLLYMENAN

J R NUNN AND H PAROLIS
Chemistry Department, Rhodes University, Grahamstown (South Africa)

(Received November 25th, 1969)

ABSTRACT

Methylation studies of phyllymenan and desulphated phyllymenan indicate that the D-galactosidic linkages are essentially α-(1→3) and β-(1→4) This information, together with previous data, has led to a partial structure for the polysaccharide.

INTRODUCTION

Previous work on phyllymenan[1], a sulphated polysaccharide from the red seaweed *Phyllymenia cornea*, revealed that the polymer contains D-galactose, 6-*O*-methyl-D-galactose, 4-*O*-methyl-L-galactose, 2-*O*-methyl-D-galactose, xylose, and ester sulphate, that all of the sulphate ester groups are attached to units linked through position 3, and that the polymer yields a high proportion of 4-*O*-β-D-galactopyranosyl-2-*O*-methyl-D-galactose and a lesser amount of 4-*O*-β-D-galactopyranosyl-D-galactose residues on hydrolysis In this paper, we report the results of methylation studies of desulphated phyllymenan and phyllymenan itself.

RESULTS AND DISCUSSION

The positions of the glycosidic linkages were deduced by methylation analysis of desulphated phyllymenan Phyllymenan was desulphated[1] with methanolic hydrogen chloride The desulphated polymer was partially methylated in methyl sulphoxide with methyl sulphate and sodium hydroxide, and methylation was completed in *N*,*N*-dimethylformamide with methyl iodide and silver oxide After hydrolysis, the products were fractionated by gradient elution from a charcoal–Celite column and characterised (molar ratios in brackets) as 2,3,4,6-tetra-*O*-methylgalactose (1 6), 2,4,6-tri-*O*-methyl-D-galactose (10 1), and 2,3,6-tri-*O*-methyl-D-galactose (8 7).

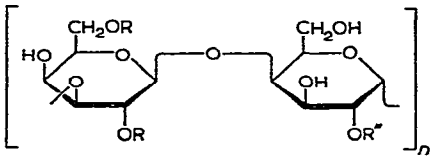
The presence in the native and desulphated polymers of a high proportion of monomethyl ethers of galactose renders difficult an unambiguous interpretation of the results of the methylation analysis. Nevertheless, some conclusions can be drawn if these results are considered together with those of partial hydrolysis, periodate oxidation, and alkali-treatment studies of phyllymenan and desulphated phyllymenan[1].

The 2,3,6-tri-*O*-methyl-D-galactose is considered to have arisen from (1→4)-linked D-galactose and (1→4)-linked 2-*O*-methyl-D-galactose residues in the polymer The isolation of 4-*O*-*β*-D-galactopyranosyl-D-galactose and 4-*O*-*β*-D-galactopyranosyl-2 *O*-methyl-D-galactose (**1**) from a partial hydrolysate of phyllymenan supports this conclusion, and indicates that at least the majority of the (1→4) linkages have the *β*-D configuration The 2,4,6-tri-*O*-methyl-D-galactose is considered to have arisen from (1→3)-linked D-galactose and (1→3)-linked 6-*O*-methyl-D-galactose residues in the polymer The presence of galactose and 6-*O*-methylgalactose in the hydrolysate of desulphated, periodate-oxidised phyllymenan, coupled with the absence of branching in desulphated phyllymenan (as indicated by the absence of di- or mono-*O* methylgalactoses in the hydrolysate of methylated, desulphated phyllymenan), supports this conclusion The isolation, on partial hydrolysis of phyllymenan, of a small proportion of a disaccharide[2], to which the structure 3-*O*-*α*-(2-*O*-methyl-D-galactopyranosyl) D-galactose (**2**) has been assigned, suggests that at least some of the (1→3) linkages have the *α*-D configuration

In common with aeodan[3] and other polysaccharides, phyllymenan was difficult to methylate Repeated treatment with methyl sulphate and aqueous sodium hydroxide afforded a partially methylated product. Further methylation was achieved by dispersing the product in methyl sulphoxide and treating the dispersion with Purdie's reagents[4] The infrared spectrum of the final product showed a small hydroxyl peak. The methylated polysaccharide was hydrolysed, and the components were separated by cellulose-column chromatography 2,3,4,6-Tetra-*O*-methylgalactose, 2,3,6-tri-*O*-methyl-D-galactose, 2,4,6-tri-*O*-methylgalactose, 2,6- and 4,6-di-*O*-methyl-D-galactose, 2-*O*- and 4-*O*-methyl-D-galactose, and D-galactose were isolated

The results of the methylation of phyllymenan must be treated with some caution because of the extreme difficulty encountered in methylating the polysaccharide, and hence the uncertainty of the extent of methylation The major methyl ether isolated, *viz*, 4,6-di-*O*-methyl-D-galactose could only have arisen from (1→3)-linked D-galactose 2-sulphate (or the corresponding 2-sulphated 6-*O*-methyl derivative) residues in the polymer The isolation of the 2,4,6-tri-*O*-methyl derivative suggests that not all of the (1→3)-linked D-galactose and/or 6-*O*-methyl-D-galactose residues are sulphated The 2,3,6-tri-*O*-methyl-D-galactose is considered to have arisen from (1→4)-linked D-galactose and (1→4)-linked 2-*O*-methyl-D-galactose residues in phyllymenan The 2,6-di-*O*-methyl-D-galactose could have arisen from (1→3)-linked D-galactose and/or 6-*O*-methyl-D-galactose residues carrying sulphate at position 4 The alternative units, *viz*, (1→4)-linked D-galactose 3-sulphate and the corresponding 6-*O*-methyl derivative, are alkali labile and are therefore ruled out. On the other hand, it may be due to undermethylation The D-galactose and mono-*O*-methyl-D-galactoses are almost certainly due to undermethylation, since their presence is not consistent with the following observations (*a*) the ester sulphate content of phyllymenan, (*b*) the absence of branching in the macromolecule, and (*c*) the high yield of 2,3,6-tri-*O*-methyl-D-galactose in the hydrolysate of methylated, desulphated phyllymenan.

At present, no unique structure can be proposed for phyllymenan However, the following partial structure is consistent with the accumulated evidence



R = H and less frequently CH_3, R′ = SO_3^- and less frequently H, R″ = CH_3 and less frequently H

A linear, repeating structure is shown, not because an alternating sequence of (1→3) and (1→4) linkages has been conclusively proved, but because the presence of approximately equal proportions of (1→3) and (1→4) linkages in the desulphated polymer is suggestive of an alternating structure The recent isolation in this laboratory of a trisaccharide[2], which furnishes **1** and **2** on partial hydrolysis, indicates that at least some regions of alternating structure do exist

EXPERIMENTAL

Concentration of solutions was carried out at 40°/20 mm, and specific rotations were measured in water Paper chromatography was carried out on Whatman No 1 paper with (*1*) 8 2 1 ethyl acetate–pyridine–water and (*2*) 18 3 1 4 ethyl acetate–acetic acid–formic acid–water *p*-Anisidine hydrochloride[5] and aniline–diphenylamine–phosphoric acid[6] are sprays *a* and *b*, respectively. Thin-layer chromatography (t l c) was carried out on glass plates coated with Silica Gel G containing calcium sulphate as binder, employing 85 7 butanone–water as solvent R_{Gal} and R_{TMG} values refer to the rates of travel of sugars relative to galactose and 2,3,4,6-tetra-*O*-methylgalactose, respectively. Infrared spectra were recorded on a Beckman IR-8 spectrophotometer

Desulphation of phyllymenan — Phyllymenan (10 g, $[\alpha]_D^{16}$ +63 3°, SO_4^{2-}, 19 62%) was shaken with 0 15M methanolic hydrogen chloride (750 ml) for 48 h at room temperature, and the desulphated polysaccharide was isolated in 72% yield as described previously[1] (Found SO_4^{2-}, 0 0%)

Methylation of desulphated phyllymenan — The polysaccharide (2 0 g) in methyl sulphoxide (50 ml) was treated with methyl sulphate (7 5 ml) in the presence of powdered sodium hydroxide (15 g) under an atmosphere of nitrogen The methyl sulphate was added in two equal portions at intervals of 1 h Further addition of methyl sulphate and sodium hydroxide was made after 2 h, and the mixture was vigorously stirred overnight, diluted with water, neutralised, and extracted with chloroform The washed chloroform extract was dried (Na_2SO_4) and evaporated to a glass (1 61 g) (Found OMe, 23 5%) Paper chromatography (solvent *1*) revealed the presence of galactose, 2-*O*- and 6-*O*-methylgalactose, 2,6- and 4,6-di-*O*-methylgalactose, 2,3,6- and 2,4,6-tri-*O*-methylgalactose, and 2,3,4,6-tetra-*O*-methylgalactose The partially methylated polymer (1 2 g) was dissolved in *N*,*N*-dimethylformamide (15 ml) and vigorously stirred with methyl iodide (15 ml) and silver oxide (15 g) for

24 h at room temperature. The product (1 04 g) was isolated after a further addition of methyl iodide and silver oxide (Found: OMe, 43.8%). A very small hydroxyl peak was observed in the i r spectrum (KBr disc)

Hydrolysis of desulphated, methylated phyllymenan, and separation of the products. — The methylated polysaccharide (0 54 g) was hydrolysed with N sulphuric acid for 6 h, neutralised ($BaCO_3$), and concentrated to a syrup (0 45 g) Paper chromatography (solvent *1*) revealed the presence of 2,3,6- and 2,4,6-tri-*O*-methyl- and 2,3,4,6-tetra-*O*-methylgalactose The syrup was applied to a column (48 × 4 8 cm) of charcoal–Celite and eluted with a linear gradient, from 0–2%, of aqueous butanone (5 4 litres) followed by 2–3% aqueous butanone (4 0 litres) Fractions (45 ml) were collected and, on the basis of paper chromatography (solvent *1*), were recombined into the following three fractions.

Fraction 1 (206 mg) was eluted with 2 02–2 18% aqueous butanone (630 ml) and was chromatographically indistinguishable (R_{TMG} 0 73, solvent *1*) from 2,4,6-tri-*O*-methylgalactose The syrup, $[\alpha]_D^{16}$ +88 3°, crystallised as the hydrate, m p 83–85°, on standing The aniline derivative, after recrystallisation from ethyl acetate, had m p 168–169° and mixed m p (with an authentic sample of 2,4,6-tri-*O*-methyl-*N*-phenyl-D-galactosylamine[3]) 169–170°

Fraction 2 (178 mg) was eluted with 2 25–2 4% aqueous butanone (580 ml) and was chromatographically indistinguishable (R_{TMG} 0 79, solvent *1*) from 2,3,6-tri-*O*-methylgalactose The syrup, $[\alpha]_D^{18}$ +97.3°, was converted into the lactone, which had m p and mixed m p (with authentic 2,3,6-tri-*O*-methyl-D-galactono-1,4-lactone[3]) 99–100°.

Fraction 3 (36 mg) was eluted with 2 65–2 80% aqueous butanone (600 ml) and was chromatographically indistinguishable (R_{TMG} 1; solvent *1*) from 2,3,4,6-tetra-*O*-methylgalactose The aniline derivative, after recrystallisation from ethanol, had m p. and mixed m p (with authentic 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine[7]) 188–189°.

Methylation of polysaccharide — The polysaccharide (26 2 g, $[\alpha]_D^{16}$ +63 3°, SO_4^{2-}, 19 62%) in water (500 ml) was treated slowly and simultaneously (under an atmosphere of nitrogen) with methyl sulphate (150 ml) and 30% aqueous sodium hydroxide (450 ml) with vigorous stirring during 7 h The mixture was then stirred for a further 17 h After repeating the methylation procedure a further six times, the mixture was dialysed against running tap-water (10 days) and concentrated to *ca* 500 ml, and the addition of the above reagents was repeated a further six times The polysaccharide (24 g) was isolated from the mixture by freeze-drying, after dialysis and concentration T l c (spray *b*) of a neutralised acid-hydrolysate revealed galactose together with a large number of its methyl ethers The methylation procedure was therefore repeated a further five times on a portion (16 g) of the partially methylated material The polysaccharide (15 8 g) was isolated as above (Found OMe, 17.1; N, 0 0; SO_4^{2-}, 16 38%) T l c of a hydrolysate revealed the presence of galactose, 4-*O*-methyl-, 2-*O*-methyl-, 2,6-di-*O*-methyl-, 4,6-di-*O*-methyl-, 2,3,6-tri-*O*-methyl-, and 2,4,6-tri-*O*-methyl-galactose, together with minute traces of 2,3,4-tri-*O*-methyl-

and 2,3,4,6-tetra-*O*-methyl-galactose The partially methylated polymer (2 3 g) was mixed with dry methyl sulphoxide and vigorously stirred for 2 h at room temperature to disperse the polysaccharide Silver oxide (30 g) and methyl iodide (60 ml) were added, and the mixture was stirred for 24 h at room temperature Two further additions of Purdie's reagents[4] were made, and each time the mixture was stirred for 24 h at 50° A large volume of water was added to precipitate the salts, the mixture was centrifuged and dialysed, and the polysaccharide (1 1 g) was isolated by freeze-drying (Found OMe, 24 7; N, 0 27; SO_4^{2-}, 6 7%) A very small hydroxyl peak was observed in the i r spectrum (KBr disc)

Hydrolysis of methylated polysaccharide and separation of the products — The methylated polysaccharide (1 g) and N sulphuric acid (15 ml) were heated on a boiling water-bath for 16 h The hydrolysate was neutralised with barium carbonate, filtered, and concentrated to a brown syrup (0 9 g), which was applied to a cellulose column (45 × 5.4 cm) and the methylated sugars were eluted with solvent *2*. Each fraction (*ca* 60 ml) was analysed for sugars by t l c (spray *b*), and similar fractions were combined, evaporated to dryness under reduced pressure, and extracted with ethyl acetate or methanol The extract, after removal of the solvent, was placed under high vacuum to remove the last traces of acetic acid, the following products were subsequently identified

Tubes 1–5 contained the degradation products (86 mg)

Tubes 6 and 7 contained 2,3,4,6-tetra-*O*-methylgalactose (28 mg) The syrup was decolourised with charcoal and converted into a crystalline aniline derivative which, after recrystallisation from ethanol, had m p and mixed m p (with authentic 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine) 188–189°, lit[8] m p 189–190°.

Tube 8 gave a syrup (30 mg), $[\alpha]_D^{17}$ +89° (*c* 0 56), chromatographically (R_{TMG} 0 86, grey spot, spray *b*) identical with 2,3,6-tri-*O*-methylgalactose The derived lactone had m p and mixed m p. (with authentic 2,3,6-tri-*O*-methyl-D-galactono-1,4-lactone[3]) 99–100°

Tubes 9 and 10 contained a mixture (102 mg) of 2,3,6-tri-*O*-methylgalactose (R_{TMG} 0 86, grey spot, spray *b*) and 2,4,6-tri-*O*-methylgalactose (R_{TMG} 0 66, blue-green spot, spray *b*) The 2,3,6-isomer constituted *ca* 80% of the mixture

Tubes 11–14 afforded a syrup (67 mg) which was shown by paper and thin-layer chromatography to be a mixture of 2,3,6-tri-, 2,4,6-tri-, and 2,6-di-*O*-methylgalactose, together with a trace of 2,3,4-tri-*O*-methylgalactose and an unidentified sugar (R_{TMG} 0 7, Whatman No 1, carmine spot, spray *a*).

Tube 15 contained no carbohydrate

Tube 16 afforded a syrup (51 mg, R_{TMG} 0 53; grey spot, spray *b*) which crystallised from ethyl acetate and, after recrystallisation from the same solvent, had $[\alpha]_D^{16}$ +47 1 (2 min) → +85 8° (*c* 0 7), m p. and mixed m p (with authentic 2,6-di-*O*-methyl-D-galactose) 128–129°, lit[8] $[\alpha]_D$ +45→ +88°, m p 128 5–130°.

Tubes 17 and 18 contained a mixture (103 mg) of 2,6- (R_{TMG} 0 53) and 4,6-di-*O*-methylgalactose (R_{TMG} 0 33, blue spot, spray *b*) The 4,6-dimethyl ether, which

constituted *ca.* 70% of the mixture, readily crystallised when the syrup was mixed with ethyl acetate

Tubes 19–24 afforded a syrup (103 mg) which crystallised (charcoal) from ethyl acetate The product (m p 140–142°), after two recrystallisations from the same solvent, had m p 146–147°, $[\alpha]_D^{16}$ +131 2 (2 min)→ +72° (*c* 0 64) The mixed m p with a sample of 4,6-di-*O*-methyl-D-galactose[3] isolated from methylated aeodan was 142° The infrared spectra of the two sugars were identical The derived phenylozasone had m p and mixed m p 154–155°

Tubes 25–35 contained no carbohydrate

Tubes 36–41 contained 2-*O*-methylgalactose (58 mg, R_{Gal} 2 2, solvent *2*) which crystallised from ethanol–ethyl acetate and, after recrystallisation from the same solvent, had $[\alpha]_D^{16}$ +85 2° (*c* 0 74), m p and mixed m p (with authentic 2-*O*-methyl-D-galactose[1]) 147–149°

Tube 42 contained no carbohydrate

Tubes 43–54 afforded a chromatographically pure syrup (99 mg, R_{Gal} 1 80, solvent *2*) which crystallised from ethanol and, after recrystallisation from ethanol–ethyl acetate, had $[\alpha]_D^{17}$ +49 (5 min)→ +73 0° (*c* 0 63), m p and mixed m p (with authentic 4-*O*-methyl-D-galactose[3]) 202–204°

Tubes 55–56 contained no carbohydrate

Tubes 57–63 contained galactose (28 mg), $[\alpha]_D^{16}$ +81° (*c* 0 66), m p and mixed m p 166–167° The derived galactaric acid had m p and mixed m p 212–213°

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Carbohydrate Research
Elsevier Publishing Company Amsterdam
Printed in Belgium

IDENTIFICATION OF LINKAGES OF A GALACTOMANNAN ISOLATED FROM SEED OF *Caesalpinia pulcherima*

A M UNRAU AND Y M CHOY
Department of Chemistry, Simon Fraser University, Burnaby 2, British Columbia (Canada)
Received November 29th, 1969)

ABSTRACT

A galactomannan, purified by repeated complex formation with copper acetate, was shown to be composed of D-mannose and D-galactose in a molar ratio of 3 1 From methylation experiments, the following partially methylated sugars were identified 2,3,4,6-tetra-*O*-methyl-D-mannose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-mannose, 2,4,6-tri-*O*-methyl-D-mannose, 3,4,6-tri-*O*-methyl-D-mannose, and 2,3-di-*O*-methyl-D-mannose (molar ratio 1 27 60 4 2 32) Ordinary and sequential (Smith) periodate degradation indicated that the D-galactose units occupied single unit branch points linked (1→6), approximately one in every twenty D-mannose units was resistant to periodate attack, the occurrence of small and equimolar quantities of 2-*O*-β-D-mannopyranosyl-D-erythritol and *O*-β-D-mannopyranosyl-(1→3)-*O*-β-D-mannopyranosyl-(1→2)-D-erythritol indicates a low frequency of isolated (1→3) linkages and consecutive (1→3) linkages A relatively simple structure may be assigned, based upon the experimental results

INTRODUCTION

A rather large number of gums of varying composition and varying degrees of structural complexity have been isolated from seed of legumes Ordinarily, the neutral gums mostly found in the endosperm are less complex structurally (composed of D-mannose and D-galactose in varying ratios) than the acidic polysaccharides which are most characteristically associated with seed coats and usually contain more than two different building units

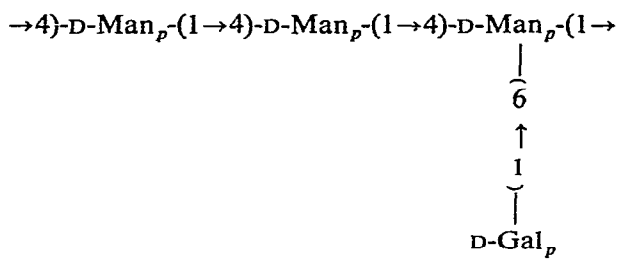
DISCUSSION

Extraction of fractured seed of *Caesalpinia pulcherima* with hot water resulted, upon cooling, in a highly viscous solution Precipitation with ethanol gave a characteristic greyish, stringy precipitate The polysaccharide was purified by repeated complex formation with copper acetate[1, 2], $[\alpha]_D^{22}$ +6° in 0 1 M sodium hydroxide

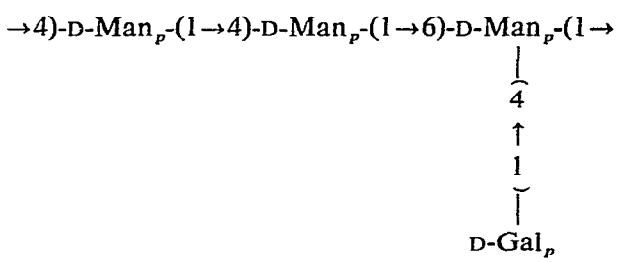
This low value suggests that the glycosidic linkages in the main chain have a β-D anomeric configuration Since the galactomannan was unaffected by prolonged treatment with emulsin, it would appear that the D-galactose units are attached by α linkages Ultracentrifugation showed a single peak, thus indicating that the polysaccharide was relatively homogeneous Hydrolysis of the polysaccharide gave D-mannose and D-galactose in a molar ratio of 3 1 The D-galactose content is higher than that found in some of the more commonly known galactomannans, such as guar gum (16% D-galactose), Carob bean (14–27% D-galactose), and Kentucky coffee bean (20% D-galactose), although some galactomannans are known to contain up to 50% D-galactose[3].

Hydrolysis of the fully methylated gum gave the following partially methylated sugars 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,4,6-tetra-*O*-methyl-D-mannose, 2,4,6-tri-*O*-methyl-D-mannose, 2,3,6-tri-*O*-methyl-D-mannose, 3,4,6-tri-*O*-methyl-D-mannose, and 2,3-di-*O*-methyl-D-mannose in the molar ratio 27 1 4 60 2 32 (by g l c) The isolation of these partially methylated sugars is in agreement with the periodate oxidation results (see next paragraph), namely approximately one out of every twenty D-mannose units is linked (1→3) In addition, the occurrence of a small number of (1→2) linkages was indicated by the identification of the requisite trimethyl sugar All of the D-galactose units occupy terminal non-reducing positions, since only 2,3,4,6-tetra-*O*-methyl-D-galactose was found, and the ratio of tetra-*O*-methyl-D-galactose to di-*O*-methyl-D-mannose was essentially 1 1 (the slightly lower proportion of tetra-*O*-methyl-D-galactose, as reported in Table I, is probably due to inadvertent loss during evaporation) The absence of 2,4-di-*O*-methyl-D-mannose indicated the absence of (1→3)-linked mannose units associated with branching at C-6 The approximate D P of the repeating unit based on the amount of tetra-*O*-methyl-D-mannose found was 120 The behavior on ultracentrifugation of the polysaccharide indicated an approximate molecular weight of 60,000, hence three repeating units of an approximate D P of 120 each could account for one single polymeric molecule Further structural details were obtained from ordinary and sequential (Smith) periodate degradation studies.

Treatment of the polysaccharide with periodate at 4–5° resulted in periodate consumption of 1 2 mole per hexose unit Reduction of the polyaldehyde with borohydride, followed by mild acid hydrolysis (Smith degradation) gave glycerol, erythritol (no threitol), and two non-reducing components having paper chromatographic mobilities lower than that of D-galactose Analysis of the hydrolyzate by the phenol–sulfuric acid method[4] indicated that 4 to 6% of hexoses had survived the periodate attack The appearance of glycerol can be attributed to terminal, non-reducing D-mannose and D-galactose units, mostly the latter, since no D-threitol was detected D-Threitol would arise from internal D-galactose units linked (1→4) The major quantity of erythritol produced shows that the main chain of the polysaccharide is composed of D-mannopyranose units predominantly linked (1→4) D-Galactose units are linked (1→6) to approximately every third D-mannose unit of the main chain, *e g.*,



or (1→4) to a D-mannose unit linked at C-6, *e g* ,



However, structural studies of a considerable number of galactomannans have shown that the D-galactose units are ordinarily linked (1→6) to the D-mannose units of the main chain[3] The presence of two non-reducing oligosaccharide fractions in a molar ratio approximately 1 1 indicated that a very small proportion of linkages other than (1→4) and (1→4)–(1→6) existed Hydrolysis of component I (R_{Man} 0.64), gave D-mannose and erythritol in a molar ratio of 1 1 This finding indicates that a small proportion of D-mannose units were linked (1→3) and these units were flanked by (1→4)-linked units, *e g* ,

$$\text{-D-Man}_p\text{-(1}\rightarrow\text{4)-D-Man}_p\text{-(1}\rightarrow\text{3)-D-Man}_p\text{-(1}\rightarrow\text{4)-D-Man}_p\text{-}$$

Hydrolysis of oligosaccharide II (R_{Man} 0 35) gave D-mannose and erythritol in a ratio of 2 1 This indicated that a small proportion of D-mannose units were consecutively linked (1→3), *e g* ,

$$\text{-D-Man}_p\text{-(1}\rightarrow\text{4)-D-Man}_p\text{-(1}\rightarrow\text{3)-D-Man}_p\text{-(1}\rightarrow\text{3)-D-Man}_p\text{-(1}\rightarrow\text{4)-D-Man}_p\text{-}$$

Sequential (Smith) periodate degradation of II gave a non-reducing component (shown by hydrolysis to consist of D-mannose and glycerol in a 1 1 molar ratio), glycerol, and formaldehyde Formaldehyde would be expected from the C-4 fragment of the erythritol moiety of II, and glycerol from the terminal, "non-reducing" (1→3)-linked D-mannose unit while the internal (1→3)-linked D-mannose unit would survive

The low incidence of (1→2) linkages in the main D-mannose chain, as indicated by the identification of the requisite 3,4,6-tri-*O*-methyl-D-mannose (less than 2%) in methylation experiments is at least one factor which prevents a detailed representation of the possible molecular structure In the absence of detailed sequential linkage analysis, a highly randomized structure would appear the most appropriate consideration

EXPERIMENTAL

General methods — All evaporations were performed under reduced pressure Whatman No. 1 and 3M paper was used in qualitative and quantitative identifications The solvent systems used were *A*, 4 1 1 ethylacetate–acetic acid–water, *B*, 8 2 1 ethylacetate–pyridine–water, *C*, butanone–water (azeotrope), *D*, 4 1 5 butyl alcohol–ethanol–water, and *E*, 85 15 1 benzene–ethanol–water Chromatograms were sprayed with *p*-anisidine–trichloroacetic acid (*F*)[6] or Tollens solution (*G*)[7] The mobilities are reported relative to the front of the solvent (R_F) or to the mobility of 2,3,4,6-tetra-*O*-methyl-D-glucose (R_{TG}) Melting points reported are uncorrected

Isolation of the galactomannan — A slurry of fractured seed in boiling water was stirred for 3–4 h, after which the solids were removed by centrifugation The cooled, viscous solution was slowly poured, with vigorous stirring, into 4 vol of ethanol whereupon a light greyish, stringy precipitate formed The solid material was removed by filtration, washed with acetone, and dried in a desiccator

Purification by precipitation with copper acetate — To a portion (8 g) of polysaccharide in 0 5M sodium hydroxide (100 ml) was added with stirring 5% copper acetate (300 ml) The gelatinous precipitate that formed was removed by centrifugation Further addition of an equal volume of copper acetate solution to the supernatant and addition of an equal volume of ethanol (95%) resulted in precipitation of a copper complex which was not investigated at this time The gelatinous complex was suspended in 95% ethanol (200 ml) and acidified with 2M hydrochloric acid (20 ml), and after maceration in a Waring blender, the fluffy, greyish precipitate was removed by centrifugation and washed free of acid with alcohol Complex formation with Cu^{2+} was repeated twice more, as described, resulting in no observable change in the ratio of the sugar components (see succeeding sections), yield 5 g, $[\alpha]_D^{22}$ +6° (*c* 0 5, 0 1M sodium hydroxide)

Ultracentrifugation — Solutions of galactomannan (0 5 to 1%) in alkali (1 to 5M sodium hydroxide) were centrifuged for 2 h, at a rotor speed of 60,000 r p m The sedimentation pattern of 0 8% galactomannan in 5M sodium hydroxide indicated an approximate molecular weight of 60,000

Hydrolysis of polysaccharide — (*a*) *With* M *sulfuric acid* A sample (250 mg) of the polysaccharide in M sulfuric acid (25 ml) was heated for 8 h at reflux temperature After neutralization with barium carbonate, the filtrate was passed through weak anion (Duolite A-4) and cation (IR-120) exchange-resin columns, and the solution was evaporated The syrupy residue was chromatographed on Whatman No 1 paper in solvents *A* and *B* After spraying with reagent *E*, D-mannose and D-galactose were identified as the only sugars present Separation on Whatman No 3MM of a portion (100 mg) of the mixture with solvent *B* gave D-mannose (60 mg) which was characterized as the phenylhydrazone, m p 199°, $[\alpha]_D^{22}$ +33° (*c* 0 8, pyridine) and D-galactose (25 mg), $[\alpha]_D^{22}$ +80° (*c* 0 7, water) The molar ratio of the two sugars was determined by elution of each sugar from a single chromatogram followed by analysis with the phenol–sulfuric acid method[4], and by g l c of the mixture of trimethylsilyl derivatives[7]

on a 5 ft × ¼ in stainless steel column packed with 20% SE-30 on HMDS treated chromosorb W. A D-mannose to D-galactose ratio of 3 1 was obtained in all cases

(*b*) *Hydrolysis with emulsin* To a sterile solution of galactomannan (25 mg) (15 ml of phosphate buffer, pH 7.0) was added emulsin (10 mg) The solution was kept for 3 days at 25° No observable change in the reducing power (3,5-dinitrosalicylic acid reagent) was observed The solution was evaporated and the residue was shown, by paper chromatography (Whatman No 1) in solvents *A* and *B* and using reagents *F* and *G*, to contain no detectable D-galactose (or D-mannose) and no evidence of smaller oligomers

Methylation analysis — A portion (2 0 g) of the galactomannan was initially treated with 30% sodium hydroxide and methyl sulfate, followed by treatment with sodium hydride in methyl sulfoxide and methyl iodide[8] The fully methylated polymer (1 5 g) (no OH-absorption evident in the i r spectrum of liquid film) was dissolved in cold 75% sulfuric acid (15 ml) The mixture was diluted with ice–water to approximately M acid concentration, and the solution was boiled for 7 h[9] A portion (900 mg) of the syrupy mixture (1 2 g), which was obtained after neutralization (barium carbonate) and evaporation of the filtrate, was analyzed by paper chromatography (Whatman No 1) using solvents *C*, *D*, and *E* The molar ratio of the partially methylated sugars was determined by the phenol–sulfuric acidproced ure and by g l c of the trimethylsilyl derivatives[10] (Table I) The mixture was separated on Whatman 3MM

TABLE I

GAS-LIQUID CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES OF PARTIALLY METHYLATED SUGARS[a]

Trimethylsilyl derivatives	*Retention time (min)*	*Molar ratio*
2,3,4,6-Tetra-*O*-methyl-D-mannose	16 5	1
2,3,4,6-Tetra-*O*-methyl-D-galactose	15 2 (α) 18 9 (β)	27
2,4,6-Tri-*O*-methyl-D-mannose	12 0	4
3,4,6-Tri-*O*-methyl-D-mannose	10 4	2
2,3,6-Tri-*O*-methyl-D-mannose	13 4	60
2,3-Di-*O*-methyl-D-mannose	8 6	32

[a]Neopentylglycolsuccinate (20%) on Gas Chrom 900, 3/16 in by 12 ft copper column, operating temp 180°, flame-ionization detector

paper in solvent *C* (Table II), and the individual, partially methylated sugars were characterized as follows

Component 1 and synthetic 2,3,4,6-tetra-*O*-methyl-D-mannose showed identical R_F (solvent *C*, 0 79), R_{TG} (solvent *D*, 0 96), and retention time of the trimethylsilyl derivative (16 5 min)

Component 2 gave, upon treatment with aniline in dry ethanol containing a trace of glacial acetic acid, 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine, m p

TABLE II
PAPER CHROMATOGRAPHY OF PARTIALLY METHYLATED SUGARS

Component	*Weight (mg)*	*Butanone–water azeotrope R_F*	*Butyl alcohol–ethanol–water R_{TG}*[a]	*$[\alpha]_D$ (degrees)*[b]	*Molar ratio*[c]	*Proposed compound*[c]
1	—	0 79	0 96	—	1	2,3,4,6-tetra-*O*-methyl-D-manno
2	120	0 69	0 88	+118		2,3,4,6-tetra-*O*-methyl-D-galacto
3	11	0 56	0 82	+150	2	2,4,6-tri-*O*-methyl-D-mannose
4	210	0 50	0 81	−9		2,3,6-tri-*O*-methyl-D-mannose a 3,4,6-tri-*O*-methyl-D-mannose
5	120	0 22	0 57	−16	1	2,3-di-*O*-methyl-D-mannose

[a]Mobility relative to 2,3,4,6-tetra-*O*-methyl-D-glucose [b]In water [c]From chromatographic results in butanon water azeotrope

190–191°, $[\alpha]_D^{22}$ +40° (*c* 0 5, acetone)[11] Component 2 and authentic 2,3,4,6-tetra-*O*-methyl-D-galactose showed identical R_F (solvent *C*, 0 69), R_{TG} (solvent *D*, 0 88), and retention time of trimethylsilyl derivative (15 2 min, α-anomer, 18 9 min, β-anomer)

Component 3 was treated with aniline, as described for component 2, to give 2,4,6-tri-*O*-methyl-*N*-phenyl-D-mannosylamine, m p 133–135°, $[\alpha]_D^{22}$ −8° (*c* 0 3, methanol)[12] The identity of component 3 was further established by the following treatments A small portion (about 6 mg) was reduced with sodium borohydride (24 h, room temp.) followed by treatment with methanol–hydrogen chloride (1%) to remove borate ions The product was treated with periodic acid (0 1M) for 2 days at 5° The solution (10 ml) was treated with barium carbonate to remove iodate and periodate ions, and then reduced with sodium borohydride as described in the preceding paragraph Borate ions were removed, and after evaporation, the residue was demethylated with 35% hydrobromic acid for 60 min at 100° D-Mannitol was identified by comparative paper chromatography and g l c of the corresponding trimethylsilyl derivative

A solution of component 4 in dry pyridine was treated with a 10% molar excess of *p*-nitrobenzoyl chloride After being heated for 30 min to 65°, the mixture was cooled, and after addition of a saturated solution of sodium hydrogen carbonate, extracted with chloroform Evaporation and recrystallization of the residue from methanol gave 1,4-di-*p*-nitrobenzoyl-2,3,6-tri-*O*-methyl-D-mannose (95% yield), m p 187–189°, $[\alpha]_D^{22}$ +33° (*c* 1 0, chloroform)[13] T l c on silica gel, showed 3 components in solvent *C* (R_{TG} 0 78, 0 76, and 0 68) and in solvent *E* (R_{TG} 0 52, 0 50, and 0.45), thus indicating three tri-*O*-methyl sugars G l c of trimethylsilyl derivatives showed the presence of 2,4,6-tri-*O*-methyl-D-mannose, 12 0 min (minor proportion), 3,4,6-tri-*O*-methyl-D-mannose, 10 4 min (minor proportion), and 2,3,6-tri-*O*-methyl-D-mannose, 13 4 min (major proportion) A portion (100 mg) of component 4 was subjected to the following series of reactions reduction with borohydride, oxidation with periodic acid, reduction with borohydride, demethylation with 35% hydrobromic

acid, deionization, and identification by paper and g l c of the degradation products Ethylene glycol and erythritol indicated the presence of 2,3,6-tri-*O*-methyl-D-mannose D-Arabinitol was identified by comparison with authentic D-arabinitol, the retention time of the trimethylsilyl derivative of authentic D-arabinitol and that obtained from component 4 were identical (3 4 min, 170°, other conditions as described previously) D-Arabinitol indicated the presence of 3,4,6-tri-*O*-methyl-D-mannose (or 2,3,4-tri-*O*-methyl-D-mannose, however, the absence of this sugar was established previously by g l c , see Table II) D-Mannitol was identified by comparison with an authentic compound, the trimethylsilyl derivatives of both having retention times of 4 4 min (170°, other conditions as described previously) D-Mannitol indicated the presence of 2,4,6-tri-*O*-methyl-D-mannose.

Component 5 gave upon treatment with *p*-nitrobenzoyl chloride, as previously described, 1,4,6-tri-*O*-*p*-nitrobenzoyl-2,3-di-*O*-methyl-D-mannose, m.p. 192–194°, $[\alpha]_D^{22}$ +65° (*c* 0 5, chloroform)[3]

Sequential periodate (Smith) degradation — To a portion (1 g) of galactomannan dissolved in water (400 ml) was added 0 1M periodic acid (100 ml), and the solution was kept at 5° Periodic acid consumption reached 1 2 mole per hexose unit in 3 days with little change thereafter To the polyaldehyde was added sodium borohydride (1 g), and the solution was kept overnight at room temperature Analysis of the solution of polyalcohol with phenol–sulfuric acid indicated an intact hexose content to the extent of 4–6%, in terms of D-mannose, of the original polysaccharide The solution was passed through a column of Amberlite IR-120 (H^+) and evaporated, and the borate ions were removed by repeated evaporation with methanol The residue was dissolved in 0 5M hydrochloric acid (50 ml), and the solution was kept for 8 h at room temperature The solution was deionized with ion-exchange resins Paper chromatography in solvents *A* and *B* indicated the presence of glycerol and erythritol, in the molar ratio 1 2 85 as determined by the periodate–chromotropic acid method[14], and of two non-reducing compounds having R_{Man} 0 64 (Compound I) and R_{Man} 0 35 (Compound II) The compounds were separated by paper chromatography (solvent *B*, Whatman 3MM paper) and characterized as follows

Glycerol (yield about 50 mg) was dissolved in dry pyridine A 10% molar excess *p*-nitrobenzoyl chloride was added, and the mixture was heated for 30 min to 75° After cooling to room temperature, an excess saturated sodium hydrogen carbonate solution was added, and the precipitate was collected and washed with water The tri-*p*-nitrobenzoate, after recrystallization from ethanol, had m p 191° (undepressed mixed m p)

Erythritol (yield about 140 mg) was treated in the same manner as glycerol. The tetra-*p*-nitrobenzoate had m p 250° (undepressed mixed m p)

Compound I (yield about 4 mg) was hydrolyzed in M sulfuric acid (10 ml) (reflux temp , 6 h) The hydrolyzate was shown by paper chromatography (Whatman No 1) in solvents *A* and *B* and spray reagent *G*, to contain D-mannose and erythritol in a 1 1 molar ratio (D-mannose, by phenol–sulfuric acid, erythritol, determined as formaldehyde by the chromotropic acid procedure)

Compound II (yield about 7 mg) A portion (about 3 mg) was hydrolyzed as previously described for compound I, and the hydrolyzate was shown to contain D-mannose and erythritol in a 2 1 molar ratio The remaining portion of II was degraded with periodate (Smith degradation, as previously described) whereupon 1 molar proportion of formaldehyde was produced (analyzed before borohydride reduction of polyaldehyde) Glycerol and a non-reducing compound III having a slightly faster mobility than I were formed upon mild acid hydrolysis of the polyalcohol Complete hydrolysis of III, as described for I, gave D-mannose and glycerol in a 1 1 molar ratio (glycerol determined as formaldehyde by the chromotropic acid procedure)

ACKNOWLEDGMENT

The authors gratefully acknowledge financial support from the National Research Council of Canada

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Carbohydrate Research
Elsevier Publishing Company Amsterdam
Printed in Belgium

ETHYNYLATION OF 1,2-*O*-ISOPROPYLIDENE-α-D-*xylo*-PENTODIALDOSE DERIVATIVES A SYNTHETIC ROUTE TO URONIC ACIDS*†

D HORTON AND FRANCES O SWANSON
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210 (U S A)

(Received December 16th, 1969)

ABSTRACT

Ethynylation of periodate-oxidized 1,2-*O*-isopropylidene-α-D-glucofuranose gave 6,7-dideoxy-1,2-*O*-isopropylidene-β-D-*ido*-hept-6-ynofuranose (**2**) together with a proportion of the α-D-*gluco* analog **6**, ozonolysis of **2** and **6** gave 1,2-*O*-isopropylidene-β-L-idofuranurono-6,3-lactone (**8**) and 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone (**7**), respectively The heptyne **2** gave a crystalline 3,5-dibenzoate **3**, which on ozonolysis gave 3,5-di-*O*-benzoyl-1,2-*O*-isopropylidene-β-L-idofuranuronic acid (**4**), saponification of **4** with methanolic sodium methoxide led to epimerization at C-5 Ethynylation of 3-*O*-benzyl-1,2-*O*-isopropylidene-5-*aldehydo*-D-*xylo*-pentodialdo-1,4-furanose (**9**) (characterized as its *p*-nitrophenylhydrazone, **13**) gave a separable, 3 4 mixture of 3-*O*-benzyl-6,7-dideoxy-1,2-*O*-isopropylidene-α-D-*gluco*-hept-6-ynofuranose (**14**) and the β-L-*ido* epimer (**10**), ozonolysis of **14** and **10** followed by hydrogenolysis gave the lactones **8** and **7**, respectively Crystalline 5-benzoates **11** and **15** were obtained from **10** and **14**, respectively Ozonolysis of **11** gave 5-*O*-benzoyl-3-*O*-benzyl-1,2-*O*-isopropylidene-β-L-idofuranuronic acid (**12**), similar treatment of **15** gave the α-D-*gluco* analog (**16**) of **15** Hydrogenolytic cleavage of the benzyl groups, and catalytic de-esterification, of **12** and **16** led to mixtures of the lactones 7 and **8** through epimerization at C-5

INTRODUCTION

The addition of unsaturated Grignard reagents to *aldehydo* sugar derivatives was reported in 1965 by this laboratory[1,2] The reaction is useful in synthetic sugar chemistry because the resultant acetylenic and vinylic derivatives are capable of undergoing a wide range of useful transformations, leading to higher aldoses[1,2,4,5], aldonic acids[1,2,4,5] unsaturated aldehydes[1,2], alditols[2,5,6], 1,2-dideoxyalditols[1,2,4,5], 1-deoxyketoses[2,6], 1,2-anhydroalditols[2], allenes[6], and various other products[7]

*Part VIII in the series "Extension of Sugar Chains Through Acetylenic Intermediates" For preliminary reports of part of this work see references 1 and 2 For part VII of this series see reference 3

†Supported in part, by the National Institute of General Medical Sciences, National Institutes of Health, U S Public Health Service Grant No GM-11976-04 (The Ohio State University Research Foundation Project 1820)

The present paper describes ethynylation of *ω-aldehydo* sugar derivatives and the conversion of the resultant sugar derivatives having the ethynyl group as the non-reducing terminus into uronic acids The fact that the secondary alcohol group adjacent to the ethynyl group is not epimerized by acid or base under the normal conditions of glycoside hydrolysis or saponification of acyl groups[7] makes the ethynylated derivatives attractive as intermediates for synthesis of uronic acids and their derivatives The great facility with which uronic acids and their derivatives are epimerized, in the presence of base, at the position α to the carboxylate group, can be troublesome in synthetic sequences. With the use of the ethynyl group as a "latent" carboxylic acid group it should be possible to retain configurational integrity even under basic conditions, and the carboxylic acid group can be generated by ozonolysis of the acetylene under neutral conditions as a late step in the synthetic sequence

The examples presented describe ethynylation of the products of periodate oxidation of 1,2-*O*-isopropylidene-α-D-glucofuranose and its 3-benzyl ether, separation of the 5-epimeric, 7-carbon acetylenic sugars produced, and conversion of the products into derivatives of D-glucuronic and L-iduronic acids

DISCUSSION

In an earlier report[1] it was shown that ethynylmagnesium bromide reacts to give a propargylic alcohol with the product formed[8] by oxidation of 1,2-*O*-isopropylidene-α-D-glucofuranose with lead tetraacetate, even though the oxidation product is not a free aldehyde but is a mixture of a dimer[9,10] (**1**) and an adduct[9] with formaldehyde for which the structure **5** has been proposed[11]. In the present work the oxidation was performed with aqueous sodium metaperiodate in the presence of a large excess of formaldehyde As judged by t l c the product was approximately a 9 1 mixture of the dimer **1** and the formaldehyde adduct **5** This mixture was treated with

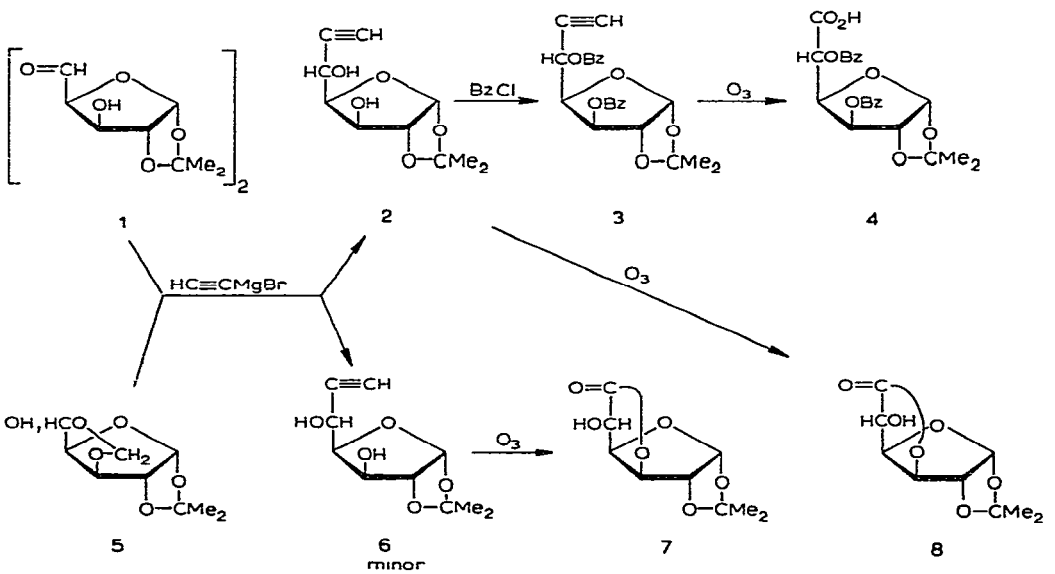


TABLE I

CHEMICAL SHIFTS MEASURED AT 100 MHz IN CHLOROFORM-*d*

Compound	*Solvent*	*Chemical shifts* (τ) *from* 100-MHz *spectra*[a]									
		H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-7*	*Aryl*	*Benzylic*	CMe_2 (*s*)	*Other*
3	C_6D_6	4 22d	5 66d	4 00d	4 91q	3 45q	8 16d	1 8, 2 8		8 55, 8 92	
4	$CDCl_3$	3 92d	5 32d	4 35d	5 08q	4 24d		2 05, 2 64		8 45, 8 69	0 40[b]
9	$CDCl_3$	3 94d	5 39d	5 72d	5 48q	0 37d		2 76	5 42, 5 61[c]	8 58, 8 72	
10	C_6D_6	4 17d	5 67d	5 98d	5 53q	5 15q[d]	7 88d	2 82	5 70s	8 66, 8 90	7 18[b]
11	C_6D_6	4 18d	5 65d	5 83d	5 12q	3 58q	8 00d	1 9, 2 9, 2 83	5 69s	8 58, 8 90	
12	$CDCl_3$	3 98d	5 37d	4 39d	5 11q	4 28d		2 09, 2 7	6 33s	8 49, 8 71	1 75[b]
13	$(CD_3)_2CO$	4 03d	5 18d	5 90d	5 22q	2 60d		1 88, 2 68, 2 88	5 25, 5 45[c]	8 52, 8 68	−0 25[e]
14	C_6D_6	4 16d	5 72d	5 88d	5 62q	5 21q[d]	7 95d	2 85	5 73s	8 68, 8 93	6 93[b]
15	C_6D_6	4 13d	5 62d	5 99d	5 19q	3 70q	7 87d	2 05, 2 90	5 75, 5 94[c]	8 56, 8 83	
16	$CDCl_3$	3 97d	5 34d	4 33d	5 22q	4 54d		2 1, 2 65	6 60s	8 45, 8 70	2 31[b]

[a]First-order values are given Peak multiplicities d, doublet, q, quartet, s, singlet [b]One proton, OH, disappears on deuteration [c]2-Proton AB system, J_{AB} 11 Hz [d]After deuteration [e]NH proton

ethynylmagnesium bromide in tetrahydrofuran to give a syrupy mixture of 5-epimeric, acetylenic sugar derivatives (**2** and **6**). Ozonolysis of this mixture gave one product having the same characteristics by t l c as 1,2-*O*-isopropylidene-β-L-idofuranurono-6,3-lactone (**8**), together with a second product having t l.c characteristics of 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone (**7**) Fractional distillation of the mixed acetylenic derivatives (**2** and **6**) gave one epimer essentially pure, as a liquid in 34% yield The latter was identified as the β-L-*ido* derivative **2** by the fact that upon ozonolysis in carbon tetrachloride it gave the β-L-*ido* lactone (**8**), identical by mixed m p , X-ray powder diffraction pattern, and t l c with an authentic sample, the absence of the α-D-*gluco* lactone (**7**) was clearly established by t l c

The β-L-*ido* acetylenic derivative (**2**) gave a crystalline 3,5-dibenzoate (**3**) that melted sharply at 195–196° and gave a satisfactory elemental analysis It showed i r spectral absorptions at 3 06 and 4 71 μm, typical of ≡C–H and C≡C stretching frequencies The n m r spectrum of **3** showed a 1-proton doublet at τ 8 16 for the acetylenic proton (H-7) showing a coupling of 2 Hz with H-5 (full details of 100 MHz n m r data are given in Tables I and II) The signal for H-5 is observed at low field (τ 3 45) because of the deshielding effect of the 5-benzoyloxy group, the signal is a sharp quartet showing the $J_{5,7}$ spacing and also a spacing of 9 0 Hz corresponding to $J_{4,5}$ and indicating that H-4 and H-5 are antiparallel in the favored conformation of **3** The evidence clearly establishes that the product is the single 5-epimer **3** Compound **3** was identical by X-ray powder diffraction pattern with a product previously prepared by J B Hughes[1], and the tentative configurational assignment[1] at C-5 on the latter product, made from chromatographic data, must be reversed on the basis of present evidence

TABLE II

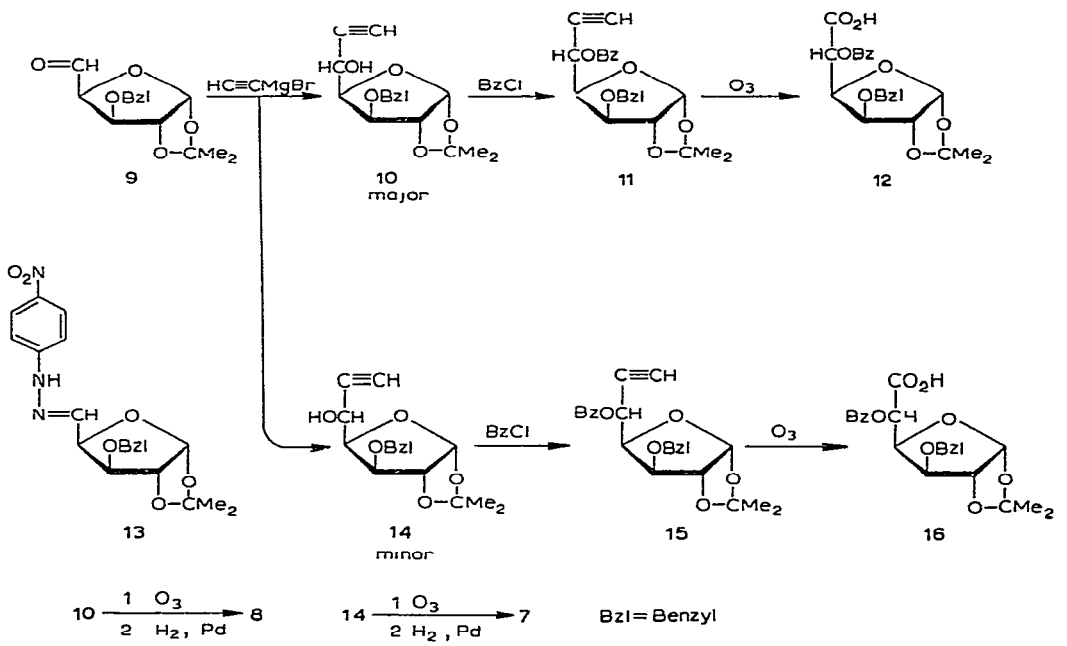
FIRST-ORDER COUPLING CONSTANTS (Hz) FROM 100-MHz SPECTRA

Compound	*Solvent*	*Coupling constants (Hz) from 100-MHz spectra*				
		$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,7}$
3	C_6D_6	4 0	0	3 0	9 0	2 0
4	$CDCl_3$	4 0	0	3 5	6 0	
9	$CDCl_3$	4 0	0	3 8	2 0	
10	C_6D_6	4 0	0	3 0	8 0	2 0
11	C_6D_6	4 0	0	3 0	9 0	2 0
12	$CDCl_3$	4 0	0	3 5	7 0	
13	$(CD_3)_2CO$	4 0	0	3 5	7 0	
14	C_6D_6	4 0	0	3 0	7 0	2 0
15	C_6D_6	4 0	0	3 0	9 0	2 0
16	$CDCl_3$	3 5	0	3 0	9 0	

Ozonolysis of the acetylenic dibenzoate **3** gave crystalline 3,5-di-*O*-benzoyl-1,2-*O*-isopropylidene-β-L-idofuranuronic acid (**4**) The n m r spectrum of **4** showed an exchangeable proton resonating at τ 0 40, characteristic of carboxylic acids The

remainder of the spectrum could be analyzed on a first-order basis Attempts to debenzoylate **3** with methanolic sodium methoxide, and convert the product into the *β*-L-*ido* lactone **8**, gave instead a mixture that appeared to contain also the α-D-*gluco* lactone (**7**), the base used for saponification is evidently strong enough to cause epimerization at C-5

The reaction sequence performed on the periodate-oxidation product from 1,2-*O*-isopropylidene-α-D-glucofuranose was also evaluated with the aldehyde (**9**) obtained from 3-*O*-benzyl-1,2-*O*-isopropylidene-α-D-glucofuranose by cleavage of the 5,6-glycol group The C-3 protecting group in **9** prevents cyclization reactions leading to products such as **5**, and the 3-substituent can be removed by hydrogenolysis without recourse to basic conditions The aldehyde **9** was first prepared[12] with the use of lead tetraacetate as the oxidant In our hands, sodium periodate in aqueous methanol proved to be more convenient and **9** was obtained as a syrup in 92% yield The product was a true aldehyde, as evidenced by the carbonyl band at 5 75 μm in the i r spectrum and the low-field (τ 0 37) narrow doublet ($J_{4,5}$ 2 0 Hz) for the aldehyde proton (H-5) in the n m r spectrum The syrupy aldehyde **9** was further characterized as its crystalline *p*-nitrophenylhydrazone (**13**), the n m r spectrum of the latter (see Tables I and II) was fully consistent with the structure assigned



Ethynylation of the aldehyde **9** gave a 5-epimeric mixture of acetylenic derivatives that could be separated by chromatography on a column of silica gel. The faster-moving component, identified by subsequent conversions as the α-D-*gluco* derivative (**14**), was obtained as a syrup in a 26 5% yield. The slower-moving, major

component was obtained as a syrup in a 33% yield and subsequent conversions showed that it was the β-L-*ido* acetylenic derivative (**10**) Both products showed narrow doublets for H-7 in their n m r spectra, at τ 7 95 for **14** and τ 7 88 for **10**, and a mixture of the epimers showed two distinct doublets for the acetylenic protons

Ozonolysis of the minor, faster-migrating acetylenic derivative, followed by hydrogenolysis of the benzyl group gave 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone (**7**), identical with an authentic sample by mixed m p and X-ray powder diffraction pattern, and none of the β-L-*ido* lactone (**8**) could be detected chromatographically This conversion establishes the structure of the acetylenic precursor as 3-*O*-benzyl-6,7-dideoxy-1,2-*O*-isopropylidene-α-D-*gluco*-hept-6-ynofuranose (**14**) Similarly, ozonolysis of the major, slower-migrating acetylenic product, followed by hydrogenolysis, gave 1,2-*O*-isopropylidene-β-L-idofuranurono-6,3-lactone (**8**), identical with an authentic sample by mixed m p and X-ray powder diffraction pattern, and none of the α-D-*gluco* lactone (**7**) could be detected chromatographically The precursor therefore has the structure 3-*O*-benzyl-6,7-dideoxy-1,2-*O*-isopropylidene-β-L-*ido*-hept-6-ynofuranose (**10**)

Benzoylation of the α-D-*gluco* derivative (**14**) gave the crystalline 5-benzoate (**15**), melting sharply at 139–140°, and the β-L-*ido* derivative (**10**) also gave a crystalline 5-benzoate (**11**), which melted sharply at 124 5–125°, a mixture of **15** and **11** showed a large melting-point depression The 5-benzoates **15** and **11** were prepared most conveniently by benzoylation of the original mixture of acetylenes (**14** and **10**) and separating the products by fractional crystallization As with the precursors, the acetylenic benzoates **15** and **11** showed H-7 resonances as narrow doublets at different field positions (τ 7 87 and 8 00, respectively), and they could readily be detected in admixture by this means The H-5 signal, the lowest-field signal of all protons on the sugar chain because of the deshielding by the benzoyloxy group, was observed as a quartet through spin-coupling with H-7 ($J_{5,7}$ = 2 Hz in each case) and H-4 Although the H-5 signals for **15** and **11** were observed at different field positions (τ 3 70 and 3 58, respectively) the $J_{4,5}$ couplings were identical (9 0 Hz), indicating that the favored conformation in both compounds is that having H-4 and H-5 antiparallel It is not possible, therefore, to use spin-coupling data for assigning configurations to these epimeric acetylenic derivatives, such assignments are possible when the starting aldehyde is attached to a dioxolane ring or when the acetylenic derivative is acyclic[2,4,5]

Ozonolysis of 5-*O*-benzoyl-3-*O*-benzyl-6,7-dideoxy-1,2-*O*-isopropylidene-β-L-*ido*-hept-6-ynofuranose (**11**) gave crystalline 5-*O*-benzoyl-3-*O*-benzyl-1,2-*O*-isopropylidene-β-L-idofuranuronic acid (**12**), and similar ozonolysis of the D-*gluco* acetylenic derivative (**15**) gave 5-*O*-benzoyl-3-*O*-benzyl-1,2-*O*-isopropylidene-α-D-glucofuranuronic acid (**16**) as a syrup The two acids **12** and **16** were characterized by n m r spectroscopy and **12** by elemental analysis Debenzylation of **12** and **16** could be effected readily by hydrogenolysis over palladium black However, removal of the benzoyl group by saponification with methanolic sodium methoxide led to epimerization at C-5 in each case, mixtures of 1,2-*O*-isopropylidene-α-D-glucofuranurono- and -β-L-idofuranurono-6,3-lactones (**7** and **8**) were obtained from each reaction

The ozonization reactions described in this paper were performed on a small scale, mainly for the purpose of stereochemical characterization, and yields of uronic acids or their lactones were not optimized for preparative purposes However, the ozonolytic cleavage of α-hydroxyacetylenes to α-hydroxyacids can be effected on a larger scale in near-quantitative yields[4,13,14], and the procedure thus offers a useful general route for preparation of uronic acids The established route to uronic acids, by way of a cyanohydrin reaction with an *ω-aldehydo* sugar derivative[9,15,16], requires alkaline conditions and gives an epimeric mixture of products, whereas the present route gives intermediates that are not prone to epimerization, and generation of the carboxylic acid can be effected without epimerization at the adjacent carbon atom.

EXPERIMENTAL

General methods — Solutions were concentrated under diminished pressure on a rotary evaporator at 30° Specific rotations were measured in a 2-dm tube. Melting points were determined with a Thomas–Hoover "Unimelt" apparatus I r. spectra were measured with a Perkin–Elmer Model 137 "Infracord" i r spectrophotometer. N m r spectra were measured with a Varian HA-100 spectrometer operating at about 28°. Chemical shifts are given on the τ-scale with tetramethylsilane (τ = 10 00) as the internal standard Microanalyses were made by W. N. Rond. X-ray powder diffraction data give interplanar spacings, Å, for CuK_α radiation The relative intensities were estimated visually m, moderate, s, strong, v, very; w, weak The strongest lines are numbered (1, strongest), double numbers indicate approximately equal intensities T l c was performed with Silica Gel G (E Merck, Darmstadt, Germany) as the stationary phase Detection was effected by spraying with sulfuric acid unless specified otherwise Petroleum ether used in recrystallization was a fraction having the boiling range 30–60°. Ozone was generated by using a Welsbach ozonator, model T-408 (The Welsbach Company, Philadelphia, Pennsylvania, U S A)

Preparation of 1,2-O-isopropylidene-α-D-xylo-pentodialdo-1,4-furanose dimer (**1**) *and 1,2-O-isopropylidene-3,5-O-methylene-α-D-xylo-pentodialdo-1,4-furanose aldehydrol* (**5**) — To a stirred solution of 1,2-*O*-isopropylidene-α-D-glucofuranose (6 g, 27 3 mmoles) in 100 ml of 37% aqueous formaldehyde at 0° was added sodium metaperiodate (6 g, 28.0 mmoles) in water (80 ml) over a period of 30 min The solution was stirred for 1 h at room temperature Ethylene glycol (0 3 ml) was then added to decompose any excess periodate. The solution was concentrated under diminished pressure to one half the original volume and the sodium iodate that separated was filtered off. The solution was extracted five times with 50-ml portions of chloroform. The chloroform extracts were dried (magnesium sulfate) and then concentrated to a syrup, yield 3 2 g (59%) of a mixture of **5** and **1**, R_f (ether) 0 70 and 0 37, respectively[11] in approximately 9.1 proportion

6,7-Dideoxy-1,2-O-isopropylidene-β-L-ido-hept-6-ynofuranose (**2**) *and its α-D-gluco epimer* (**6**) — Ethylmagnesium bromide was prepared from magnesium (2 5 g,

102 mmoles) and ethyl bromide (12 g) in dry tetrahydrofuran (150 ml) Acetylene was passed slowly through dry tetrahydrofuran (250 ml) at room temperature by means of a gas-dispersion tube, and the liquid was stirred magnetically. After 1 h the solution of ethylmagnesium bromide was added dropwise The resulting solution gradually turned bright red and then a dark reddish-brown. The resultant solution of ethynylmagnesium bromide was stirred for 1 h with acetylene still passing through the solution. A solution of the periodate-oxidized product (**5**+**1**, 5 g, 25 3 mmoles) in dry tetrahydrofuran was then added dropwise. After the addition had been completed, the stream of acetylene was maintained for an additional 3 h The tetrahydrofuran was then evaporated off, ethyl ether (250 ml) was added to the residue, and the mixture was shaken with cold, aqueous, saturated ammonium chloride After separation of the layers, the aqueous phase was extracted with ether The combined ether extracts were washed with saturated aqueous sodium chloride, dried over magnesium sulfate, and evaporated The resultant syrupy mixture contained compound **2** (R_F 0 41, ether) and compound **6** was also present Fractional vacuum distillation gave **2**, yield 1.85 g (8 6 mmoles, 34%), b p 125–135° (0 075 torr, bath temp 175–185°), λ_{max}^{film} 3 08, 4 70 (C≡C–H), 7 23 μm (doublet, CMe_2)

*1,2-O-Isopropylidene-β-*L*-idofuranurono-6,3-lactone* (**8**) — Ozone was passed for 45 min through a solution at 0° of 6,7-dideoxy-1,2-*O*-isopropylidene-β-L-*ido*-hept-6-ynofuranose (**2**, 500 mg, 2 34 mmoles) in carbon tetrachloride The solution was evaporated and several small portions of toluene were added to the residual syrup and evaporated Crystallization of the syrup from acetone–ether gave the lactone **8**; yield 80 mg (15%), m p 135–136° (lit [16] m p. 137–138°), R_F 0 25 (9 1 benzene–methanol), X-ray powder diffraction data 6 47 s (2), 5 57 w, 4 97 s (1), 4 45 m, 4 21 m (3), 4 02 w, 3 90 w, 3 63 w, 3 41 m, 3 23 w, 3.12 vw, 3 02 vw, 2 81 w, 2 68 m, 2 52 vw, 2.47 m

The product was identical with an authentic sample of **8** by mixed m p and comparative X-ray powder diffraction pattern No 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone (**7**) could be detected either in the crystalline product or in the mother liquors, although by t l c compound **7** (R_F 0 40 in 9 1 benzene–methanol) can be clearly differentiated from **8**.

Ozonolysis of a small amount of the undistilled product of the preceding ethynylation experiment, by the same procedure used for the conversion of **2** into **8**, gave a product that by t l c (9 1 benzene–methanol) showed the β-L-*ido* lactone **8** as a major component (R_F 0 25), and the α-D-*gluco* lactone **7** (R_F 0 40) as a minor component

*3,5-Di-O-benzoyl-6,7-dideoxy-1,2-O-isopropylidene-β-*L*-ido-hept-6-ynofuranose* (**3**) — Benzoyl chloride (3 ml) in dichloromethane (10 ml) was added slowly to a solution at 0° of 6,7-dideoxy-1,2-*O*-isopropylidene-β-L-*ido*-hept-6-ynofuranose (**2**, 1.85 g, 8.6 mmoles) in dry pyridine (10 ml), and the solution was stirred overnight. The reaction mixture was poured into ice–water (50 ml) and stirred for 1 h. The product was extracted with dichloromethane, and the extract was washed sequentially with 20 ml of 10% aqueous hydrochloric acid, two 20-ml portions of 10% aqueous

sodium hydrogen carbonate, and water The extract was dried (magnesium sulfate) and evaporated to a syrup that crystallized from absolute methanol to give **3**, yield 1 80 g (49%), m p 195–196°, $[\alpha]_D^{18}$ −16 ±3° (*c* 1, chloroform), R_F 0 90 (3 1 chloroform–ether), 0 48 (5 5 2 benzene–petroleum ether–ether), λ_{max}^{KBr} 3 06, 4 71 (C≡C–H), 5.80 (C=O), 7 40 (doublet, CMe_2), 13 15, 14 15 μm (aryl), X-ray powder diffraction data 12 62 m, 7 03 vw. 6 21 s (1), 5 59 w. 4 97 w, 4 68 m (3), 4 41 m, 4 05 s (2), 3 62 vw. 3 45 vw, 3 18 vw

Anal Calc for $C_{24}H_{22}O_7$ C, 68 25, H, 5 21 Found C, 68 35, H. 5 34 Further recrystallization gave no change in the m p of this product The X-ray powder diffraction pattern was identical with that of a product[1] having m p 191–193° earlier believed to be the D-*gluco* analog

*3,5-Di-O-benzoyl-1,2-O-isopropylidene-β-*L*-idofuranuronic acid* (**4**). — Ozone was passed for 45 min through a solution of **3** (500 mg, 1 2 mmoles) in carbon tetrachloride (100 ml) that was kept at 0° The solution was evaporated water (50 ml) was added to the residue, and the mixture was evaporated again Crystallization of the resultant syrup from ether–petroleum ether gave **4** yield 106 mg (20%), m p 176- 179° (decomp), $[\alpha]_D^{21}$ +1 1 ±1° (*c* 0 9, chloroform), λ_{max}^{KBr} 3 0 (CO_2H), 5 75 (C=O) 7 25 (doublet, CMe_2), 14 1 μm (aryl), X-ray powder diffraction data 10 46 s (1), 8 30 w, 7 02 vw, 6 44 m, 5 94 vw, 5 45 vw, 5 19 s (2), 4 72 s (3), 4 45 w, 4 25 s

Anal Calc for $C_{23}H_{22}O_9$ C, 62 44, H, 5 01 Found 62 36, H, 4 91

A solution of **4** in abs methanol was treated with ~1 1 molar equivalents of sodium methoxide solution After 1 h at 30°, the solution was neutralized with acetic acid. evaporated, and toluene was added twice to the residue and evaporated T l c of the product showed components having R_f 0 40 and 0 25 (9 1 benzene–methanol) corresponding to 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone (**7**) and the β-L-*ido* analog (**8**)

*Preparation of 3-O-benzyl-1,2-O-isopropylidene-α-*D*-glucofuranose* — To a solution of 5,6-di-*O*-acetyl-3-*O*-benzyl-1,2-*O*-isopropylidene-α-D-glucofuranose[17] (7 0 g, 17 7 mmoles) in abs methanol (50 ml) was added a catalytic amount of sodium methoxide After 4 h at room temperature, the solution was neutralized with a few drops of acetic acid, and then evaporated The resultant thick syrup was dissolved in warm benzene and the solution was filtered through Celite Evaporation of the solution gave the product as a colorless syrup, yield 5 27 g (96%), R_f 0 17 (3 1 chloroform–ether), λ_{max}^{film} 2 90 (OH), 7 25 (doublet, CMe_2), 13 35, 14 30, 14 70 μm (aryl), carbonyl absorption absent.

*Preparation of 3-O-benzyl-1,2-O-isopropylidene-5-*aldehydo-α-D-xylo-*pertodialdo-1,4-furanose*[12] (**9**) — A solution of 3-*O*-benzyl-1,2-*O*-isopropylidene-α-D-glucofuranose (5 27 g, 17 mmoles) in abs methanol (50 ml) was stirred at 0°, and sodium metaperiodate (4 g, 18 mmoles) in water (50 ml) was added dropwise After the addition had been completed, the solution was stirred for 1 5 h at 0° Ethylene glycol (0 5 ml) was added to decompose any excess of oxidant and the methanol was evaporated off The aqueous solution was extracted with 3 × 50-ml portions of chloroform, and the dried (magnesium sulfate) extract was evaporated to give **9** as a chromato-

graphically homogeneous syrup, yield 4 33 g (92%). R_F 0.42 (3 1 chloroform–ether) The product was distilled, b.p. 145–155° (0.02 torr, bath 200°), λ_{max}^{film} 5 75 (aldehyde C=O), 7 25 (doublet, CMe_2), 13 50, 14 30 μm (aryl).

*3-O-Benzyl-1,2-O-isopropylidene-5-*aldehydo-D-xylo-*pentodialdo-1,4-furanose* p-*nitrophenylhydrazone* (**13**) — To a solution of the aldehyde **9** (500 mg, 1 8 mmoles) in methanol (5 ml) was added *p*-nitrophenylhydrazine hydrochloride (500 mg) in a mixture of water (5 ml) and pyridine (1 ml) The mixture was kept for 24 h at room temperature and then concentrated to a thick syrup. The syrup was dissolved in benzene (50 ml) and the solution was extracted with 25-ml portions of water, 5% sodium hydrogen carbonate, 10% sulfuric acid, and water The benzene solution was dried (magnesium sulfate) and evaporated The resultant syrup was crystallized from benzene–ethanol to give **13** as yellow crystals, yield 570 mg (79%), m p. 141–142°, $[\alpha]^{23}$ −143 ±3° (*c* 1 2, chloroform), R_F 0 55 (3 1 chloroform–ether), λ_{max}^{KBr} 3 06 (N–N), 6 24 (C=N), 6 50, 7 55 (NO_2), 7 25 (doublet, CMe_2), 13 28, 14 37 μm (aryl), X-ray powder diffraction data 10 64 vw, 9 57 m (2), 7 98 w, 7 29 vw, 6 84 w, 6 49 w, 6 12 vw, 5 59 m (2), 5 01 s (1), 4 51 m

Anal Calc for $C_{21}H_{23}N_3O_6$ C, 61 01, H, 5 61, N, 10 16 Found C, 60 97, H, 5 82, N, 10 43

*3-O-Benzyl-6,7-dideoxy-1,2-*O-*isopropylidene-α-*D-gluco-*hept-6-ynofuranose* (**14**) *and its β-*L-ido *epimer* (**10**) — Ethynylmagnesium bromide (80 mmoles) was prepared as before from magnesium (2 g) and ethyl bromide (9 g) in tetrahydrofuran (300 ml) A solution of the aldehyde **9** (10 84 g, 39 mmoles) in dry tetrahydrofuran (50 ml) was added with stirring and with passage of a continuous, slow stream of acetylene to the solution of Grignard reagent during 30 min at room temperature After a further 3 h the solution was evaporated, ether (250 ml) was added to the residue, and the resulting solution was shaken with cold, saturated, aqueous ammonium chloride (200 ml) The separated aqueous phase was extracted with ether, and the combined ether extracts were washed with saturated aqueous sodium chloride (75 ml) The dried (magnesium sulfate) ether extract was evaporated to give a mixture of **10** and **14** as a yellow syrup, yield 8 41 g, R_F 0 43 and 0 64 (3 1 chloroform–ether) A suspension of 300 g of silica gel (Silica Gel Davison, Grade 950, 60–120 mesh, a product of the Davison Division of the W R Grace Chemical Co , Baltimore, Md , U. S A.) in 3 1 dichloromethane–ether was poured into a column (2 8 × 43 cm) and allowed to settle The syrupy mixture of **10** and **14**, dissolved in 5 ml of 3 1 dichloromethane–ether, was placed on top of the column Elution with 3 1 dichloromethane–ether gave the two components resolved completely from each other The first product to be eluted, the D-*gluco* epimer (**14**) was obtained as an oil, yield 3 14 g (27%), R_F 0 64 (3 1 chloroform–ether) The second component to be eluted, the L-*ido* epimer (**10**), was also obtained as an oil, yield 3 96 g (33%), R_F 0 43 (3 1 chloroform–ether)

*Conversion of 3-O-benzyl-6,7-dideoxy-1,2-*O-*isopropylidene-α-*D-gluco-*hept-6-ynofuranose* (**14**) *into 1,2-*O-*isopropylidene-α-*D-*glucofuranurono-6,3-lactone* (**7**) — Ozone was passed for 45 min through a solution of **14** (140 mg, 0 47 mmoles) in carbon tetrachloride (100 ml) at 0° The solution was shaken with water (50 ml) and

the mixture concentrated to a syrup The syrup was dissolved in abs ethanol (75 ml) and hydrogenated at atmospheric pressure for 24 h with palladium black (15 mg) as catalyst The mixture was filtered through Celite, the filtrate was evaporated, and small portions of toluene were added to the resultant syrup and evaporated Crystallization from acetone–ether gave the D-*gluco* lactone (**7**), yield 20 mg (20%), m p 118–119° (lit [16] m p 120°), X-ray powder diffraction data 10 84 m (3), 7 03 w, 5 49 s (1), 4 64 m (2), 4 08 w, 3 91 vw, 3 51 w, 3 26 vw, 3 15 vw, 2 97 w

The product was identical with an authentic sample[16] by mixed m p and X-ray powder diffraction pattern None of the 5-epimeric lactone **8** could be detected in the mother liquors by t l c

*Conversion of 3-O-benzyl-6,7-dideoxy-1,2-O-isopropylidene-β-*L*-ido-hept-6-ynofuranose* (**10**) *into 1,2-O-isopropylidene-β-*L*-idofuranurono-6,3-lactone* (**8**) — Compound **10** (100 mg, 0 33 mmoles) was subjected to the sequence of reactions of the preceding experiment, and the crystalline *β-*L*-ido* lactone (**8**) was obtained, yield 24 mg (35%), m p 135–136° (lit [16] 137–138°), identical with authentic **8** by mixed m p and X-ray powder diffraction pattern The 5-epimeric lactone (**7**) could not be detected in the mother liquors by t l c

*5-O-Benzoyl-3-O-benzyl-6,7-dideoxy-1,2-O-isopropylidene-β-*L*-ido-hept-6-ynofuranose* (**11**) *and its* α-D-gluco *epimer* (**15**) — To an unseparated mixture of 3-*O*-benzyl-6,7-dideoxy-1,2-*O*-isopropylidene-α-D-*gluco*-hept-6-ynofuranose (**14**) and the *β-*L*-ido* epimer (**10**) (2 0 g, 6 58 mmoles) in dry pyridine (10 ml) was added benzoyl chloride (2 g) slowly with stirring After 12 h ice and water (50 ml) were added, and the mixture was stirred for 2 h The product was extracted with two 30-ml portions of dichloromethane, and the combined extracts were washed successively with 20 ml of 10% hydrochloric acid, 2 × 20 ml of aqueous sodium hydrogen carbonate, and 2 × 20 ml of water The dried (magnesium sulfate) extract was evaporated, and an ether solution of the product was decolorized with activated charcoal Evaporation of the solvent gave a pale-yellow syrup that crystallized from abs methanol to give a white, crystalline mixture of **11** and **15**, yield 1 39 g (52%), R_F 0 76 (3 1 chloroform–ether)

Fractional recrystallization of the mixture from abs methanol gave the two separate epimers The less-soluble epimer was the *β-*L*-ido* derivative (**11**), which formed rectangular prisms, yield 510 mg (19%), m p 124 5–125°, $[\alpha]_D^{20}$ −32 ±3° (*c* 1, chloroform), R_F 0 38 (5 5 1 petroleum ether–benzene–ether), λ_{max}^{KBr} 3 10, 4 72 (C≡C–H), 5 80 (C=O), 7 22 (doublet, CMe_2), 13 65, 14 05 μm (aryl), X-ray powder diffraction data 12 44 m (3), 9 02 vw, 7 06 vw, 6 15 s (1), 5 61 w, 4 70 m (2), 4 41 w, 4.11 w, 3 85 vw, 3 65 w

Anal Calc for $C_{24}H_{24}O_6$ C, 70 57, H, 5 92 Found C, 70 30, H, 5 93

The more-soluble epimer, the α-D-*gluco* derivative (**15**) was obtained as rectangular prisms, yield 470 mg (18%), m p 139–140°, $[\alpha]_D^{23}$ −79 ±3° (*c* 1 1, chloroform), R_F 0 29 (5 5 1 petroleum ether–benzene–ether); λ_{max}^{KBr} 3.10, 4 72 (C≡C–H), 5 80 (C=O), 7 25 (doublet, CMe_2), 13 45, 14 05 μm (aryl), X-ray powder diffraction data 11 89 m,

8 95 s (2), 7 51 w, 6 56 vs (1), 5 99 s, 5 14 s (3), 4 77 m, 4 25 s, 4 05 s, 3 36 m, 3 20 w, 3 02 w.

Anal Calc for $C_{24}H_{24}O_6$ · C, 70 57, H, 5 92 Found C, 70 50, H, 5 91

A mixture of **11** and **15** melted over the range 115–139° When the separated epimers were benzoylated individually **10** gave **11**, and **14** gave **15**

5-O-Benzoyl-3-O-benzyl-1,2-O-isopropylidene-β-L-idofuranuronic acid (**12**) — Ozone was passed for 30 min through a solution of **11** (100 mg, 0 25 mmoles) in carbon tetrachloride (100 ml) at 0°. The solution was shaken with water (50 ml), and the mixture was evaporated to a syrup that crystallized from ether–petroleum ether to give **12**, yield 51 mg (51%), m p. 157–158°, X-ray powder diffraction data· 10 39 s (1), 8 16 w, 6 42 w, 5 88 vw, 5 17 m (3), 4 75 m (2), 4 38 w, 4 19 w, 3 74 vw, 3 41 vw, 3 22 vw, 3 09 vw

Anal. Calc for $C_{23}H_{24}O_8$ C, 64 47, H, 5 61 Found C, 64 91, H, 5 54

Compound **12** was hydrogenated in ethanol for 24 h in the presence of palladium black. Treatment of the product in abs methanol with sufficient sodium methoxide to split off the benzoyl group, with subsequent neutralization with acetic acid, evaporation of the solution, and evaporation of toluene from the residue, gave a product that, by t l c , contained two components, migrating as 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone (**7**) and the *β-L-ido* analog (**8**), indicating epimerization at C-5 in the debenzoylation step.

5-O-Benzoyl-3-O-benzyl-1,2-O-isopropylidene-α-D-glucofuranuronic acid (**16**) — Compound **15** (300 mg, 0 7 mmoles) was ozonized as in the preceding experiment, to give **16** as a syrup, yield 98 mg (33%) When this product was successively debenzylated, saponified, and lactonized by the procedure used with **12**, a mixture of the two lactones **7** and **8** was again obtained, indicating that epimerization at C-5 had occurred during the saponification step

ACKNOWLEDGMENTS

The authors thank P L Durette and J H Lauterbach for recording the n m r. spectra, and J B Hughes for preliminary experimental work

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

BRANCHED-CHAIN SUGARS

PART XI* A SYNTHESIS OF 6-DEOXY-4-*C*-VINYL-L-TALOSE AND 5-DEOXY-3-*C*-VINYL-L-LYXOSE

B M GOUGH, S W GUNNER, W G OVEREND, AND N R WILLIAMS
Chemistry Department, Birkbeck College (University of London), Malet Street, London W. C 1 (Great Britain)

(Received December 19th, 1969)

ABSTRACT

The preparation is described of the title compounds as intermediates in a projected synthesis of streptose Treatment of methyl 6-deoxy-2,3-*O*-isopropylidene-α-L-*lyxo*-hexopyranosid-4-ulose with vinylmagnesium bromide gave methyl 6-deoxy-2,3-*O*-isopropylidene-4-*C*-vinyl-α-L-talopyranoside from which 6-deoxy-4-*C*-vinyl-L-talose (**5**) was obtained by sequential deacetonation and glycoside hydrolysis Oxidation of **5** with 1 equivalent of lead tetra-acetate gave 5-deoxy-3-*C*-vinyl-L-lyxose

INTRODUCTION

As part of our programme of investigation of methods for the conversion of glycosiduloses into branched-chain sugars, we have examined the possibility of preparing the naturally occurring, branched-chain sugar streptose from methyl 6-deoxy-2,3-*O*-isopropylidene-α-L-*lyxo*-hexopyranosid-4-ulose (**1**), which is readily obtainable from L-rhamnose Although an acceptable conversion was not achieved, and a more satisfactory alternative route has since been reported[1], several points of interest emerged from our investigation and are now reported

DISCUSSION

Compound **1**[2], on treatment with vinylmagnesium bromide in ether, yielded methyl 6-deoxy-2,3-*O*-isopropylidene-4-*C*-vinyl-α-L-talopyranoside (**2**). Analysis of the reaction product by g l c indicated that the ratio of *talo–manno* isomers was >10 1, and, after acid hydrolysis, paper-chromatographic examination showed only one major component, the principal minor product being rhamnose. Deacetonation of compound **2** with Amberlite IR-120(H^+) resin in methanol afforded methyl 6-deoxy-4-*C*-vinyl-α-L-taloside (**3**). Sequential catalytic hydrogenation and deacetonation of compound **2** gave methyl 6-deoxy-4-*C*-ethyl-α-L-talopyranoside (**4**). Evidence for the *talo* configuration of compounds **2**, **3**, and **4** is summarized in Table I.

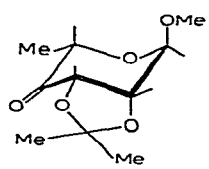
*Part X S W Gunner, R D. King, W G Overend, and N R Williams, *J Chem Soc* (*C*), in the press

TABLE I

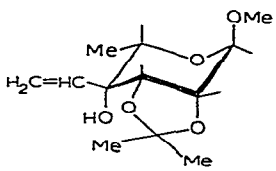
CHROMATOGRAPHIC BEHAVIOUR AND INFRARED SPECTRAL PROPERTIES OF COMPOUNDS 2, 3, AND 4

Compound	R_F[a]	R_F[b]	ΔR_F(%)	$\nu_{max}(cm^{-1})$[c]
2	—	—	—	3567
3	0 73	0 90	23	3570, 3516
4	0 79	0 92	16	3560, 3512
Methyl α-D-rhamnopyranoside	0 52	0 52	0	3560

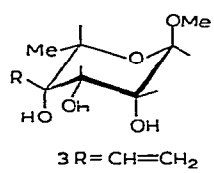
[a]4 1 5 Butyl alcohol–ethanol–water, organic phase [b]4 1 5 Butyl alcohol–ethanol–water + 1% benzeneboronic acid [c]For 5mM solutions in carbon tetrachloride



1



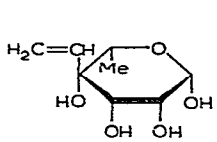
2



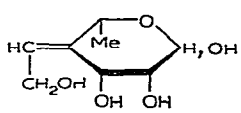
3 R = CH=CH$_2$
4 R = Et

The increased R_F value obtained on paper chromatography in the presence of benzeneboronic acid, together with the i r band at *ca* 3520 cm^{-1} indicating strong intramolecular hydrogen bonding, are characteristic of a *cis, cis-ax-eq-ax* triol system[3] which can occur only with the *talo* isomer This assignment is further supported by the small rotational shift observed on formation of the cuprammonium complex[4] with compound **3** (−175°) when compared with methyl L-rhamnopyranoside (−1618°), thereby suggesting that, in **3**, the 2,3- and 3,4-diol pairs are of opposite and compensating chirality. The *talo* configuration would be anticipated on steric grounds, since in the expected *1C*(L) chair conformation of compound **1**, as depicted, attack by the Grignard reagent from the axial side would be hindered by the axial O-2, whereas no hindrance is offered to equatorial attack in this conformation

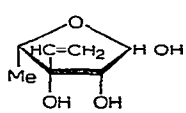
Acid hydrolysis of compound **3** led to 6-deoxy-4-*C*-vinyl-L-talose (**5**) as a crystalline monohydrate, its mutarotation {$[\alpha]_D$ +20 (initial)→ −24° (24 h)} indicated that it was the β-L anomer Under the acidic conditions employed for this hydrolysis, allylic rearrangement of the tertiary alcohol to the hydroxyethylidene derivative **6** was considered a possibility However, the n m r spectrum of compound **5** in deuterium oxide indicated clearly the presence of three vinylic protons as required by **5**, not one as required by **6**



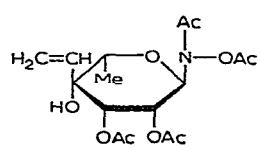
5



6



7



8

The degradation of compound **5** to the corresponding pentose, 5-deoxy-3-*C*-vinyl-L-lyxose (**7**), provides a sugar which, on ozonolysis of the vinyl group to give a formyl residue, should yield streptose However, the degradation proved to be difficult Oxidative procedures described by Ruff[5] and by MacDonald and Fischer[6] were not applicable, since the vinyl group would be expected to undergo oxidation simultaneously Attempts to utilise modifications of the Wohl degradation proved unsatisfactory. 6-Deoxy-4-*C*-vinyl-L-talose oxime, although chromatographically homogeneous, could not be obtained crystalline and appeared to be unstable Treatment[7] of the crude material with 1-fluoro-2,4-dinitrobenzene afforded in one preparation a crystalline compound in 5% yield after purification by preparative paper chromatography, but this could not be definitely characterised as **7** In some experiments using sodium acetate and acetic anhydride on the oxime, according to Zemplén's modification[8] of the Wohl degradation, a crystalline compound was isolated in 5–15% yield. On the basis of its analysis, and i r and n m r. spectra, this was considered to be *N*-acetoxy-*N*-acetyl-2,3-di-*O*-acetyl-6-deoxy-4-*C*-vinyl-α-L-talopyranosylamine (**8**), the α-L configuration being suggested by the low specific rotation, but no material corresponding to **7** could be detected Compounds of this type have been discussed by Deulofeu[9] Oxidation of compound **5** with lead tetra-acetate (1 mol equiv) in glacial acetic acid, a reagent which Perlin *et al* [10] have observed to cleave preferentially the C-1–C-2 diol system in other aldoses, did eventually afford compound **7** as a syrup (30% yield after column chromatography) which was characterised as its crystalline toluene-*p*-sulphonylhydrazone Subsequently, compound **7** was obtained also by mild, acid hydrolysis of 5-deoxy-1,2-*O*-isopropylidene-3-*C*-vinyl-L-lyxofuranose[1] Ozonolysis of compound **7** yielded several products (paper chromatography), possibly including streptose, but no material corresponding to a crystalline derivative of streptose could be obtained from this mixture and this route was not investigated further

EXPERIMENTAL

Melting points are uncorrected Infrared spectra were measured with a Perkin–Elmer Infracord Spectrophotometer, model 137 For high-resolution measurements near 3500 cm^{-1}, a Unicam S P 700 spectrophotometer was employed, with 5 mM solutions in carbon tetrachloride and 1-cm silica cells N m r spectra were measured with a Varian A-60 spectrometer for solutions in deuteriochloroform (unless stated otherwise) containing tetramethylsilane as internal reference For paper chromatography, Whatman No 1 paper was used For preparative separations, Whatman No 3 paper was employed Except where otherwise indicated, the organic phase of a mixture of 4 1 5 (v/v) butyl alcohol–ethanol–water was used as solvent Spots were visualized by alkaline silver nitrate[11] When benzeneboronic acid was added to the developing solvent, the paper was treated with hydrogen fluoride in aqueous acetone before treatment with silver nitrate[12] Thin-layer chromatography (t l c) was performed with Silica Gel G (Stahl) and (*a*) 3 1 chloroform–ethanol or (*b*) 3 1 chloroform–light

petroleum (b p 40–60°) Spots were visualized by the anisaldehyde reagent[13]. Gas–liquid chromatography (g l c.) was conducted at 150° by using a column (4 feet) of Apiezon L stationary phase (10%) on Celite Unless contra-indicated, all solvents were removed under reduced pressure at 35–40°, last traces of solvent were removed by drying *in vacuo*.

Methyl 6-deoxy-2,3-O-isopropylidene-4-C-vinyl-α-L-talopyranoside (**2**) — A solution of methyl 2,3-*O*-isopropylidene-α-L-*lyxo*-hexopyranosid-4-ulose[2] (**1**, 10 g) in anhydrous ether (100 ml) was added to a suspension of vinylmagnesium bromide in a mixture of anhydrous tetrahydrofuran and ethyl ether prepared[14] from vinyl bromide (25 ml) and magnesium (8 6 g) The mixture was stirred and heated for 4 h under reflux After cooling, the mixture was treated with distilled water sufficient to coagulate the precipitate, the ethereal solution was decanted, and the residue washed with ether (2 × 100 ml) The combined, ethereal solution was dried ($MgSO_4$), and the solvent was removed to give a clear, yellow syrup which crystallised on storage Residual syrup was removed on a porous tile, and the solid was purified by sublimation at 40°/0 55 mmHg The syrup, extracted from the tile with ether, yielded a further crop of crystals that were similarly purified Compound **2** was obtained as colourless needles (7 g, 62%), m p 45–45 5°, $[\alpha]_D^{20}$ −13° (*c* 0 4, methanol), ν_{max} 1650 cm^{-1} (C=C)

Anal. Calc for $C_{12}H_{20}O_5$. C, 59 0, H, 8 25. Found C, 58 8, H, 8 4

The material gave a positive, unsaturation test with bromine G l c analysis of the crude syrup showed that the crystalline compound was the major product In addition, small amounts (<4%) of methyl 6-deoxy-2, 3-*O*-isopropylidene-α-L-manno- and -talo-pyranoside and two unidentified products (possibly including the C-4 epimer of compound **2**) were noted A similar pattern of products was observed in the paper-chromatographic analysis (butyl alcohol–pyridine–water, 6 2 5, v/v) of the hydrolysate obtained by treating the crude syrup in water with Amberlite IR-120 (H^+) resin. 6-Deoxy-4-*C*-vinyl-L-talose, the major component, showed R_F 0 65 6-Deoxy-L-mannose (R_F 0 38) and 6-deoxy-L-talose (R_F 0 48), together with another substance (R_F 0.74), were also present.

Methyl 6-deoxy-4-C-vinyl-α-L-talopyranoside (**3**) — Compound **2** (0 5 g) was dissolved in methanol (25 ml) and Amberlite IR-120 (H^+) resin (2 g) was added The mixture was shaken for 48 h at room temperature The supernatant was decanted, and the resin was washed with methanol (3 × 15 ml) The combined solution was concentrated to a syrup which crystallised on trituration with ethyl acetate Recrystallisation from ethyl acetate gave compound **3** (0 34 g, 81%) as colourless prisms, m p 80–81°, $[\alpha]_D^{20}$ −77° (*c* 0 44, methanol), ν_{max} 1650 cm^{-1} (C=C)

Anal. Calc for $C_9H_{16}O_5$ C, 52 9, H, 7 9 Found C, 53 3, H, 8 0.

Methyl 6-deoxy-4-C-ethyl-α-L-talopyranoside (**4**) — Compound **2** (0 49 g) was reduced with hydrogen over Adams' platinum catalyst in the usual way, uptake being complete in 20 min at room temperature The syrupy product (0 46 g), $[\alpha]_D^{20}$ −34 4° (*c* 0.6, methanol), was then treated with a suspension of Amberlite IR-120 (H^+) resin in methanol (15 ml), as described above The product was recrystallised from ethyl

acetate to give compound **4** as fine needles (0 23 g, 56%), m p 66–67°, $[\alpha]_D^{21}$ −84° (*c* 0 35, methanol), there was no absorption at 1650 cm^{-1} (C=C)

Anal. Calc for $C_9H_{18}O_5$ C, 52 4, H, 8 8. Found C, 52 5, H, 8 7

6-Deoxy-4-C-vinyl-L-talose monohydrate (**5**) — A solution of compound **2** (15 g) in water (525 ml) was heated under reflux for 7 h with Amberlite IR-120 (H^+) resin (100 g) and, after cooling, the supernatant liquor was decanted and the residue washed with hot water (3 × 75 ml) The combined, aqueous solution was clarified with a small amount of DeAcidite FF (OH^-) resin and filtered, and the filtrate was concentrated to a solid which was recrystallised from ethyl acetate Compound **5** (8 8 g, 69%) had m p 110–111°, $[\alpha]_D^{21}$ −4 7° (3 min)→ −23 7° (24 h) (*c* 1.69, water), ν_{max} 1660 cm^{-1} (C=C), R_L 0 63, and 0 46 (chloroform–ethanol, 3 1); n m r. data (D_2O) 4 protons between τ 4 07 and 4 91 (3 vinyl H and H-1), with a 2-proton signal at τ 4 31 ($C=CH_2$)

Anal Calc for $C_8H_{14}O_5$ H_2O C, 46 15, H, 7.75 Found C, 46 2, H, 7 15

Much poorer yields were obtained using either M hydrochloric acid or 0 5M sulphuric acid for the hydrolysis

Degradation of 6-deoxy-4-C-vinyl-L-talose — (*a*) *Weygand–Wolz procedure*[7]. Compound **5** (2 g) was added to a solution of hydroxylamine hydrochloride (1 g) in water (10 ml) at room temperature, and the solution was adjusted to pH 4 0, and maintained at this pH, with 0 5M sodium hydroxide After 24 h, the solution was neutralised, the solvent removed, and the residue dried by distilling ethanol from it The residue was extracted with ethyl acetate (3 × 20 ml), and the extracts were evaporated to yield a deliquescent, amorphous solid (2 g), assumed to be 6-deoxy-4-*C*-vinyl-L-talose oxime, this showed a single spot (R_F 0 60) on paper chromatography This material in methanol (2 ml) and water (10 ml) was treated with sodium hydrogen carbonate (2 g) in water (45 ml) The solution was heated to 65°, and 1-fluoro-2,4-dinitrobenzene (4 g) in propyl alcohol (20 ml) was added in three portions (16, 2, and 2 ml) over 2 h Throughout the reaction, a stream of carbon dioxide was passed through the solution and then through acidified silver nitrate solution to remove hydrogen cyanide [silver cyanide (0 44 g) was collected] The cooled solution was acidified to pH 3 with dilute hydrochloric acid, and the precipitated 2,4-dinitrophenol was removed by filtration The filtrate (neutralised with dilute sodium hydroxide) was evaporated, and a solution of the residue in water was treated with charcoal to absorb excess of fluorodinitrobenzene The filtered solution was reconcentrated, and the residue was extracted with ethyl acetate (3 × 20 ml) Removal of the solvent gave a residue which was shown by paper chromatography to contain a product (R_F 0 66) and impurities (lower R_F values), but no starting material Purification by preparative, paper chromatography afforded a compound, which, after successive recrystallisation from benzene and ethyl ether, had m p 79–80°, $[\alpha]_D^{22}$ +72 (initial)→ −20 5° (24 h) (*c* 1 5, ethyl acetate), R_F 0 66, λ_{max} 6 05 μm ($CH=CH_2$) The yield was 75 mg (5%).

Anal Calc. for $C_7H_{12}O_4$. C, 52 5, H, 7 55, O, 40 0 Found C, 52 6, H, 8 6, O, 40 5

(*b*) *Wohl–Zemplén procedure*[8] 6-Deoxy-4-*C*-vinyl-L-talose monohydrate (**5**) (2 8 g) was dissolved in a solution of hydroxylamine hydrochloride (9 g) in water (20 ml) The pH was adjusted to 4 0 with M sodium hydroxide, and the solution was stirred for 20 h at room temperature and then for 4 h at 80°, the pH being kept at 4 0. The solution was cooled, neutralised with M sodium hydroxide, and evaporated to dryness Fused sodium acetate (4 g) and redistilled acetic anhydride (25 ml) were added to the crude oxime, and the mixture was heated for 5 h at 80° and then for 30 min at 140° After cooling, the mixture was poured onto crushed ice (100 ml) and left for 12 h The aqueous solution was extracted with chloroform (4 × 50 ml), and the extract was washed sequentially with saturated aqueous sodium hydrogen carbonate and water, and dried over anhydrous magnesium sulphate Removal of the solvent yielded a pale-yellow syrup which, on storage, deposited a small amount of crystalline material This material (0 25 g), after recrystallisation (twice) from benzene–light petroleum (b p 60–80°), had m p 184 5–185 5° $[\alpha]_D^{20}$ −35° (*c* 0 83, chloroform), ν_{max} 3600 (OH), 1750 (C=O), 1705 (NAc), 1670 cm^{-1} (C=C) N m r data τ 7 82 (s) (3H), 7 90 (s) (3H), 7 94 (s) (6H) (acetyl protons), 4 58–4 97 (4H, 3 vinyl protons and H-1), with a 2-proton signal at 4 58 ($C=CH_2$), total proton count 23

Anal Calc for $C_{16}H_{23}NO_9$ C, 51 5, H, 6 2, N, 3 75 Found C, 51 2, H, 6 1, N, 3 7.

This material is considered to have structure **8**, it was recovered unchanged after further treatment with sodium acetate and acetic anhydride for 3 h at 140°

The supernatant syrup, assumed to contain the required 2,3,5-tri-*O*-acetyl-4-*C*-vinyl-L-talononitrile, was dissolved in chloroform and treated with ice-cold, methanolic sodium methoxide After 20 min at 0°, the solution was diluted with ice–water and acidified to pH 5 with M sulphuric acid After washing with chloroform, the aqueous layer was evaporated to dryness, the residue was taken up in water, and the solution was evaporated (procedure adopted twice) to remove hydrogen cyanide A solution of the residual syrup in water was de-ionised with a mixed-bed resin, and the product was purified by preparative, paper chromatography. However, apart from starting material (40%), only small quantities of degraded materials were obtained, and these could not be characterised

(*c*) *With lead tetraacetate* Following the procedure described by Perlin[10], a solution of lead tetraacetate (7 4 g) in freshly distilled, glacial acetic acid (250 ml) was added to a solution of compound **5** (3 4 g) in redistilled, glacial acetic acid (400 ml) The resultant solution was stirred vigorously and, after 45 sec, gave a negative starch–iodide reaction. After a further 30 sec, oxalic acid dihydrate [2.1 g in acetic acid (30 ml)] was added, and the mixture was stirred for 30 min After filtration and solvent removal, a cloudy syrup (2 75 g) was obtained which was clarified in ethyl acetate solution Paper-chromatographic analysis of the syrup indicated that it comprised starting material (R_F 0 63), together with a major constituent (R_F 0 75), a minor product (R_F 0 82), and a trace component (R_F 0 66) By use of the hydroxylamine–ferric chloride reagent[15], the components having R_F 0 75 and 0 82 were shown to be formic esters An initial attempt to separate this mixture on a cellulose column was com-

plicated by concomitant, partial hydrolysis of the formic ester groups, so preliminary hydrolysis was carried out by heating the recovered fractions in water on a steam bath for 3 h The formic acid liberated was neutralised with 2M sodium hydroxide and, after solvent removal, the fractions were separately subjected to chromatography on a cellulose column with butyl alcohol half-saturated with water as eluant In this way, the major component, 5-deoxy-3-*C*-vinyl-L-lyxose (7), was obtained as a colourless syrup (0 655 g, 30% based on oxidised material), $[\alpha]_D^{20}$ −45° (*c* 0 22, methanol), ν_{max} 1645 (C=C), 1720 cm^{-1} (C=O), R_F 0.66 and 0 63 (chloroform–ethanol, 3 1) Starting material (5) (0 57 g) was also recovered Although compounds **5** and **7** had very similar R_F values when examined in 4 1 5 butyl alcohol–ethanol–water, they could be distinguished by t l c. using 3 1 chloroform–light petroleum (b p 40–60°) and detection with anisaldehyde Compound **5** had R_F 0 46 and appeared as a dark-purple spot, whereas compound **7** appeared as a yellow–brown spot with R_F = 0.63

Compound **7** (80 mg) and toluene-*p*-sulphonylhydrazine (80 mg) in methanol (1 5 ml) were heated together under reflux for 1 h Water (0 5 ml) was added to the cooled solution The crystals obtained at 0° were recrystallised twice from aqueous methanol to give 5-deoxy-3-*C*-vinyl-L-lyxose toluene-*p*-sulphonylhydrazone (81 mg, 50%) as colourless plates, m p 152 5–153°, $[\alpha]_D^{20}$ −33 9° (*c* 1 6, methanol), ν_{max} 1650 (C=C, C=N), 860, 1600 (phenyl), 1320 (S=O), 1150 cm^{-1} (RSO_2N)

Anal Calc for $C_{14}H_{20}N_2O_5S$ C, 51 2, H, 6 1, N, 8 5, S 9 8 Found C, 50 5 5, H, 6 1, N, 8 7; S, 9 8

Ozonolysis of 5-deoxy-3-C-vinyl-L-lyxose (7) — A solution of compound **7** (200 mg) in dry, distilled ethyl acetate (10 ml) was subjected to ozonolysis at room temperature Uptake of ozone was complete in 0 3 h The solution was shaken with hydrogen over Adams' catalyst until rapid uptake of hydrogen ceased Work-up in the usual way afforded a colourless syrup (200 mg) which, after drying *in vacuo*, showed ν_{max} 1730 (CHO) and 1645 (trace) cm^{-1} (C=C) Paper-chromatographic examination revealed a major component (R_F 0 69), a minor component (R_F 0 35), and a small amount of starting material (R_F 0 66) After storage for several days at 0°, the syrup underwent change, paper-chromatographic examination showed a continuous streak between R_F 0 72 and 0 30, and the material showed only a small absorption at 1730 cm^{-1}.

An attempt to oxidise freshly ozonolysed material (100 mg) with bromine in the presence of barium carbonate to obtain streptosonic acid monolactone was unsuccessful, a small amount (15 mg) of crystalline material, m p 85–87°, ν_{max} 1790 (γ-lactone), 1650 cm^{-1} (H-bonded acid?), R_F 0.72 (Found C, 51 4; H, 7 0%), was obtained but not characterised

ACKNOWLEDGMENT

The authors thank the Governors of Birkbeck College and the Medical Research Council for financial support of this investigation

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

CHEMICAL MODIFICATION OF TREHALOSE

PART III[1]. THE INTRODUCTION OF AZIDO AND AMINO SUBSTITUENTS AT THE 4,4′ AND 6,6′ POSITIONS

Y ALI*, L HOUGH, AND A C RICHARDSON
Department of Chemistry, Queen Elizabeth College (University of London), London, W 8 (Great Britain)

(Received December 24th, 1969)

ABSTRACT

The displacement reactions of 2,3-di-*O*-benzoyl-4,6-di-*O*-mesyl-α-D-glucopyranosyl 2,3-di-*O*-benzoyl-4,6-di-*O*-mesyl-α-D-glucopyranoside (**1**) with azide anion have been studied In hexamethylphosphoric triamide, all four sulphonate groups were replaced to give the tetra-azide **4** with inversion of configuration at the 4,4′ positions When *N*,*N*-dimethylformamide was used as the solvent, selective substitution of only the primary sulphonate groups in **1** occurred The tetraazide **4** was debenzoylated to give 4,6-diazido-4,6-dideoxy-α-D-galactopyranosyl 4,6-diazido-4,6-dideoxy-α-D-galactopyranoside (**5**), which was reduced to the corresponding tetraamine **6**, characterized as the tetra-*N*-acetyl derivative

Similarly, 2,3,6-tri-*O*-benzoyl-4-*O*-mesyl-α-D-glucopyranosyl 2,3,6-tri-*O*-benzoyl-4-*O*-mesyl-α-D-glucopyranoside (**2**) underwent replacement of both sulphonate groups by azide to give, after debenzoylation, 4-azido-4-deoxy-α-D-galactopyranosyl 4-azido-4-deoxy-α-D-galactopyranoside (**8**).

The p m r spectra of the tetra-azido derivatives **4** and **5** were interpretable by first-order analysis and clearly showed that both rings had the *galacto* configuration and that both adopted the C_1^4 conformation

INTRODUCTION

In part II[1] of this series, trehalose derivatives having mesyl substituents at the 4,4′,6,6′ and at the 4,4′ positions were described, all other hydroxyl groups being protected by benzoyl substituents Both of these derivatives underwent nucleophilic displacement by benzoate anions to give the D-*galacto* isomer of trehalose as the octabenzoate

Amino derivatives of trehalose are of interest, since they may possess antibiotic activity[2]; consequently, we have studied the replacement reactions of the sulphonic esters by using the azide anion as nucleophile

*Present address Agricultural College, Abu Ghyraib, Iraq

RESULTS AND DISCUSSION

2,3-Di-*O*-benzoyl-4,6-di-*O*-mesyl-α-D-glucopyranosyl 2,3-di-*O*-benzoyl-4,6-*O*-mesyl-α-D-glucopyranoside (**1**) reacted smoothly with sodium azide in hexameth phosphoric triamide at 100° to afford a tetra-azide in high yield By analogy with t behaviour[3] of methyl 2,3-di-*O*-benzoyl-4,6-di-*O*-mesyl-α-D-glucopyranoside unc similar conditions, the tetra-azide was assigned the D-*galacto* structure **4**, wh would arise by a direct S_N2 replacement reaction This structure was confirmed analysis of the p m r. spectrum (see below) Mainly selective replacement of t primary sulphonate groups of **1** by azide occurred when *N*,*N*-dimethylformamide v used as solvent at 100° The tetra-azide **4** was conveniently reduced either by cataly hydrogenation or by the action of a small proportion of Raney nickel catalyst in t presence of hydrazine The syrupy tetra-amine **6** so formed was converted into t crystalline tetra-*N*-acetyl derivative **7**

The 4,4′-dimethanesulphonate **2** was similarly treated with sodium azide hexamethylphosphoric triamide to give an impure product which, after debenzoylati and silica-gel chromatography, afforded the 4,4′-diazide **8** in 45% overall yield Redu tion of azide **8** by a variety of methods afforded a diamine which was unstable a was transformed into a water-insoluble product during the isolation procedu Attempts to obtain the di-*N*-acetyl derivative also failed to yield a pure product

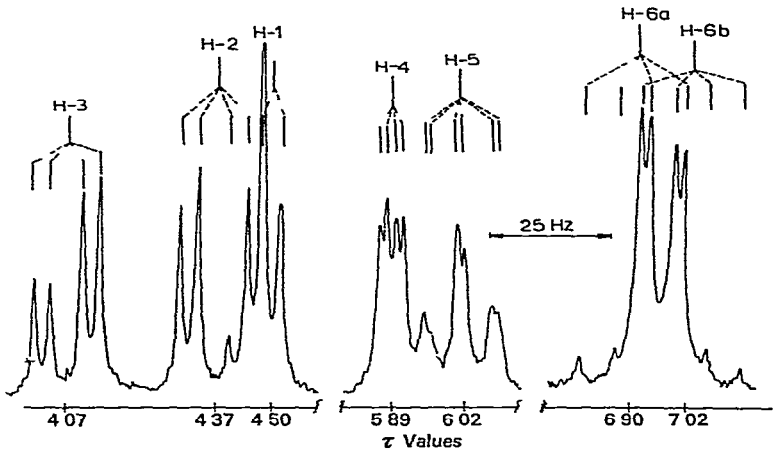


Fig 1 Partial p m r spectrum ($CDCl_3$) of **4** at 100 MHz, showing resonances due to ring prot only

Unequivocal support for the structure of the tetra-azides **4** and **5** was provid by the p m r spectra which were both amenable to first-order analysis The spectru of the tetrabenzoate **4** (Fig. 1) was strikingly similar to that of methyl 4,6-diazid 2,3-di-*O*-benzoyl-4,6-dideoxy-α-D-galactopyranoside[3], except that the resonance f H-1 was at lower field and those for H-6 were at higher field, showing that t two compounds were analogous and that, in the disaccharide, the two rings w

identical The two low-field quartets at τ 4.07 (J 10 2 and 3.4 Hz) and 4 37 (J 10.2 and 3 8 Hz) were assigned to H-3,3′ and H-2,2′, respectively, because of the known tendency of benzoyloxy groups to strongly deshield adjacent methine protons[4], and because the H-1,1′ doublet at τ 4 50 had the same splitting (3 8 Hz) as the quartet at higher field. Of the remaining single-proton resonances, a pair of overlapping quartets at τ 6 90 and 7 02 were obviously due to the AB part of an ABX system and were assigned to the protons at C-6 (and C-6′) The narrow, but well-resolved, quartet, at τ 5 89 (J 3 4 and 1 5 Hz) was characteristic of H-4 of a galactoside[3,5], for which $J_{4,5}$ is usually abnormally small The remaining resonance at τ 6 02, which must be due to H-5,5′, occurred as a sextet made up of a triplet with each limb split by the very small coupling between H-4 and H-5 The observed, first-order, coupling constants ($J_{1\ 2}$ 3 8; $J_{2,3}$ 10 2; $J_{3,4}$ 3 4, $J_{4,5}$ 1 5, $J_{5\ 6a}$ 7 5, $J_{5,6b}$ 7 0, $J_{6a\ 6b}$ 14 0 Hz) confirm that the tetra-azide has the *galacto* configuration and that, as would be expected, both rings are identical and exist in the C_1^4 conformation

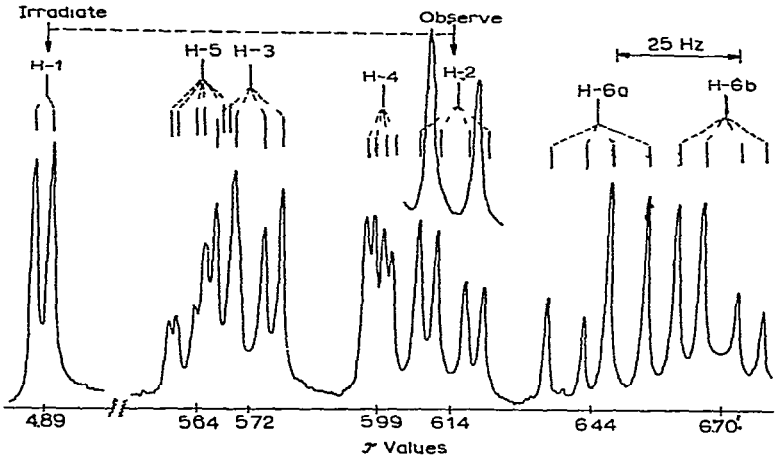
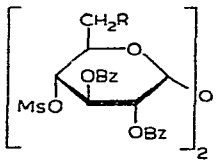


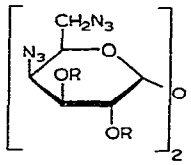
Fig 2 Partial p m r spectrum (acetone-d_6) of 5 at 100 MHz, showing resonances due to ring protons only, and the result of decoupling the H-1 resonance

As a result of the deshielding effect of the benzoate groups in 4, the resonances for the ring protons were spread over 300 Hz (at 100 MHz), but for the debenzoylated derivative 5, the same number of resonances was contained in a width of 130 Hz There was very little overlap (Fig 2), and coupled resonances were sufficiently far apart to permit a first-order interpretation The doublet (τ 4 89, J 3.6 Hz) far H-1,1′ was readily recognised at lower field than any of the others, and irradiation at this frequency collapsed the quartet at τ 6 4 (J 3 6 and 9 9 Hz) into a doublet (J 9 9 Hz), which was therefore assigned to H-2,2′ As in the spectrum of the tetrabenzoate 4, the resonance for H-4,4′ was observed as a narrow, well-resolved quartet at τ 5 99 (J 3 6. and 1.6 Hz) and that for the protons at C-6 (and C-6′) as a pair of separate quartets at τ 6 44 and 6 70 The remaining resonances were a partly overlapped quartet at τ 5 72 (J 9 9 and 3 6 Hz) and a sextet at 5 64 due to H-3,3′ and H-5,5′, respectively.

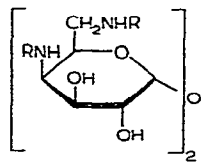
The first-order coupling constants ($J_{1,2}$ 3 6, $J_{2\,3}$ 9 9; $J_{3\,4}$ 3 6, $J_{4,5}$ 1 6, $J_{5\,6a}$ 7.5, $J_{5,6b}$ 5 5, $J_{6a,6b}$ 12 5 Hz) show once again the *galacto* configuration and the C_1^4 conformation of the two rings



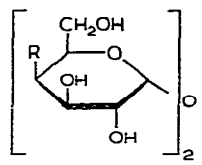
1, R=OMs
2, R=OBz
3, R=N_3



4, R=Bz
5, R=H

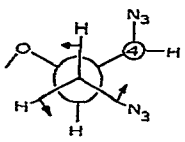


6, R=H
7, R=Ac

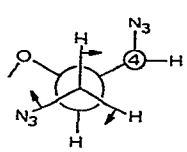


8, R=N_3
9, R=NH_2
10, R=NHAc

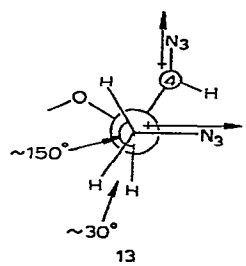
It is notable that for azides **4** and, particularly, **5** the two H-6 resonances are separated, and that approximately first-order coupling constants are available For **5**, the two 5,6 coupling constants are 7 5 and 5 5 Hz In open-chain derivatives, the coupling between *gauche* hydrogen atoms (60°) in the staggered arrangement should be *ca* 3–4 Hz, and for *anti* hydrogens atoms the value should be *ca* 8–9 Hz[6], although values of about 2 and 9 5 Hz have been suggested[7] For the tetra-azides **4** and **5**, the most-stable conformation of the exocyclic carbon atom, from steric considerations, would be either **11** or **12**



11



12



13

However, for each of these conformations, coupling constants of *ca* 3 5 and 8 5 Hz would be expected In order to explain the observed difference in values, rotation of the C-5–C-6 bond must occur from the completely staggered arrangements to the extent of *ca* 30°, an anticlockwise rotation is required in **11** and a clockwise rotation in **12*** In **12**, such a rotation will increase the dipolar interaction between the azide substituent and the dipole associated with the ring oxygen atom, whereas in **11** rotation through *ca* 30° would hold the C-6–N_3 and C-4–N_3 bond at 90° and hence minimise dipolar interaction This can be best visualised by using molecular models of the Dreiding type and viewing along the C-6–C-4 axis It is therefore postulated that in **5** the position of the azide group is as shown in **13**

*Such a rotation will decrease the H-H dihedral angles from 60° and 180° to about 30° and 150°, respectively, and hence increase the smaller coupling constant and decrease the larger value [M Karplus, *J Phys Chem*, 30 (1959) 11]

EXPERIMENTAL

Unless otherwise stated, all rotations are in chloroform solution at $21 \pm 2°$ The infrared spectra of all of the new compounds were in accord with the structures assigned

4,6-*Diazido*-2,3-*di*-O-*benzoyl*-4,6-*dideoxy*-α-D-*galactopyranosyl* 4,6-*diazido*-2,3-*di*-O-*benzoyl*-4,6-*dideoxy*-α-D-*galactopyranoside* (**4**) — The tetrasulphonate[1] **1** (8 g) was dissolved in dry, warm hexamethylphosphoric triamide (32 ml) and heated at 100° (bath) with sodium azide (12 g) for 2 h when t l c (ether) indicated that the reaction was complete The product was precipitated by the addition of water, filtered off, and washed well with water The moist solid was dissolved in methylene chloride, and the solution was washed well with water, dried ($MgSO_4$), and evaporated to a syrup which crystallised in the presence of a little ethanol The tetra-azide was recrystallised by slowly adding ethanol to a solution in a little boiling ether When nearly all of the ether had boiled off, the product crystallised to give **4** (5 g, 78%), m p 62–66°, $[\alpha]_D$ +196° (*c* 1 4) The analytical sample was obtained by a similar recrystallisation, except that methylene chloride was used in place of ether, m p 65–67°, $[\alpha]_D$ +190° (*c* 2) (Found C, 55 4, H, 4 3, N, 19 3 $C_{40}H_{34}N_{11}O_{12}$ calc C, 55 9, H, 3 9, N, 20 5%)

6-*Azido*-2 3-*di*-O-*benzoyl*-6-*deoxy*-4-O-*mesyl*-α-D-*glucopyranosyl* 6-*azido*-2,3-*di*-O-*benzoyl*-6-*deoxy*-4-O-*mesyl*-α-D-*glucopyranoside* (**3**) — The tetrasulphonate[1] **1** (10 g) was heated in *N*,*N*-dimethylformamide (50 ml) with sodium azide (4 g) for 3 75 h at 100° (bath) Monitoring of the reaction mixture by t l c (ether) indicated that a slower-moving material (probably the mono-azide) was formed initially, which was transformed into the diazide Subsequently, a faster-moving component appeared, which was probably a triazide The reaction mixture was diluted with water, and the solid product was collected and washed well with water and ethanol Recrystallisation was achieved by slow addition of methanol to a boiling solution of the product in a small volume of methylene chloride When most of the methylene chloride had evaporated, crystallisation of the product (7 1 g, 78%) took place T l c indicated that the diazide was contaminated with *ca* 5–10% of the faster-moving triazide Two further recrystallisations by the same procedure, with the exception that the crystals were filtered off from the boiling solution, afforded the pure diazide free of the impurity, m p 173–175°, $[\alpha]_D$ +239° (*c* 1 6) (Found C, 52 4, H, 4 1, N, 8 5 $C_{42}H_{40}N_6O_{17}S_2$ calc C, 52 4, H, 4 1, N, 8 7%)

P m r data (100 MHz) τ 4 44 (doublet, H-1,1′), 4 72 (quartet H-2,2′), 3 92 (triplet, H-3,3′), 5 06 (triplet, H-4,4′), 5 99 (1 2 1 1 2 1 sextet, H-5,5′), 7 1 (complex multiplet, H-6,6′), 7 2 (singlet, CH_3SO_2), 1 8–2 8 (complex multiplet, aromatic protons) First-order coupling constants $J_{1,2}$ 3 6, $J_{2,3}$ 10 0, $J_{3,4}$ 9 8, $J_{4,5}$ 9 8, $J_{5,6a}$ ~3–4, $J_{5\ 6b}$ ~3–4/Hz

4,6-*Diazido*-4,6-*dideoxy*-α-D-*galactopyranosyl* 4,6-*diazido*-4,6-*dideoxy*-α-D-*galactopyranoside* (**5**). — The tetrabenzoate **4** (3 g) was suspended in methanol (15 ml), and 0 5M sodium methoxide (2 ml) was added The mixture was heated under reflux for a few minutes when dissolution was rapidly achieved The mixture was then evapor-

ated under a stream of compressed air until crystallisation commenced The product was collected, and washed well with methanol and ether to give 0 88 g (57%) of the tetra-azide, m p. 194 5–195°, $[\alpha]_D$ +122° (*c* 1 5, methanol)

The mother liquors were deionised with Amberlite IR-120 (H^+) resin and concentrated to dryness Recrystallisation of the buff-coloured solid from ethanol gave **5** (0 37 g, 24%), m p 195°, $[\alpha]_D$ +124° (*c* 1, methanol) (Found C, 32 4, H, 4 5, N, 37 0. $C_{12}H_{18}N_{12}O_7$ calc C, 32 6, H, 4 1, N, 37 9%)

4,6-*Diacetamido*-4,6-*dideoxy*-α-D-*galactopyranosyl* 4,6-*diacetamido*-4,6-*dideoxy*-α-D-*galactopyranoside* (**7**) — A solution of the tetra-azide **5** (0 34 g) in boiling ethanol (25 ml) containing a little Raney nickel catalyst was treated slowly with hydrazine hydrate (2 ml) so as to maintain a steady effervescence The mixture was boiled for a further 15 min, filtered, and concentrated to dryness The resulting syrup was taken up into a little methanol, some insoluble material was filtered off, and the filtrate was treated with acetic anhydride (1 5 ml) and then concentrated until crystals began to appear. After cooling, the tetra-*N*-acetyl derivative was collected as a monohydrate (0 205 g, 52%) and washed well with ethanol and acetone, m p 294–297° (decomp) with softening and pre-melting at 210–220° (loss of hydration), $[\alpha]_D$ +176° (*c* 0 8, water) The analytical sample was prepared by recrystallisation from aqueous methanol (Found C, 45 6, H, 6 9, N, 10 7 $C_{20}H_{36}N_4O_{12}$ calc . C, 45 8, H, 6 9, N, 10 7%)

The same product was obtained in about the same yield by catalytic hydrogenation of the tetra-azide with 10% palladium-on-charcoal It had m p 289–293° and $[\alpha]_D$ +175° (*c* 0 5, water).

4-*Azido*-4-*deoxy*-α-D-*galactopyranosyl* 4-*azido*-4-*deoxy*-α-D-*galactopyranoside* (**8**) — The 4,4′-dimethanesulphonate **2** (5 g) was dissolved in warm hexamethylphosphoric triamide (25 ml) and heated at 100° (bath) with sodium azide (2 g) for 2 h The product, precipitated by the addition of water, was extracted with ether The dried ethereal solution was evaporated to give a syrup that was composed (t l c , methylene chloride–methanol, 4 1) of a major component and two minor components

The syrup was suspended in hot methanol (50 ml) and treated with 0 5N sodium methoxide (3 ml), and the mixture was boiled until dissolution was achieved (a few minutes), when examined by t l c , the presence of a single, major component and two minor impurities (of which one moved with the solvent front) was indicated The faster-moving component was removed during the subsequent work-up which involved the dilution of the reaction mixture with water and ether extraction Deionisation and subsequent concentration of the aqueous layer afforded the diazide contaminated with a slower moving impurity, as indicated by t l c The syrup was purified on a column of silica gel with 4 1(v/v) methylene chloride–methanol to give a syrup which slowly crystallised in the presence of a little acetone Recrystallisation from acetone gave the diazide **8** (0 78 g, 45%), m p 205–206° (dec), $[\alpha]_D$ +172° (*c* 1, water) The analytical sample was further crystallised from acetone–chloroform, m p 204–205° (Found C, 36 0, H, 5 1, N, 21 2 $C_{12}H_{20}N_6O_9$ calc C, 36 75, H, 5 1; N, 21.4%)

Attempted reduction of the diazide **8** in methanolic solution by using either hydrazine–Raney nickel, 10% palladium-on-charcoal–hydrogen at 50 pounds/in^2, or

Adams' catalyst–hydrogen at 60 pounds/in^2 afforded, in each case, a seemingly crystalline residue after filtration and concentration Upon attempted recrystallisation, it changed into an amorphous solid that was insoluble in water and alcohols

In one case, the reaction mixture, after the removal of the catalyst, was concentrated to small bulk and treated with acetic anhydride, whereupon a white, powdery solid was precipitated which decomposed without melting at 265–270°, but which was only partially soluble in water and completely insoluble in methanol (Found C, 44 5, H, 6 7, N, 5 8 $C_{16}H_{28}N_2O_{11}$ calc C, 45 3, H, 6 6, N, 6 6%)

A further reduction, with Adams' catalyst in a mixture of water and acetic anhydride, failed to give a characterisible product

ACKNOWLEDGMENTS

We thank Bristol Laboratories for generous financial support, and the Physico-Chemical Measurements Unit at Harwell for 100-MHz spectra

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

THE ENZYMIC HYDROLYSIS OF SUBSTITUTED PHENYL β-D-XYLOPYRANOSIDES

F VAN WIJNENDAELE AND C K DE BRUYNE
H I K W, Lab Anorg Scheikunde A, State University, Ghent (Belgium)
(Received November 1st, 1969, in revised form, January 1st, 1970)

ABSTRACT

The influence of the substituents on the enzymic hydrolysis of substituted phenyl β-D-xylopyranosides by a β-D-xylosidase has been investigated Electronic, steric, and hydrophobic parameters were used, with regression analysis, to analyse the structure–activity relationship The K_M and V_M values were correlated with the electronic substituent constants The *meta* and *para* substituents behaved as separate series Hydrophobic and steric influences were of minor importance The influence of the substituents on V_M depends on the temperature of the experiments as compared to the isokinetic temperature and on the place of the substituent

INTRODUCTION

In recent years, there have been many successful attempts to correlate biological activity with chemical constitution Hansch and his co-workers[1–6] introduced the concept of new parameters and the use of multiple regression analysis They succeeded in correlating the biological activity with a linear combination of electronic (Hammett σ), hydrophobic (π), and steric (E_s) parameters An especially interesting case was their analysis of data[7] for the hydrolysis of substituted phenyl β-D-glucosides by emulsin[3] Other examples include choline-esterase inhibitors[8], monoamineoxidase inhibitors[9], and pancreatic lipase[10].

Our investigation is concerned with the influence of the substituents on the enzymic hydrolysis of a series of phenyl β-D-xylopyranosides by a β-D-xylosidase[11] of fungal origin

Under the conditions used, all substrates followed simple Michaelis–Menten or Briggs–Haldane[12] kinetics Thus, the Michaelis constants (K_M) and maximal velocity values (V_M) were determined from Hanes' plots[13] by weighted regression analysis According to Koshland[14], the enzymic hydrolysis proceeds by a double-displacement mechanism with retention of configuration In our case, the first step should lead to a xylosyl–enzyme complex, presumably by a concerted attack of an electrophile on the glycosyl oxygen atom and a nucleophile on C-1 of the glycosyl group with heterolysis of the glycosyl–oxygen bond The second step is then a nucleophilic attack of the acceptor molecule (in this case water) on C-1 of the glycosyl group, with subse-

quent desorption Polar factors should be important in the first step and in the push-pull mechanism itself, whereas hydrophobic and steric factors should influence the adsorption/desorption steps We tried, thus, to correlate K_M and V_M with the electronic, hydrophobic, and steric parameters

RESULTS AND DISCUSSION

Influence of the substituents on K_M — K_M and pK_M ($= -\log K_M$) values and estimated standard deviations are presented in Table I It is clear from these data that

TABLE I

K_M VALUES AT 30° AND 40°

Substituent	σ	$10^4 K_M(M)$				pK_M	
		40°		*30°*		*40°*	*30°*
None	0	6 12	±0 33	5 73	±0 25	3 230	3 242
p-Methyl	−0 17	2 61	0 20	2 32	0 18	3 583	3 634
p-Chloro	+0 227	2 94	0 20	2 56	0 17	3 531	3 592
p-Methoxy	−0 268	3 10	0 20	3 00	0 20	3 510	3 523
p-Nitro	+1 27	3 47	0 18	3 33	0 10	3 459	3 478
m-Chloro	+0 373	4 50	0 20	3 99	0 13	3 346	3 398
m-Methyl	−0 069	5 32	0 21	4 99	0 10	3 274	3 302
m-Nitro	+0 710	3 77	0 10	3 20	0 10	3 453	3 496
p-Chloro-*m*-methyl	+0 158	3 64	0 10	3 07	0 12	3 438	3 511
3,4-Dimethyl	−0 23	4 05	0 21	3 58	0 14	3 392	3 446

the substituents have only a limited effect on K_M When a correlation of pK_M with σ and/or π was attempted, this proved unsuccessful, both for single and multiple parameter functions The correlation coefficient for the regression function, pK_M *versus* σ, is less than 0 1. However, if *para* and *meta* substituents are taken as separate series, an excellent correlation is obtained for both groups Regression analysis yields the equations

for *para* substituents, $\log K_M\,(40°) = -3\,561 + 0\,0826\sigma$, with reaction constant $\rho = +0\,083 \pm 0\,013$, the standard error of the estimate $s_{y\,x} = 0\,014$, and the correlation coefficient $r = 0\,99$, and $\log K_M\,(30°) = -3\,616 + 0\,108\,\sigma$, with $\rho = +0\,108 \pm 0\,001$, $s_{y,x} = 0\,001$ and $r = 0\,99$

for *meta* substituents, $\log K_M\,(40°) = -3\,281 - 0\,226\sigma$, with $\rho = -0\,23 \pm 0\,04$, $s_{y\,x} = 0\,024$, and $r = 0\,98$, and $\log K_M\,(30°) = -3\,315 - 0\,246\sigma$, with $\rho = -0\,25 \pm 0\,02$, $s_{y,x} = 0\,011$, and $r = 0\,99$

The disubstituted substrates do not fit either of these equations Although the K_M values do not differ much from each other, and the high correlation is in part due to the fact that few points were used in the regression, there seems to be no doubt that *meta* and *para* substituents cannot be treated together as one single series These equations also reveal that hydrophobic bonding (as measured by π) or steric factors

play little role in the *relative* activity of the substrates, since neither π nor E_s is necessary in the equations Thus, the relative changes in K_M are due to polar effects, but even these effects are of minor importance as can be seen from the low value of the reaction constant ρ.

The fact that *meta* and *para* substituents must be taken as separate series and that the reaction constants have different signs reveals the special nature of the enzyme catalysis In contrast to chemical (*e g* , acid or base) catalysis, the spatial requirements of the active site introduce a supplementary factor that influences the formation of the enzyme–substrate complex and the reaction itself A basic simplification of the Hammett theory, that *meta* and *para* substituents are held rigidly at such large distances from the reaction centre that no change in steric interactions occurs and that the polar effects of *meta* and *para* substituents can be treated together, does not hold *a priori* for enzyme catalysis Even if one considers the very simple situation in which the effect of the substituent is solely polar in nature, its magnitude can be altered by the proximity of a group on the enzyme surface If this is the case, any *meta* substituent may be influenced so that the Hammett σ value no longer reflects its actual polar effect in the enzyme–substrate complex, whereas the same substituent in the *para* position may not be altered A somewhat similar situation occurs with the well-known steric inhibition of resonance In this case too, a neighbouring group disturbs the substituent, so that its normal polar effect (σ) is altered Thus, it seems quite normal that, in enzymic reactions, *meta* and *para* substituents react in a somewhat different way

The same argument explains why disubstituted substrates do not fit the regression lines If the polar effects were additive, as is the case in chemical catalysis, these derivatives should fit the equations Actually, the K_M values for these substrates are a mean of the values for the corresponding monosubstituted xylosides A further illustration of the spatial requirements and influences is found in the fact that phenyl β-D-xyloside, without substituent groups, also deviates from the equations

If, according to Exner[15], $\log K_M$ (40° = T_1) is plotted *versus* $\log K_M$ (30° = T_2), all points fit the same isokinetic line Regression analysis yields the equation

$\log K_M$ (40°) = −0 258 +0 914 $\log K_M$ (30°), with slope b = 0 91 ±0 05, $s_{y,x}$ = 0 02, T_2/T_1 = 0 968, and r = 0 99 The isokenitic temperature β = 481 °K = 208°, and is thus far above the experimental temperature With $b < T_2/T_1$ and $\beta > T_1$, this represents case 3/*a* or compensation in the Exner classification In this case, the selectivity of the reaction decreases with increasing temperature As can be seen from the Hammett plot, this is indeed the case, since the ρ-value decreases with increasing temperature both for *para* and *meta* substituents Because the derivatives fit the same isokinetic line, it seems very probable that the same fundamental mechanism operates for all of these substrates

A different character in the activity of *meta* and *para* substituents was found by Fuller *et al.*[9] for the inhibition of monoamineoxidase, by Hansch *et al.*[8] for choline-esterase inhibitors, and for the hydrolysis of phenyl β-D-glucosides by emulsin[3] In the latter case, however, the same equation could be derived for the effects of both *para* and *meta* derivatives, if it is accepted that hydrophobic forces are

very important for a *para* substituent but not for the same group in a *meta* position The coefficients of the σ-parameter (ρ) were large and had the same sign, which means that polar effects were important in the case of emulsin Our results reveal the absence of hydrophobic and steric effects and prove that even polar effects are of limited importance Thus, there is a large difference in the design of the active site of the β-D-xylosidase and the emulsin, not only with respect to the glycosyl group but also to the aglycon group

Influence of the substituents on V_M — Tables II and III summarize the maximum velocities (expressed as μmoles of phenol per min and per mU of enzyme activity) and

TABLE II

MAXIMUM REACTION VELOCITY (V_M/mU) AT 30°

Substituent	σ	$V_M/mU \times 10^3$		$-log\ V_M$ *observed*	$-log\ V_M$	
					recalc [a]	*recalc* [b]
None	0	0 55	±0 01	3 26	3 26	3 16
p-Methyl	−0 17	0 53	0 02	3 28	3 27	3 14
p-Chloro	+0 227	0 57	0 01	3 24	3 25	3 18
p-Methoxy	−0 268	0 54	0 01	3 26	3 27	3 13
p-Nitro	+1 27	0 63	0 02	3 20	3 20	3 28
m-Chloro	+0 373	0 62	0 02	3 20	3 24	3 20
m-Methyl	−0 069	0 71	0 02	3 15	3 26	3 15
m-Nitro	+0 71	0 60	0 01	3 22	3 22	3 23
p-Chloro-*m*-methyl	+0 158	0 57	0 01	3 25	3 25	3 17
3,4-Dimethyl	−0 230	0 59	0 01	3 23	3 27	3 14

[a]Calculated from the regression equation for *para* substituents [b]Calculated from the regression equation for *meta* substituents

TABLE III

MAXIMUM REACTION VELOCITY (V_M/mU) AT 40°

Substituent	σ	$V_M/mU \times 10^3$		$-log\ V_M$ *observed*	$-log\ V_M$	
					recalc [a]	*recalc* [b]
None	0	1 03	±0 03	2 98	2 98	2 86
p-Methyl	−0 17	0 98	0 03	3 01	3 00	2 85
p-Chloro	+0 227	1 09	0 03	2 96	2 97	2 87
p-Methoxy	−0 268	0 99	0 04	3 00	3 00	2 85
p-Nitro	+1 27	1 27	0 04	2 89	2 88	2 93
m-Chloro	+0 373	1 28	0 04	2 89	2 96	2 88
m-Methyl	−0 069	1 38	0 06	2 86	2 99	2 86
m-Nitro	+0 710	1 25	0 03	2 90	2 93	2 90
p-Chloro-*m*-methyl	+0 158	1 19	0 05	2 92	2 97	2 87
3,4-Dimethyl	−0 23	1 13	0 03	2 95	3 00	2 85

[a]Calculated from the regression function for *para* substituents [b]Calculated from the regression function for *meta* substituents

the estimated standard deviations Although the enzyme concentration is not known, the maximum velocity, since it is calculated on the basis of one and the same enzyme activity, is proportional to the overall reaction constant k_2 in the scheme

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

As can be seen from Tables II and III, the substituent groups have a very small effect on V_M and, thus, on the reaction itself As in the determination of the K_M values, the *meta* and *para* derivatives had to be treated separately to obtain a good correlation

Regression analysis yields the following equations

for *para* substituents, log V_M (40°) = $-2\,984 + 0\,075\sigma$, with $\rho = +0\,075 \pm 0\,007$, $s_{y,x} = 0\,01$, and $r = 0\,99$, and log V_M (30°) = $-3\,259 + 0\,048\sigma$, with $\rho = +0\,048 \pm 0\,008$, $s_{y,x} = 0\,01$, and $r = 0\,96$

for *meta* substituents, log V_M (40°) = $-2\,863 - 0\,052\sigma$, with $\rho = -0\,052 \pm 0\,01$, $s_{y,x} = 0\,012$, and $r = 0\,97$, and log V_M (30°) = $-3\,161 - 0\,09\sigma$, with $\rho = -0\,09 \pm 0\,02$, $s_{y,x} = 0\,013$, and $r = 0\,97$

The log V_M values were recalculated for all derivatives by using either the *para* or the *meta* equation (Tables II and III) As can be seen from these recalculated values, the fit is excellent, except for disubstituted derivatives where the maximum velocities are a mean of the values for the monosubstituted substrates The situation is thus very similar to that found for K_M values, except for the fact that the reaction constant (ρ) for *para* derivatives increases with increasing temperature, whereas for *meta* substituents it decreases

It may seem surprising that polar effects have so little influence on the reaction If the hydrolysis is depicted as a heterolysis of the glycosyl–oxygen bond with an electron shift to the oxygen atom, it is difficult to see how a negative substituent (*e g*, the strongly electron-attracting nitro group) could have the same influence as a positive substituent (*e g*, methyl) But it is possible that the small, overall effect is due to cancellation of opposite effects, analogous to the acid-catalyzed hydrolysis of the same compounds[17] If an acid group on the enzyme takes part in the reaction mechanism by protonation of the glycosyl oxygen atom, a nitro group, by lowering the electron density around this oxygen atom, will affect this step unfavourably, but heterolysis will still be favoured The converse is true for a positive substituent

The mechanistic pathway may then involve an intermediate carbonium–oxonium ion A stabilization of this carbonium ion by a basic group on the enzyme surface could then account for the high transferase activity of this enzyme with numerous alcohols as acceptor[19] The subsequent step consists of attack, which is possibly base-assisted, by a water molecule on C-1 of the xylosyl carbonium ion, and desorption The whole mechanism is thus, in fact, the Koshland[14] double displacement

This poses the question of whether the change from *para* to *meta* for a given substituent does alter the reaction mechanism Accordingly, log V_M (40° = T_1) was

plotted *versus* log V_M (30° = T_2) Graphical analysis proves that there are indeed two isokinetic lines For *para* substituents, log V_M (40°) = −2 108 +1 563 log V_M (30°), with b = 1 563 ±0 126, $s_{y,x}$ = 0 007, and r = 0 99 The isokinetic temperature β = 287°K = 14° (±4) is below the experimental temperature With $b>1$ and $\beta<T_2$, this represents case 3/b or compensation of the parameters in the Exner classification The selectivity should increase with increasing temperature, which is indeed the case if one considers the ρ-values at 30° (0 048) and 40° (0 075)

For *meta* substituents, log V_M (40°) = −1 179 +0 534 log V_M (30°), with b = 0 534 ±0 002, $s_{y,x}$ = 0 001, T_2/T_1 = 0 936, and r = 0 98 The isokinetic temperature β = 325°K = 52° is above the temperature of the experiment With $b<T_2/T_1$ and $\beta>T_1$, this represents case 3/a or compensation of the parameters, but now the selectivity should decrease with increasing temperature The Hammett relation indicates that ρ decreases from 0 09 (30°) to 0 05 (40°)

According to Leffler[16], the reaction constant ρ becomes zero at the isokinetic temperature and changes its sign when surpassing this temperature If the Exner plot and the β-value are real, the isokinetic temperature approaches the temperature of the experiment for both *meta* and *para* derivatives, and hence ρ approaches zero This could be a reason why the polar factors, as expressed by σ/ρ, have little importance The different signs associated with the ρ-values are then attributable to the fact that the experimental temperature for *meta* is below, and for *para* is above, the isokinetic temperature of the respective series In this case, neither the sign nor the value of ρ has a clear-cut mechanistic meaning

As found for the Hammett plots, disubstituted derivatives do not fit the isokinetic lines, illustrating the fact that, because of the very special nature of enzyme catalysis, substituent effects are not always simply additive

Since *meta*, *para*, and disubstituted derivatives do not fit the same isokinetic line, the strict application of the criterion means that the hydrolysis does not proceed by the same mechanism However, this seems very improbable All of the substituted xylosides are hydrolyzed by the same main reaction type, involving compensation of the activation parameters An increase in $\Delta H^{\ddagger}$ is cancelled by an increase in $\Delta S^{\ddagger}$, so that $\Delta G^{\ddagger}$ remains essentially constant The sign of the ρ-values can be attributed to the different isokinetic temperatures, and even this difference does not surpass 40° For these reasons, we believe that all derivatives are hydrolyzed by the same basic mechanism, and that the small differences are due to the fact that these criteria are too simple for the complex nature of the enzyme catalysis

Our results can be compared with those of Hantsch *et al* for the hydrolysis of phenyl β-D-glucosides Again, the same regression function could be calculated, if it is accepted that hydrophobic forces are important in the reaction mechanism of the *para*, but not of the *meta*, derivatives This is not the case for the β-D-xylosidase A further difference is found in the fact that the ρ-values had the same sign and were much higher than our values Thus, polar factors are important for emulsin but not for β-D-xylosidase

In a study of the base-catalyzed hydrolysis[18] of the same series of substituted phenyl β-D-xylosides, it was found that polar factors have a large influence on the reaction rate ($\rho = +2.3$) Electron-attracting groups greatly increase the rate, in accordance with the accepted mechanism This involves the anchimeric assistance of the C-2 oxyanion and the formation of an intermediate 1,2-epoxide The absence of polar influences in the enzymic reaction rules out the possibility of an analogous, anchimeric effect, the enzyme providing the general base for abstraction of a proton from HO-2 of xylose

The change of the β-value for the *meta* and *para* derivatives most probably occurs in the heterolysis mechanism itself, since the nature of the aglycon group seems less important in adsorption and desorption steps A possible explanation for the different, isokinetic temperatures could then be a shift in the relative importance of the electrophile/nucleophile or general acid/base parts of the push-pull mechanism This shift should be caused by the *place* of the substituent on the phenyl ring and not by its nature

That this β-D-xylosidase binds specifically to the glycon moiety of the substrates, and that the aglycon part, in contrast, binds aspecifically, was proved by Loontiens[11] and Claeyssens[19] This, and a cancelling effect in adsorption/desorption steps, seems to be the reason why relative hydrophobic parameters are unimportant, although there can be no doubt that the aglycon group binds by hydrophobic forces to the enzyme[19]

According to steady-state kinetics, K_M is a composite constant [$K_M = (k_{-1} + k_2)/k_1$], and thus $1/K_M$ is not, *a priori*, a measure of the affinity of the enzyme for the substrate Log K_M values fit the same isokinetic line, and the isokinetic temperature is far above the temperature of the experiment The different signs of the ρ-values are thus probably due to the influence of the k_2 ($= V_M$) in the composite constant K_M This means that $1/K_M$ is not a measure of the affinity of the enzyme and makes

TABLE IV

ACTIVATION ENERGY

Substituent	*Enzymic*	E (*kcal mole*$^{-1}$)	
		0 1M HCl[a]	*4M NaOH*[b]
None	11 7	29 6	28 2
p-Methyl	11 5	28 7	28 7
p-Chloro	12 1	29 2	27 3
p-Methoxy	11 3	30 7	26 2
p-Nitro	13 3	28 3	26 4[c]
m-Chloro	13 6	28 6	27 9
m-Methyl	12 5	29 3	27 4
m-Nitro	14 0	27 6	28 1[d]
p-Chloro-*m*-methyl	13 9	29 4	27 8
3,4-Dimethyl	12 2	30 7	28 3

[a]From Ref 17 [b]From Ref 18 [c]0 05M NaOH [d]M NaOH

speculation about the influence of the substituents on the "binding" of the substrate of rather doubtful value

With the aid of the V_M values at 30° and 40°, the Arrhenius activation energy (E) was calculated and compared to the energy found for the acid- and base-catalyzed reactions (Table IV) Since only two values of V_M were used, the standard deviation on E is large (*ca* 1 kcal), which makes comparison difficult However, it is clear from the data that the activation energy for the enzymic reaction is very low compared to the chemical catalysis, and that the *meta* substituents on the whole show a higher E-value than the *para* derivatives

EXPERIMENTAL

The substituted phenyl β-D-xylopyranosides were synthesized as described previously[20–22]. The enzyme was purified from a commercial hemicellulase of fungal source by Loontiens[11], who investigated the glycon specificity and found that the enzyme also hydrolyzed, although to a much smaller extent, β-D-glucosides and α-L-arabinosides The pH optimum for the β-D-xylosidase (β-D-xyloside xylohydrolase, EC 3 2 1 37) is about 3 0–4 0

Standardisation of the enzyme activity — Since no crystalline enzyme was available, and all of the reaction rates had to be calculated for the same amount of enzyme, the activities of the enzyme solutions were carefully determined under standard conditions

The standard incubation mixture contained 16 6mM biphthalate buffer (pH 3) 2mM *p*-nitrophenyl β-D-xylopyranoside, and enzyme, and was kept at 40° At intervals, portions (2 ml) were withdrawn and added to 2 ml of aqueous sodium carbonate (15%), and the absorption was measured in a Beckman Model C Colorimeter at 400 nm (Jenaer–Schott IL/PIL filter) The reaction velocity was calculated from the slope of the line of absorption *versus* time Only the region of linear increase in absorption was used A unit (U) of xylosidase activity is defined as the amount of enzyme that catalyses the hydrolysis of one μmole of *p*-nitrophenyl β-D-xyloside per ml per minute, under the conditions described A milliunit (mU) was used for practical purposes Each standardisation value used was the estimated mean, calculated by the method of least-squares, of at least ten determinations The estimated, standard deviation amounts to 1% (maximum)

Determination of K_M *and* V_M — All determinations were carried out in 16.6mM biphthalate buffer (pH 3), since it was shown that this buffer had no inhibitory effect on the enzyme The substrate solutions were always freshly prepared

The reaction mixture consists of 16 6mM buffer (pH 3), 0 3–2 0mM substrate, and 30–50 mU of enzyme. The exact amount of enzyme varied, but was always carefully determined and was usually in the range 30–50 mU At intervals, 1-ml portions of the reaction mixture were withdrawn and added to 2 ml of aqueous sodium carbonate (20%), and the concentration of the liberated phenol was measured, nitrophenols were determined at 400 nm, and the other phenols by the method of

Folin and Ciocalteu[23]. For each phenol, a calibration curve had to be constructed under similar conditions.

The initial velocities were calculated from the slope of the graph (initial, linear portion) of μmole of phenol/ml *versus* time (min). Each value is the estimated mean of at least three determinations

When substrate concentrations higher than 2mM were used, the reaction no longer followed simple Michaelis–Menten kinetics, presumably because of a transfer reaction in which phenyl β-D-xylopyranoside acts as an acceptor[24], with the subsequent synthesis of higher saccharides.

In order to detect significant, experimental aberrations, K_M and V_M were first determined graphically from a Hanes plot[13] The more-exact values and their estimated standard deviations were then calculated by weighted-regression analysis, according to the procedure of Wilkinson[25] The essential difference is that weighted, rather than simple, means are calculated, and weighted sums of squares and products are used The variance on the observed velocity is taken as a measure of the statistical weight This more-complex method was chosen because K_M and V_M showed only slight differences with the different substrates Since V_M values had to be calculated on the same enzyme-activity basis (1 mU), the standard deviation on the enzyme standardisation was included in the calculation of the estimated, standard deviation on V_M/mU.

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Carbohydrate Research
Elsevier Publishing Company Amsterdam
Printed in Belgium

THE PREPARATION OF COMB-SHAPED POLYSACCHARIDES BY POLYMERIZATION OF A MALTOSE DERIVATIVE

B VERUOVIC* AND C SCHUERCH

Department of Chemistry, State University College of Forestry at Syracuse University, Syracuse, New York 13210 (U S A)

(Received October 27th, 1969, in revised form, January 7th, 1970)

ABSTRACT

The Lewis acid-catalyzed polymerization of 1,6-anhydro-2,3-di-*O*-benzyl-4-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl)-β-D-glucopyranose (1,6-anhydromaltose-hexabenzyl ether) leads to products of number-average molecular weights up to about 14,000 and specific rotations as high as +96–97° Variations in reaction conditions affect the molecular weight and optical rotation of the products significantly Debenzylation of the products having the highest molecular weight and optical rotation yields hydrated polysaccharides, presumably comb-shaped, of $[\alpha]_D^{25}$ +174° (or corrected to a water free condition $[\alpha]_D^{25}$ +189°) This value corresponds to a high degree of stereoregularity in the main chain with predominantly α-D linkages on both the main chain and branches

INTRODUCTION

In spite of the great potential importance of biochemical and physiological studies on stereoregular polysaccharides, there are very few methods available for their controlled synthesis Three stereoregular (1→6)-α-D-glycopyranans have been synthesized in this laboratory by the phosphorus pentafluoride-initiated polymerization of appropriate 1,6-anhydro-2,3,4-tri-*O*-benzyl-β-D-glycopyranoses, followed by debenzylation of the polymeric derivatives with sodium in liquid ammonia[1–6]. It is of interest to determine how general this reaction may be The polymerization of a perbenzylated 1,6-anhydro disaccharide should yield a comb-shaped polymer with a linear backbone and pendant monosaccharide units regularly substituted on each sugar unit in the main chain Polysaccharides of this type have not been synthesized previously and appear to be only approximated in nature This article describes the investigation of the polymerization of perbenzylated 1,6-anhydromaltose (1,6-anhydro-2,3-di-*O*-benzyl-4-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl)-β-D-glucopyranose), and the preparation of the parent polysaccharide

*Present address Katedra Pakr Chemie, VSCHT, Praha 6, Czechoslovakia

TABLE I

POLYMERIZATION OF 1,6-ANHYDRO-2,3-DI *O*-BENZYL-4-*O*-(2,3,4,6-TETRA-*O*-BENZYL-α-D-GLUCOPYRANOSYL)-β-D-GLUCOPYRANOSE[a]

Catalyst (*mole-percent*)	*Cocatalyst* (*mole percent*)	*Solvent* (*ml*)	*Temp* (*degrees*)	*Time* (*hours*)	*Yield*[b] (%)	$[\alpha]_D^{25}$ (*degrees*)	$\overline{Mn}$	$[\alpha]_D^{25}$ (*degrees*) (*pet ether solubles*)
Catalyst PF_5								
5	—	1,CH_2Cl_2	−78	100	traces	46	—	+8.0
20	—	1,CH_2Cl_2	−78	100	15	69	—	+7.9
40	—	1,CH_2Cl_2	−78	100	7	76	—	+8.0
10[c]	—	1,CH_2Cl_2	+20	100	56	47	3030	+33
20	20,C_6H_5COF	1,$C_6H_5CH_3$	+20	100	55	55	3280	+17
20[d]	20,C_6H_5COF	1,$C_6H_5CH_3$	−78	100	2	30	—	+8.0
10	—	1,C_6H_{14}	+20	100	39	26	1970	+8.7
20	—	1,CH_3NO_2	0	100	65	46	2130	+29
20	10,C_6H_5COF	0.43,CH_2Cl_2	+20	100	71	50	3380	+31
20	10,C_6H_5COF	0.43 and 1.0, CH_2Cl_2	−78	100	~10	84	—	+8.1
20[e]	20,C_6H_5COF	0.43,CH_2ClCH_2Cl	−30	100	53	85	4340	—
20	—	0.43,$C_6H_5NO_2$	+1	100	68	48	2510	—
20	15,C_6H_5COF	0.43,CH_2Cl_2	−60	100	69	98	11100	—
20	20,C_6H_5COF	0.43,CH_2Cl_2	−60	200	77	96.5	13050	—
20	20,C_6H_5COF	0.43,CH_2Cl_2	−60	100	72	96	13400	—
Catalyst BF_3								
20[b]	20,C_6H_5COF	0.43,CH_2Cl_2	+20	100	74	57	3090	—
Catalyst BF_3 $O(C_2H_5)_2$								
20[f]	—	0.43,CH_2Cl_2	+20	100	11	36	—	+20
20[f]	20,C_6H_5COF	0.43,CH_2Cl_2	+20	100	78	61	3270	—
Catalyst SbF_5								
20[g]	—	0.43,CH_2Cl_2	−60	100	8	63	—	—
20	20,C_6H_5COF	0.43,CH_2Cl_2	−60	100	9	59	—	—
20[g]	20,C_6H_5COF	0.43,CH_2Cl_2	+20	100	71	56	—	—

[a]Table refers to polymerizations of 5×10^{-4} mole of monomer (0.43 g). Figs. 1–4 summarize additional experiments on same scale. [b]Yields refer to petroleum ether-insoluble product. [c]Similar results in toluene with and without 10 mole percent of water as cocatalyst. [d]Similar results with 1 mole-percent of water as cocatalyst. [e]Similar results without cocatalyst. [f]Only traces of product at −60 and −78° with and without cocatalyst. [g]No product at −78°.

RESULTS AND DISCUSSION

The monomer was prepared from maltose by a conventional sequence of reactions[7–10] and was subjected to an exploratory series of at least 20 polymerizations, some examples of which are found in Table I. These indicated that the polymerization of this compound was more sensitive to reaction conditions than those of similar monosaccharide derivatives[1 4 5 6]. The reaction temperature, type of catalyst, its concentration, and the kind of solvent all affected the conversion and optical rotation of the products significantly and termination processes were much more severe than had been observed with monosaccharide derivatives. The specific rotations and yields of products produced appeared higher when benzoyl fluoride was used as cocatalyst than when it was omitted or water used as cocatalyst No solvent was found that was superior to methylene chloride

A detailed study of the polymerization was next undertaken in order to establish optimum conditions for high yield, stereoregularity, and molecular weight. On the basis of previous experience and these exploratory runs, the system used consisted of a monomer with phosphorus pentafluoride and benzoyl fluoride as catalyst and cocatalyst, and methylene chloride as solvent The effect of temperature of polymerization on yield, specific rotation, and molecular weight was first studied (Fig 1) since

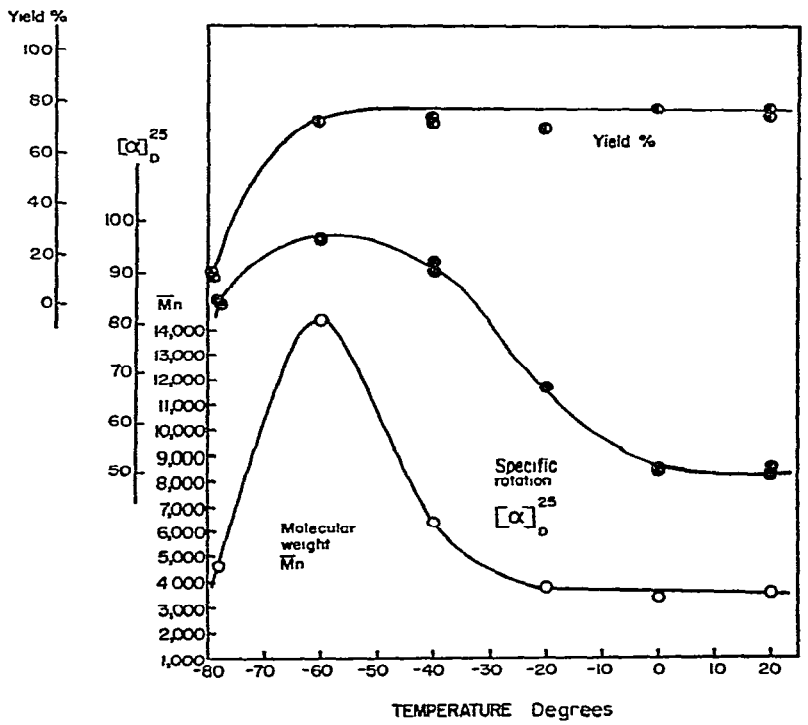


Fig 1 The effect of temperature on the polymerization of 1,6-anhydro-2,3-di-*O*-benzyl-4-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl)-β-D-glucopyranose For experimental details, see Results and Discussion

this variable was known to produce marked effects The proportions of reagents (monomer to catalyst to cocatalyst, molar ratio 1 0 2 0 2; and monomer to solvent, wt /vol ratio 1 1) and time (100 h) were chosen on the basis of the preliminary experiments Fig 1 demonstrates a marked and rather narrow optimum temperature range near −60° at which both the specific rotation and the molecular weight are at a maximum At higher temperatures, the propagating site presumably takes on a more carbonium-ion character, stereospecificity is lost, and termination processes become more important[5] At lower temperatures, the polymerization presumably is still relatively stereospecific but propagation is sluggish, and the yield of petroleum ether-insoluble polymer and its molecular weight are much lower The slightly lower optical rotation of the product produced at −78° probably reflects an end-group effect rather than decreased stereoregularity The yield of petroleum ether-insoluble product varies from about 70–80% over a temperature range from −60 to +20°, but this does not correspond to total conversion The petroleum ether-soluble material obtained from low-temperature runs had a specific rotation close to that of unchanged monomer, while the soluble material obtained from higher-temperature runs had higher specific rotations which indicated the presence of soluble reaction products Furthermore, by extending the reaction time at −60° somewhat higher yields of product can be obtained (Fig 2)

Using the optimum temperature of −60° and a 1 1 catalyst to cocatalyst molar ratio, a number of experiments were performed in which the molar ratio of catalyst

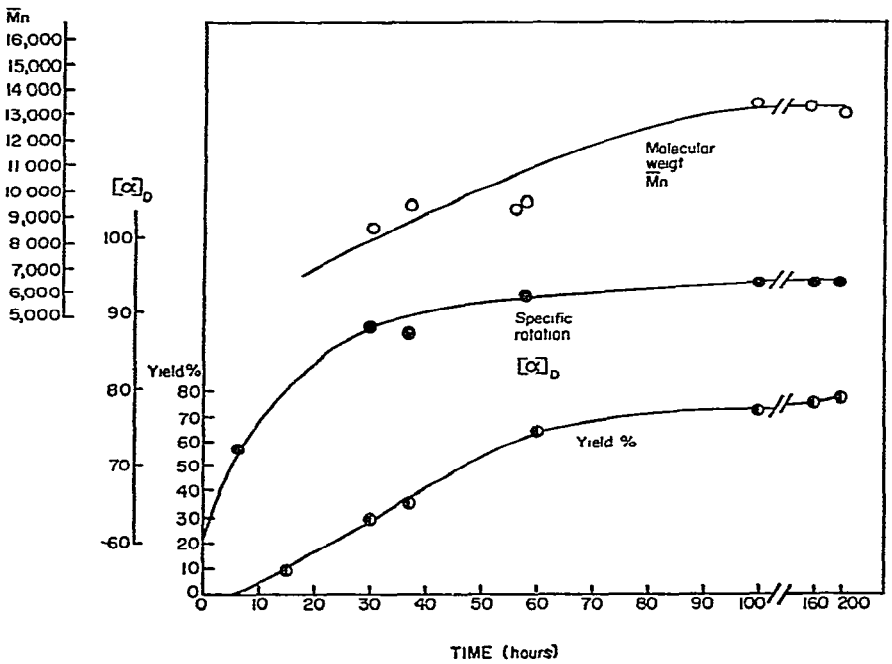


Fig 2 Rate of polymerization of 1,6-anhydro-2,3-di-*O*-benzyl-4-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl)-β-D-glucopyranose For experimental details, see Results and Discussion

to monomer was varied from 2 5% to 60% (Fig. 3) The yield of petroleum ether-insoluble polymer rose rapidly from low values at low catalyst to monomer ratios to a plateau of about 72–77% over the range of catalyst molar ratios of 20 to 60%

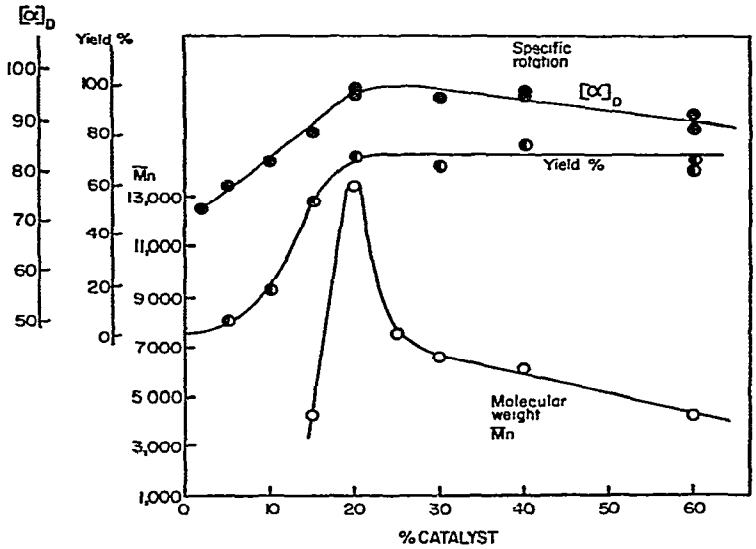


Fig 3 The effect of catalyst concentration on the polymerization of 1,6-anhydro-2,3-di-*O*-benzyl-4-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl)-β-D-glucopyranose For experimental details, see Results and Discussion

Optical rotations of petroleum ether-soluble material suggest that at the higher catalyst concentrations total conversions may be higher and some low-molecular-weight material is soluble This is also suggested by the molecular weights of the petroleum ether-insoluble material, which are at a maximum at a catalyst concentration of 20% and markedly decrease at higher concentrations. The specific rotation of the polymer and, therefore, the stereospecificity of the reaction is relatively insensitive to catalyst concentration, but also is at a maximum at 20% molar ratio At optimum conditions (temp −60°, monomer to solvent wt /vol ratio 1 1, catalyst to monomer molar ratio 20%), the amount of cocatalyst used was next varied (Fig 4) The best results were obtained at a cocatalyst to monomer molar ratio of 20% or at equivalent quantities of catalyst and cocatalyst.

The rate of this polymerization under such conditions is quite low (Fig 2) The number-average molecular weight, specific rotation, and yield of petroleum ether-insoluble material all increased significantly for the first 100 h After 100 h, the yield continued to increase at a very low rate for a second 100 h, with little change in specific rotation or molecular weight. Since the polymerization is irreversible, the increase in optical rotation must, of course, reflect a lower end-group content in the higher molecular-weight product, rather than any improvement in stereoregularity.

Three samples of polymer were prepared under the best conditions found and were debenzylated by means of sodium in liquid ammonia, dialyzed until ash free,

and recovered by freeze-drying The pulp-like products retained water tenaciously, and were readily dissolved in water The specific rotation of the samples was $[\alpha]_D^{25}$ +174° (*c* 2, water) When corrected to a theoretical carbon content, the specific rotation was 7% less than that of the synthetic (1→6)-α-D-glucopyranan of known 100% α-D configuration[11]. The stereospecificity of this polymerization is thus also very high, but the exact value must await structural investigations by Drs E. T. Reese and F W. Parrish

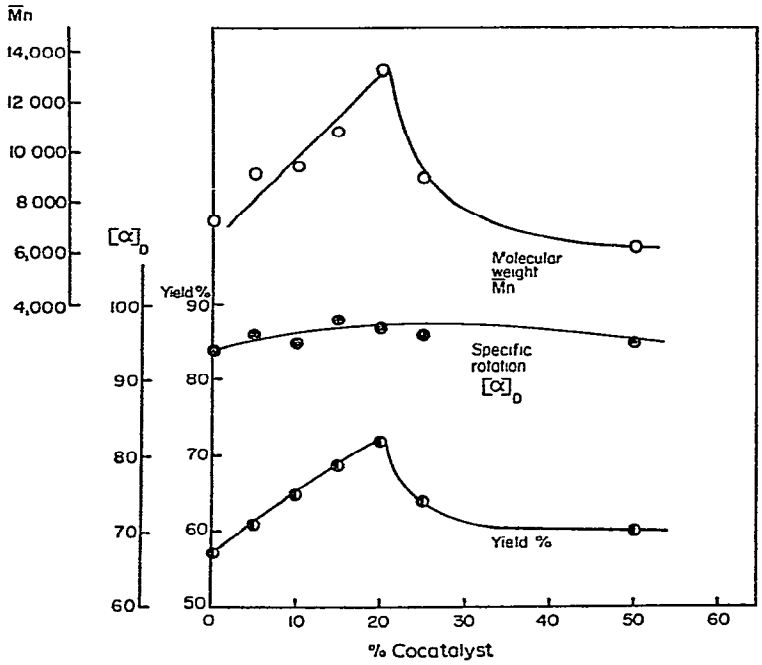


Fig 4 Effect of cocatalyst concentration on the polymerization of 1,6-anhydro-2,3-di-*O*-benzyl-4-*O*-(2 3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl)-β-D-glucopyranose For experimental details, see Results and Discussion.

It is obvious that the conditions under which the preparative runs were made were compromises It is obscure how the catalyst–cocatalyst combination operates and why such large quantities are necessary. The many oxygen functions on the monomer which can coordinate with catalyst and the bulkier size of the monomer probably decrease the rate of propagation and make side reactions or termination more probable Presumably benzoyl fluoride might act as a source of an initiating carbonium ion, but the large quantities needed and the slow initiation and propagation observed suggest that it is not very effective Furthermore, in limited tests Olah *et al* [12] found evidence of only weak interactions between these chemicals Clearly at large concentrations, the catalyst–cocatalyst combination can reduce molecular weight, presumably by termination or transfer processes It is possible that superior systems may be found in the future which will yield higher molecular-weight polymers

from this monomer or perhaps even somewhat higher stereoregularity, but exploratory runs with boron trifluoride and antimony pentafluoride gave results which were inferior to those with phosphorus pentafluoride (Table I)

EXPERIMENTAL

Synthesis of monomer. — The monomer, 1,6-anhydro-2,3-di-*O*-benzyl-4-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl)-β-D-glucopyranose, was prepared from maltose *via* acetylation[7], phenyl glycoside formation[8], cyclization[9], reacetylation, and careful crystallization[9], m p. 184–185°, $[\alpha]_D^{25}$ +48 4° (*c* 2, chloroform), and finally benzylation[10], 1,6-anhydromaltose hexaacetate (36 g) yielded 30 g of product, m p. 77–78°, $[\alpha]_D^{22}$ +7.66° (*c* 2, chloroform) Thin-layer chromatography and n m r. spectrum indicated no impurities.

Anal Calc for $C_{54}H_{56}O_{10}$ C, 74 97, H, 6 52 Found C, 74 86, H, 6 50

Reagents — Phosphorus pentafluoride was generated *in situ* from *p*-chlorobenzenediazonium hexafluorophosphate (Phosfluogene A[13], recrystallized from water at 0°) Boron trifluoride was prepared by heating calcium fluoroborate *in situ* at 350° Boron trifluoride etherate and antimony pentafluoride were distilled 3 times *in vacuo* on the vacuum line before using

Benzoyl fluoride (analytical grade, n_D^{20} 1 5048, stabilized with sodium fluoride) was used without purification Measured quantities of distilled water were used for cocatalysts as saturated solutions in spectral-analyzed toluene[14]. Methylene chloride, spectral-analyzed grade, was dried over calcium hydride and its purity was checked by n m r Ethylene dichloride, before using, was purified by washing with sulfonic acid, sodium hydrogen carbonate, and water, and then dried over calcium chloride After redistillation, ethylene dichloride was dried over calcium hydride Toluene, spectral-analyzed grade, was dried over calcium hydride Nitrobenzene was electronic grade Nitromethane was spectral grade and was dried over molecular sieves

Polymerizations — Polymerizations were carried out as described previously[1], usually on 0 43 g, 5×10^{-4} moles of monomer The polymerizations were stopped with methanol (2 ml), and the products were neutralized with sodium hydrogen carbonate solution and extracted into chloroform The dried and concentrated chloroform solutions were poured into 25 parts of petroleum ether The precipitated polymer was separated, dissolved, and freeze-dried in benzene

Physical constants — Specific rotations were measured in a 1-ml polarimeter cell in 2% chloroform (or water) solutions at room temperature (~25°) Molecular weights of polymers were determined in benzene with a Mechrolab Model M301-A vapor-pressure osmometer at concentrations of 15–60 g/l. The monomer was used as reference material

Debenzylation — Polymer 43 (Table I, 0 33 g) was dissolved in purified 1,2-dimethoxyethane (10 ml) and the solution was added to a solution of sodium (1 5 g) in liquid ammonia (100 ml) at −78° After 1 h, ammonium chloride was added until the color had completely disappeared The ammonia was evaporated under a slow

stream of nitrogen, and the white residue was slurried in dichloromethane, dissolved in water, and the solution dialyzed against running doubly-distilled water The polymer was freeze-dried, and a white, soft pulp-like material remained. Yield, 93 mg (82%), $[\alpha]_D^{25}$ +174° (*c* 2, water)

Anal. Calc for $C_{12}H_{20}O_{10}$ C, 44 43, H, 6 21 Found· C, 40 86; H, 6 53. Presumably the difference in values is due to retained water. Analogous polymerizations performed on 1 72 g of monomer and followed by debenzylation gave similar products of $[\alpha]_D^{25}$ +174 5 and 174.8°.

ACKNOWLEDGMENT

The present work has been supported by Grant GM-06168 from the National Institute of General Medical Sciences, National Institutes of Health, U. S. Public Health Service

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

CONFORMATIONS OF THE ALDITOLS

G A JEFFREY AND H S KIM
Department of Crystallography, University of Pittsburgh, Pittsburgh, Pa 15213 (U S A)
(Received January 13th, 1970)

ABSTRACT

The hypothesis that parallel C-*n*–O (or C-*n*–C) and C-(*n*+2)–O [or C-(*n*+2)–C] bonds in the alditols give rise to conformational instability accounts for the conformations observed in nine crystal-structure determinations It relates conformation to configuration in the solid state, and enables predictions to be made concerning the most stable rotamers of the isolated molecules

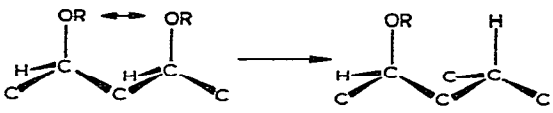
INTRODUCTION

It is usually assumed that rotation about the C–C bonds in the polyhydroxyalkyl chain of a sugar alcohol is sufficiently unrestricted that a mixture of rotamers exists in solution and in the molten state, and that this accounts for the low values for the molecular optical rotations of alditol solutions as compared with those of cyclic pentoses and hexoses[1]. These rotameric mixtures are presumed to consist mainly of those molecules in which the vicinal interactions are staggered, with an energy barrier (for passing through the eclipsed conformation) that is low enough to permit interconversion in solution at room temperature

Crystallization of alditols from solution and from the melt might, therefore, be expected to provide examples of conformational polymorphism, even though this is a comparatively rare phenomenon*. Actually, there is, as yet, no known example of such polymorphism for the alditols. Where polymorphism does occur and the structures have been studied, as for D-mannitol, the conformation of the molecules is the same[4] (three different crystal structures)

The work of Horton and co-workers[5] on the n m r. spectra of acyclic sugar derivatives in solution has provided data consistent with the hypothesis that the acyclic carbon chains have the extended, planar, zigzag arrangement, unless this would give rise to a steric interaction between substituents on alternate carbon atoms, as in I. If such an interaction is possible, the carbon chain adopts, by a 120° rotation about a C–C bond, a non-linear, or bent, chain conformation so that these interactions are avoided, as in II

*Two examples are the dimorphs of DL-methionine[2] and some unsaturated dicarboxylic acids[3]



This behavior implies a relationship between configuration and conformation that can be applied to the unsubstituted alditols in the crystalline state It will be shown that this relationship explains the conformations observed in the crystal structures that have been studied, and that it can be used to predict, with significant confidence, those which have not yet been observed. It also provides a means for predicting the most probable rotameric states existing in the liquid state.

RESULTS AND DISCUSSION

The carbon-chain conformations — Tables I and II list the results of nine crystal-structure determinations of alditols and one of an alditol complex

TABLE I

MEAN VALUES OF BOND LENGTHS AND VALENCE ANGLES IN SOME PENTITOLS AND HEXITOLS

Alditol	*C–C bond*[a] (Å)	*C–O bond* (Å)	*C–C–C angle* (*degrees*)	*C–C–O angle* (*degrees*)	$3\sigma^b$ (Å, *degrees*)	*References*
DL-Arabinitol	1 523(4)	1 434(21)	112 8(0 5)	109 7(3 7)	0 018, 1 5	7
Ribitol	1 524(14)	1 423(6)	113 3(2 8)	109 5(2 1)	0 009, 0 6	8
Xylitol	1 523(13)	1 433(9)	111 9(1 7)	109 9(3 0)	0 009, 0 6	9
D-Mannitol-A	1 52	1 43	113	110		4
D-Mannitol-B	1 516(21)	1 437(12)	112 7(1 1)	109 5(3 4)	0 030, 1 8	4
D-Mannitol-K	1 519(6)	1 426(11)	113 4(0 4)	110 2(2 0)	0 015 0 9	4
Galactitol	1 520(13)	1 432(11)	111 6(1 5)	110 0(2 3)	0 010, 0 6	10
D-Glucitol	1 520(18)	1 427(35)	113 5(4 2)	109 1(6 3)	0 015, 0 9	11
D-Glucitol–pyridine	1 521(28)	1 432(41)	113 2(1 3)	109 6(0 3)	0 050, 3 3	12
D-Iditol	1 522(31)	1 420(29)	112 2(1 8)	109 9(5 8)	0 020, 1 1	13

[a]The figures in parentheses give the difference between the smallest and greatest values in the group These variations are not significantly different from the mean values, when compared with the accuracy of their determination as indicated by the values of 3σ [b]σ is the estimated standard-deviation

From Table I, it may be seen that the molecular bond-lengths and bond-angles in these molecules form a consistent set of data There is no evidence of any stereospecific variations in bond lengths, such as have been observed for the pyranose sugars[6] (In detail, there is sometimes observed a trend toward shorter C–C or C–O distances at the ends of the molecules, but this is probably a technical aberration arising from inadequate treatment of thermal motion in the analysis of the X-ray diffraction data.)

TABLE II

CARBON-CHAIN CONFORMATIONS OF THE ALDITOLS IN THE CRYSTALLINE STATE

Alditol	*Configuration of carbon atoms*[a]	*Carbon-chain conformation*		*References*
DL-Arabinitol	LDD	extended	observed in DL racemate	7
Ribitol	DDD	bent	observed[b]	8
Xylitol	DLD	bent	observed	9
D-Mannitol	LLDD	extended	observed in three polymorphic forms	4
Galactitol	DLLD	extended	observed	10
D-Glucitol	DLDD	bent	observed in free sugar and in complex with pyridine	11 12
D-Iditol	LDLD	bent	observed	13
Allitol	DDDD	bent	predicted	15
D-Altritol	LLLD	bent	predicted	

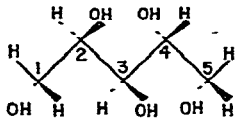
[a]In the sequence of the atomic numbering in Figs 1 and 2 [b]Also in the ribitol moiety[14] of vitamin B_2

Table II shows the conformations found in these crystal structures These results can be rationalized by the following rule: "The carbon chain adopts the extended, planar zigzag when the configurations at alternate carbon centers are different, and is bent and non-planar when they are the same".

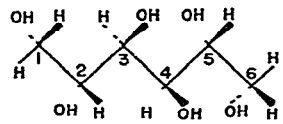
This rule permits prediction that the remaining hexitols, allitol, and D-altritol, the crystal structures of which have not yet been determined, will be found to have bent, non-planar carbon chains The rule can also be extended throughout the homologous series, to predict, for example, that D-*erythro*-L-*galacto*-octitol (LDDLDD), and D-*erythro*-L-*talo*-octitol (DDDLDD), will be found to have bent, non-planar, carbon-chain conformations, whereas, for *erythro-manno*-octitol (DDLLDD), and D-*threo*-L-*galacto*-octitol (LDDLLD), they will be extended

The stereochemical basis for this rule is clearly shown in Figs 1 and 2 For D-arabinitol, D-mannitol, and galactitol, the extended carbon-chain observed is illustrated in Fig 1 For ribitol, xylitol, D-glucitol, and D-iditol, the extended-chain conformation (not observed) is shown on the left, and the bent-chain conformation observed on the right, of Fig 2 All of these observations are consistent with the hypothesis that rotamers having parallel C-n–O and C-(n+2)–O bonds are high-energy conformational states having a small population in the solutions*, and a low probability of crystallizing The application of this hypothesis leads directly to the rule already given

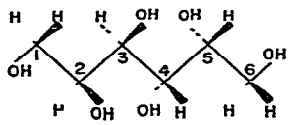
*The solutions were generally in water or alcohol–water, where solvent effects could override the energy difference between the rotamer molecules The only example where an extended zigzag chain is maintained despite a parallel C-n–O/C-(n+2)–O interaction in the crystalline state is potassium D-gluconate[16] This early structure-determination is not definitive by current standards, and we are re-investigating it



D-ARABINITOL



D-MANNITOL



GALACTITOL

Fig 1 Pentitol and hexitols having an extended, planar, carbon-chain conformation in the crystalline state

For *meso*-alditols, exemplified by galactitol, ribitol, and xylitol, three different situations occur on crystallization For galactitol (m p 188°), the extended-chain conformer has a center of symmetry* In the crystalline state, this symmetry is distorted by the crystal-field forces (*i e* , the intermolecular hydrogen bonds), so that the structure is a racemate of left- and right-handedly distorted, symmetrical conformers. These distortions, although significant, are small compared with the conformational differences between rotamers The conformation in the crystal is, therefore, a small perturbation of that of the symmetrical rotamer. Released from the constraint of the crystal field, the galactitol molecules in solution undergo thermal movements about a mean structure that is centrosymmetrical

For ribitol (m p 101°), the interaction between C-2–O and C-4–O in the extended-chain conformation can be relieved by a rotation of 120° about C-2–C-3 or about C-3–C-4, as shown in Fig 2 This affords two mirror-related, bent-chain conformers, both of which occur in the crystal structure and are related by a center of symmetry in space group $P2_1/c$

For xylitol (m p 94°), the crystal contains only bent-chain conformers having the same sense in the enantiomorphic space-group $P2_12_12_1$. Because the left- and the right-handed, bent-chain conformers must be equally probable in solution, the resolution of a racemic mixture of rotamers must occur on crystallization A crystalline form melting at 62° has been reported[17], and it is tempting to speculate that this

*Mirror symmetry, vertically through the mid-point of the middle C–C, or a center of symmetry in the Fischer projection formula, corresponds to a center of symmetry or a two-fold axis of symmetry, respectively, in the rotamer having the extended, planar, zigzag chain of carbon atoms and terminal hydroxyl groups

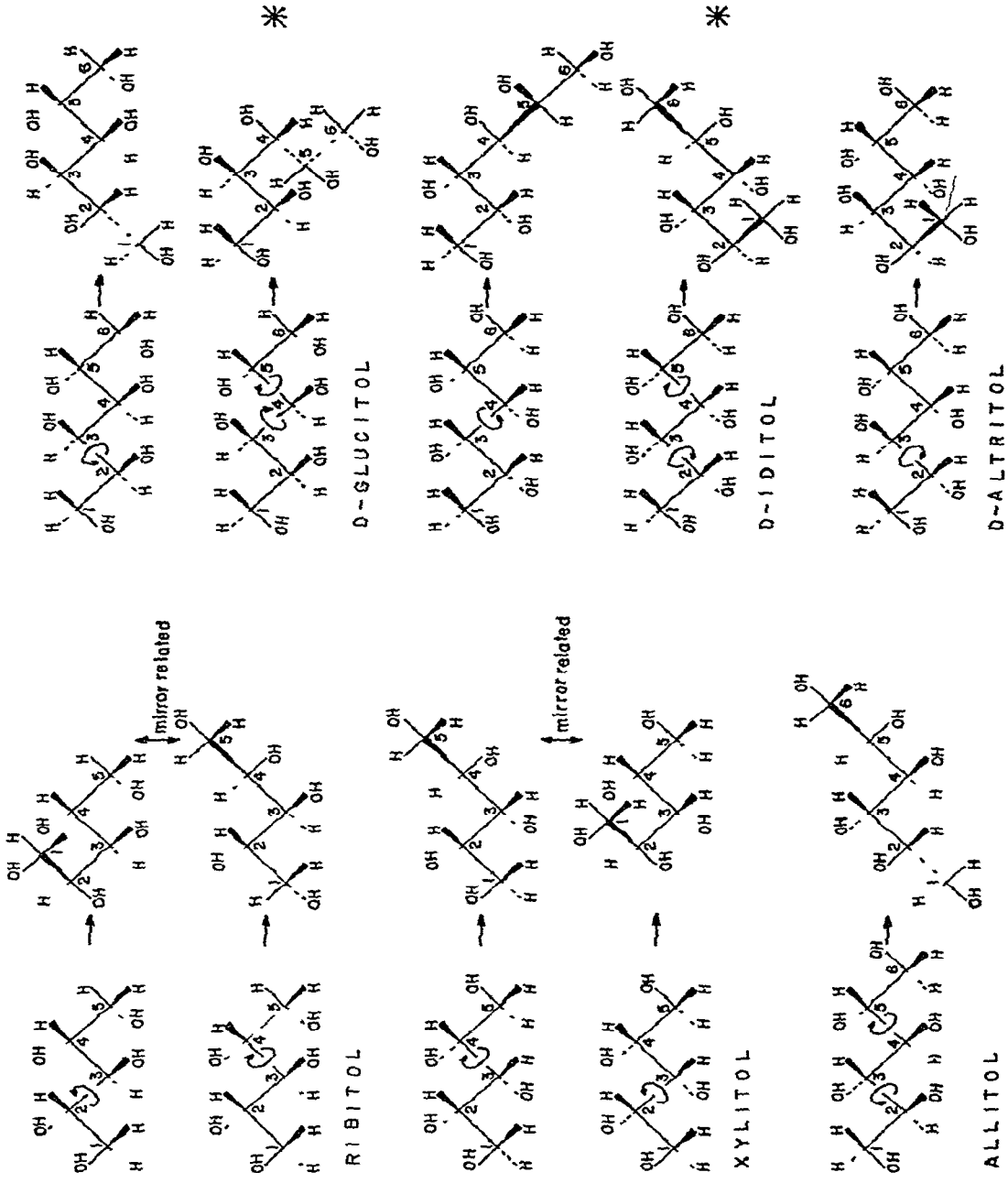


Fig. 2. Pentitols and hexitols having a bent, non-planar, carbon-chain conformation in the crystalline state. The bent-chain conformation is shown on the right-hand side, and the corresponding extended-chain on the left. Arrows indicate rotations about C–C bonds. The asterisks indicate alternative bent-chain conformations that *have not been observed*.

is the racemate (although racemates generally have a melting point higher than that of either constituent)

In a solution of ribitol or of xylitol, the low-energy rotamers will be those having the bent carbon-chains. The absence of optical activity in solution is, therefore, a consequence of an equal proportion of left- and right-handed, bent-chain species, and not of the symmetry of the extended carbon-chain species, as in galactitol

Allitol has the centrosymmetric space group $P2_1/c$, with two molecules in the unit cell[15]; hence it may be predicted that the molecules will be found to have the unique, centrosymmetrical, bent-chain conformation shown in Fig 2

DL-Arabinitol and D-mannitol have the extended carbon-chain conformation in the crystalline state, including the three polymorphic forms of D-mannitol All other rotamers invoke parallel C-n–O (or C-n–C) and C-(n+2)–O [or C-(n+2)–C] bonds It may be postulated, therefore, that the extended-chain rotamers are the low-energy states where they are observed in the crystals, and high-energy states where they are not observed This situation also holds for D-glucitol, which has a bent-chain conformation The parallel C-2–O to C-4–O interaction in the extended-chain rotamer, shown on the left in Fig 2, is avoided by a rotation of 120° about the C-2–C-3 or C-3–C-4 bond, this gives rise to the two bent-chain rotamers shown in Fig. 2, which are not symmetry-related That conformation twisted about C-2–C-3 is observed in the crystal structure of both D-glucitol and the complex of D-glucitol with pyridine It has C-2 and C-6 in a plane, with C-1 exoplanar The other bent-chain rotamer is structurally more complex The 120° rotation about C-3–C-4 brings C-5–O and C-2–C-3 into parallel alignment, requiring a further rotation of 120° about C-4–C-5 to produce the stable rotamer in which C-1 to C-4 are coplanar and C-4, C-5, and C-6 are coplanar in a different plane A structure containing this rotamer has not yet been observed

A similar discussion applies to D-iditol The parallel C-2–O/C-4–O and C-3–O/C-5–O interactions possible in D-iditol are relieved by one rotation of 120° about C-3–C-4 or by two rotations of 120° about C-2–C-3 and about C-4–C-5 The former gives a conformation in which C-1 to C-4 lie in one plane and C-3 to C-6 lie in a second, this conformation is observed in the crystal structure The latter two rotations would give a conformation in which C-2 to C-5 lie in a plane and the two terminal carbon atoms are exoplanar, this conformation has not yet been observed

In D-altritol, which has not yet been studied, it is only by rotation about C-2–C-3 that the parallel C-2–O/C-4–O interaction can be avoided The carbon-chain conformation shown at the right in Fig 2 is predicted for the crystal structure.

Orientation of the terminal groups — It is clear that, of the three vicinally staggered orientations of the terminal hydroxyl groups, only one will, in general, invoke a parallel C-1–O/C-3–O or C-(n-2)–O/C-n–O interaction Therefore, there will be four rotamers having similar energy that differ in the orientation of their terminal groups, except where the symmetry of the molecules, or a particular bent-chain conformation, lowers the number to three, two, or one (for examples, see Table III).

TABLE III

MOST-FAVORED ROTAMERIC STATES FOR THE ALDITOLS[a]

Alditol	*Carbon-chain conformations*	*Alternative orientation of terminal groups*
Pentitols	2→3→4	(1→2, 4→5)
DL-Arabinitol	A A	(A,A); (M,A), (A,P), (M,P)[b]
Ribitol	M A	(A,A), (A,P)[b] } mirror-related
	A P	(A,A), (M,A)[b] } mirror-related
Xylitol	M A	(A,A)[b], (A,M), (M,A), (M,M) } mirror-related
	A P	(A,A)[b], (P,A), (A,P), (P,P) } mirror-related
Hexitols	2→3→4→5	(1→2, 5→6)
D-Mannitol	A A A	(A,A), (A,P), (P,A), (P,P)[b]
Galactitol	A A A	(A,A)[b], (A,M), (P,M)
D-Glucitol	M A A	(A,A)[b], (A,P), (M,A), (M,P)[c]
	A P P	(A,A), (P,A)
D-Iditol	P A P	(A,A), (A,P), (P,A), (P,P)
	A M A	(A,A)[b], (A,M), (M,A), (M,M)
Allitol	M A P	(A,A)[d]
D-Altritol	P A A	(A,A), (A,M)

[a]See Fig 3 for key to nomenclature [b]Observed in the crystal structure [c]Observed in crystal structure of the complex [d]Unique and centrosymmetrical

In the crystal structures of DL-arabinitol and D-mannitol, the terminal hydroxyl groups are in the bent orientation In contrast, in galactitol, they are extended so that the oxygen atoms are in the same plane as the carbon atoms, as shown in Fig 1, this orientation is almost certainly determined by the crystal-field forces of the intermolecular hydrogen-bonding It would be expected that, on dissolution, equilibration would occur to give an approximately equal mixture of the four rotamers having extended carbon chains, and extended or bent orientations for the terminal hydroxyl groups For galactitol, only three such rotamers are possible, because of the center of symmetry.

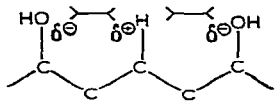
The conformation of the D-glucitol in its complex with pyridine differs from that adopted in the crystals of the parent alditol. The orientation of both of the terminal hydroxyl groups is bent in the former, and extended in the latter. Again, it is reasonable to assume that this difference is a result of the intermolecular forces in the solid state

The nature of the interaction — The O O separation corresponding to parallel C-n–O, C-(n+2)–O bonds is 2 50 Å, based on the mean distances and unstrained angles given in Table I This distance is the same as that between axially attached hydroxyl oxygen atoms on alternate carbon atoms in the cyclitols and cyclic sugars that have unstrained, six-membered rings It is commonly described[18] as a "1,3 interaction"* and is associated with an instability factor estimated[21] to be about 2 kcal/

*The term is, however, ambiguous as it is also used to describe the interaction of two atoms bonded to the same carbon atom[19] In that terminology, vicinal interactions are 1,4, and those between oxygen atoms on alternate carbon atoms in a chain are 1,5 A related interaction is the "pentane effect" in polymers, in which the repulsion between methyl groups on alternate carbon atoms lessens the conformational variety in polyisobutylenes[20]

mole for the cyclitols in aqueous solution at 25° For those ring systems for which a rotation of 120° about a C–C bond is not possible, it is presumed that a separation exceeding 2 50 Å is obtained at the expense of the angles between ring atoms, which depart from the ideal values for the unstrained ring In the crystal structure of 1,6-anhydro-β-D-glucopyranose[22], which has the *1C* (D) conformation for the pyranoid part, the O- O separation of the *exo*-axial hydroxyl groups on C-2 and C-4 is 3 30 Å However, this shape is stabilized by the anhydro ring, which distorts the pyranose ring in such a way as to favor this separation A more direct observation has been made on crystalline *epi*-inositol[23], in which the separation of the axially attached atoms O-2 and O-6 is 2 96 Å The resulting strain in the cyclohexane ring results in a flattening of C-1 towards the plane of C-2, C-4, and C-6 The conformational angles around the ring (starting with C-1–C-2) are 53, 57, 62, 60, 54, and 52°, values clearly consistent with a distortion arising from repulsion between O-2 and O-6

In addition to the repulsion interaction depicted in I, it is tempting to seek evidence that might indicate a tendency to adopt a conformation having a parallel alignment of C–O and C–H bonds, leading to an attraction at about 2 5 Å, as shown



III

in III Actually, crystals of DL-arabinitol, D-mannitol, ribitol, and D-glucitol in its complex with pyridine consist of conformations that have the maximum number of parallel C-*n*–O and C-(*n*+2)–H bonds However, crystals of galactitol, xylitol, D-glucitol, and D-iditol do not, because of the extended or *anti* orientation of the terminal hydroxyl groups We conclude, therefore, that this attraction (if it exists) is much less important than the repulsion between parallel C–O bonds and that, in the solid state, it is obscured by the intermolecular hydrogen-bonding forces

Rotameric stability in the liquid state — There is no reason why these intramolecular interactions controlling conformation in the solid state should not apply to the molecules in solution or in the molten state In fact, in the absence of the "permanent" intermolecular hydrogen-bonding of the crystalline state, they should be even more significant The replacement of the hydroxyl groups by groups that have greater mean Van der Waals radii should emphasize the energy differences between rotameric states, this conclusion is consistent with the work of Horton and co-workers[5,24] on acetylated sugars and their derivatives In Table III, the arguments just used in discussing the molecules in the crystalline state have been applied to predict the most stable rotameric states for the "free" molecules of the pentitols and hexitols It is not, however, possible to predict whether, at any given temperature, these favored rotamers would correspond to 80 percent or 99 9 percent of the molecular species present in a solution or a melt For making such quantitative predictions, there would be required, at least, a realistic estimate of the potential-energy function

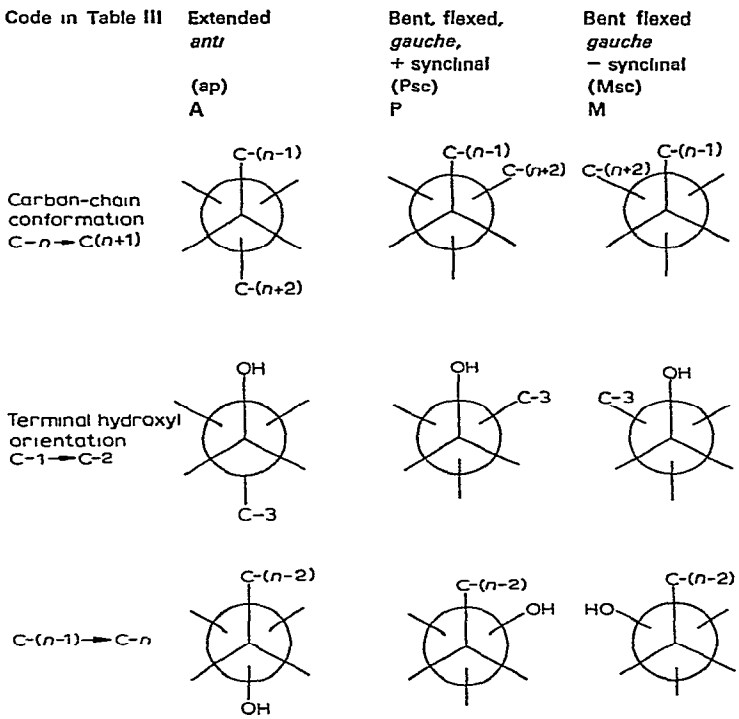


Fig 3 Boeseken projections about C–C bonds

for these non-bonding O O and O C interactions in the direction of the interaction, *i e*, at approximately 90° to the C–O or C–C bonds Although considerable progress has been made recently in deriving non-bonding, *intermolecular*, potential-energy functions for C C, C H, and H H pairs[25], an ability to derive similar information for C O and O O *intramolecular* interactions is still undeveloped In polar solvents, the added complication of solvation also operates, it could favor a particular rotamer through the mechanism of hydrogen-bonding to a functional group of the solvent However, in the absence of very strong complex-formation, it seems most probable that the rotameric population in solutions should be restricted to those shown in Table III

ACKNOWLEDGMENT

This research was supported by Grant No GM-11293 of the U S Public Health Service, National Institutes of Health

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Carbohydrate Research
Elsevier Publishing Company Amsterdam
Printed in Belgium

DERIVATIVES OF 5,6-DIDEOXY-5,6-EPIMINO-L-IDITOL

A D BARFORD* AND A C RICHARDSON
Department of Chemistry, Queen Elizabeth College (University of London), London W 8 (Great Britain)
(Received January 22nd, 1970)

ABSTRACT

The 5,6-ditosyl esters of 1,3 2,4-di-*O*-ethylidene- and 1,3 2,4-di-*O*-benzylidene-D-glucitol readily underwent selective displacement of the terminal ester groups by azide anions, and the resulting 6-azido-5-toluene-*p*-sulphonates were converted into the appropriate 5,6-epimines by the action of lithium aluminium hydride The ring-opening reactions of 1,3 2,4-di-*O*-benzylidene-5,6-dideoxy-5,6-epimino-L-iditol and some of its *N*-acyl derivatives have been studied, mainly with chloride and azide anions Predominant, if not exclusive, attack occurs at the terminal position of the epimine ring to give products having the L-*ido* configuration

INTRODUCTION

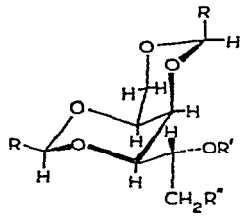
A variety of epimine derivatives of carbohydrates have been prepared in which the epimine ring is either fused to a pyranoside ring[1–11], a furanoside ring[12–14], or to an acyclic portion of the molecule[15 16] One method employed for the preparation of such epimines involves the treatment of *vic-trans*-benzamido-sulphonates with either lithium aluminium hydride or other basic reagents[3–6] However, a drawback of this method is the competing formation of oxazoline derivatives, particularly where free rotation of the benzoyl group about the carbon–nitrogen bond is possible[3]. A more-general method of epimine synthesis utilises *vic-trans*-azido-sulphonates as substrates, which, when reduced under basic conditions, afford the corresponding epimine in high yield The original reagent used for this transformation was hydrazine–Raney nickel[2], but this has the disadvantage that the first-formed epimine may undergo reductive rupture of the ring, and hence the success of this method depends upon the activity of the catalyst[13] This troublesome side reaction is avoided by the use of lithium aluminium hydride Good yields of epimines, which are not affected by the reagent[13,14 16], are usually obtained We have now utilised this latter method for the preparation of some epimino derivatives of alditols

RESULTS AND DISCUSSION

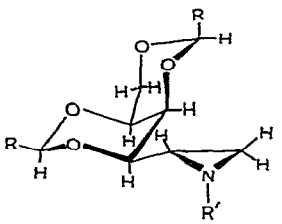
1,3 2,4-Diacetals of D-glucitol, in which the 5,6-hydroxyl groups are free, are prepared readily from the alditol and were used as starting materials for the prepara-

*Present address Chester Beatty Research Institute, Royal Cancer Hospital, Fulham Road, London S W 3, Great Britain

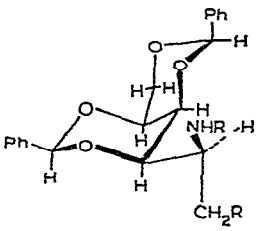
tion of the corresponding 5,6-epimines (**11** and **12**) Initially, we employed the 1,3 2,4 di-*O*-ethylidene derivative **1**, because it was readily transformed into the 5-*O*-acetyl 6-sulphonate **2** by selective monotosylation followed by acetylation[17]. Treatment o **2** with azide anions in *N*,*N*-dimethylformamide gave the 5-*O*-acetyl-6-azide **3**, whic was usually contaminated with small amounts of the 6-azido-5-sulphonate **7** Thi impurity obviously arose from small amounts of the 5,6-disulphonate **6** which con taminated **2** The structure of **7** was demonstrated by an unequivocal synthesis fron the 5-*O*-acetyl-6-azide **3** by *O*-deacetylation to give **4**, followed by tosylation. Th corresponding, syrupy 5-*O*-mesyl derivative **5** was also prepared from **4** and wa readily converted into 5,6-dideoxy-5,6-epimino-1,3 2,4-di-*O*-ethylidene-L-iditol (**11** by the action of lithium aluminium hydride



1 R=Me, R=H R''=OH
2 R=Me, R=Ac, R''=OTs
3 R=Me, R=Ac, R''=N_3
4 R=Me, R'=H, R''=N_3
5 R=Me R'=Ms, R''=N_3
6 R=Me, R'=Ts, R''=OTs
7 R=Me R'=Ts, R''=N_3
8 R=Ph, R'=Ts, R''=OTs
9 R=Ph, R'=Ts, R''=N_3
10 R=Ph, R'=Ts, R''=NHBz



11 R=Me, R=H
12 R=Ph, R=H
13 R=Ph, R=Ac
14 R=Ph, R=Bz
15 R=Ph, R'=Ms



16 R=Ac, R=Cl
17 R=Bz, R=Cl
18 R=H, R'=Cl
19 R=H, R'=N_3
20 R=Ac, R'=N_3
21 R=H, R'=NH_2
22 R=Bz, R'=NHBz
23 R=Ms, R=Cl
24 R=Ms, R=H
25 R=Ms, R'=OMe
26 R=Ms, R=OH
27 R=Bz, R=N_3

An alternative route for the preparation of precursor **4** involved the 5,6-epoxid **28** which was formed by the action of sodium methoxide on the 5-*O*-acetyl-6-sulphon ate[17] **2** The epoxide underwent terminal ring-opening when treated with a mixtur of sodium azide and ammonium chloride in 2-methoxyethanol to give the 6-azide (50% yield) which was identical with the product prepared *via* direct replacement

The synthesis of the epimine by either of these routes was, however, marre by contamination of the 5-*O*-acetyl-6-sulphonate **2** with the 5,6-disulphonate **6** an by the failure of the intermediary 6-azido-5-methanesulphonate **5** to crystallise. Th highly crystalline nature of the 6-azido-5-sulphonate **7**, originally isolated as a impurity in the 6-azido-5-acetate **3**, suggested that this might be a more-convenien precursor of the epimine **11** Accordingly, the 5,6-disulphonate **6** was prepared an readily underwent selective replacement of the primary sulphonate group by azid to give **7** in high yield Subsequent reduction of **7** with lithium aluminium hydrid afforded the 5,6-epimine (43%, *ca* 32% overall yield from **1**) The parallel sequenc of reactions (**8**→**9**→**12**) was also performed with 1,3 2,4-di-*O*-benzylidene-D-glucitol The disulphonate[18] **8** afforded the 6-azido-5-sulphonate **9** (95% yield) which gav the corresponding epimine **12** in 70% yield.

The n m r. spectra of the epimines **11** and **12** were amenable to first-order analysis, and they confirmed the terminal nature of the epimine ring (Table I) The two spectra were very similar, but that from the diethylidene derivative **11** was better resolved, and the decoupling experiments performed on this compound confirmed the assignments (Fig 1) The three single-proton resonances at highest field were assigned to the C-H protons attached to the epimine ring, since previous work[4] [19] has shown that these protons are usually at a higher field (usually above τ 7) than most other ring protons These three resonances consisted of a pair of doublets at τ 8 52 (J 3 2 Hz) and 8 14 (J 6 3 Hz) due to the C-6 protons, and a 1 1 2 2 1 1 sextet at 7 54 due to H-5 Only one limb of the doublet at τ 8 52 could be observed, since it was overlapped with the methyl resonances of the ethylidene groups, but the coupling constant (3.2 Hz) could be found from the H-5 sextet Irradiation of the H-5 sextet collapsed the H-6 doublets into singlets, and a 1 1 1 1 quartet at τ 6 89 (J 7 0 and 1 5 Hz) collapsed into a broad singlet, showing that this was the H-4 resonance Of the remaining resonances, a narrow 1 2 1 triplet at τ 6 41 and a narrow 1 3 3 1 quartet at 6 55 were assigned to H-2 and H-3, and the pair of quartets at 5 87 (J 12 7 and 1 3 Hz) and 6 16 (J 12 7 and 2 0 Hz) were obviously the AB portion of an ABX pattern and were assigned to the two protons at C-1 The H-3 resonance was shown to be that at τ 6 41 by irradia-

TABLE I

P M R DATA FIRST-ORDER CHEMICAL SHIFTS (τ VALUES) AND COUPLING CONSTANTS (Hz) AT 100 MHz

	9[a]	**10**[a]	**11**[a]	**12**[a,b]	**15**[a]	**17**[c]	**27**[d]
H-1*eq*	5 67q[f]	5 68q	5 87q	5 57q	5 64q	—	5 63q
H-1*ax*	5 86q	5 87q	6 16q	5 90q	5 95q	—	5 90q
H-2	6 06q	6 26q	6 55q	6 24q	6 29q	—	~6 1cm
H-3	6 25t	5 88t	6 41t	6 09t	6 15t	—	5 82t
H-4	4 89q	—	6 89q	6 51q	6 42q	5 69q	5 44q
H-5	4 93cm	4 86cm	7 54sex	7 44sex	6 88sex	5 50cm	5 08cm
H-6$^{e}_{trans}$	6 40cm	—	8 14d	8 20d	7 32d	—	6 10cm
H-6$^{e}_{cis}$		—	8 52d	8 45d	7 74d	—	
N-*H*	—	3 35t	9 05s	9 17s	—	1 72d	1 80d
Ar*CH*	4 41s	4 40s	—	4 34s	4 44s	4 39s	4 22s
	4 59s	4 58s	—	4 34s	4 46s	4 42s	4 32s
Ph-*Me*	7 76s	7 73s	—	—	—	—	—
$J_{1ax,1eq}$	12 5	12 5	12 7	12 5	12 5	—	12 5
$J_{1eq,2}$	1 5	1 5	1 3	~1 5	1 5	—	1 4
$J_{1ax\,2}$	1 6	1 8	2 0	~1 5	1 8	—	2 0
$J_{2,3}$	~1 5	~2 0	1 5	~1 5	~2 0	—	~2 0
$J_{3,4}$	1 8	~2 6	1 5	~1 5	2 0	1 8	1.7
$J_{4\,5}$	3 5	~5 0	7 0	~7 0	7 3	8 0	5 8
$J_{5,6trans}$	~3	~5 0	6 3	6 0	7 2	—	—
$J^{e}_{5,6cis}$	~8	~7 0	3 2	3 5	4 4	—	—
$J_{6_{trans}\ 6cis}$	—	—	0	0	0	—	—
$J_{NH\ CH}$	—	~7	—	—	—	~7	~8

[a]In chloroform-*d* [b]At 60 MHz [c]In methyl sulphoxide-d_6 at 75° [d]In pyridine-d_5 [e]*cis* and *trans* applies only to the epimines [f]s = singlet, d = doublet, t = triplet, q = quartet, sex = sextet, cm = complex multiplet

tion at the H-4 resonance, which collapsed this triplet into a narrow doublet, as well as collapsing the H-5 sextet into a quartet Hence, the quartet at τ 6 55 was assigned to H-2 The H-6 resonance, which was partly obscured in the spectrum of the ethylidene derivative, was clearly observed in the spectrum of the benzylidene compound as a doublet (J 3 5 Hz) The notable feature about these spectra is the lack of geminal coupling between the H-6 protons Such an observation is in line with other work[20], in which it has been shown that terminal epimine rings possess very small (0–2 1 Hz) geminal coupling constants, and Paulsen and Stoye[15] have reported a value of 1 Hz for a carbohydrate epimine

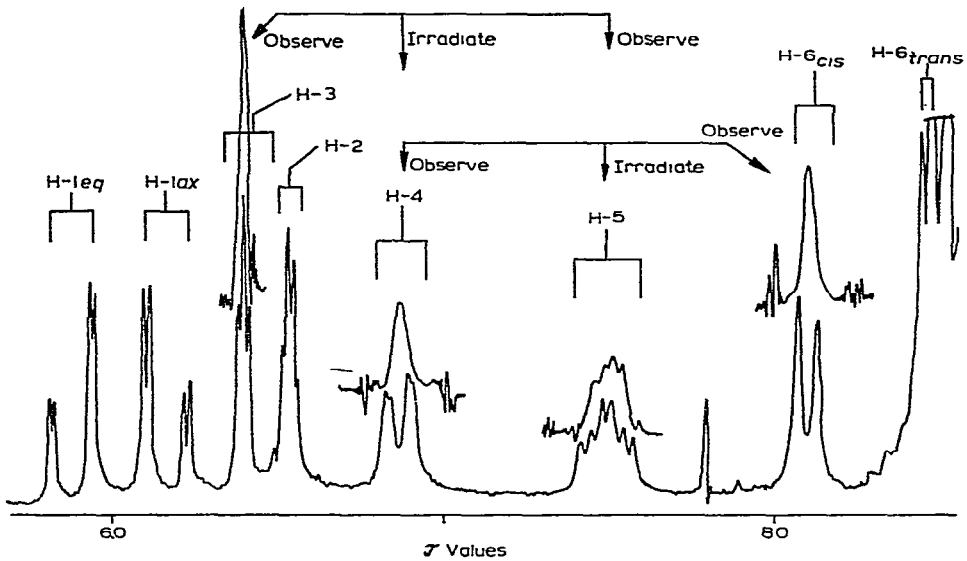


Fig 1. Section of 100 MHz n m r spectrum of 5 6-dideoxy-5 6-epimino-1,3 2,4-di-*O*-ethylidene-L-iditol (**11**) in chloroform-*d* showing the results of decoupling experiments

The assignment of the individual C-1 and C-6 protons was made as follows In a study of various 2-phenylaziridines, Brois and his co-workers[20] have found that $J_{cis} > J_{trans}$ for vicinal coupling across the aziridine ring, so that the doublet at highest field (τ 8 52) is assigned to H-6_{trans}, since it has the smallest splitting, and that at τ 8 14 must be due to H-6_{cis} For the C-1 protons, the equatorial proton H-1*eq* would be deshielded to a greater extent than the axial proton H-1*ax* by the adjacent O-2 which is *cis*-axial to H-1*eq* Hence, the lower-field resonance at τ 5 87 is assigned to H-1*eq* and that at 6 16 to H-1*ax* In agreement with this, one would expect the coupling constant between H-1*eq* and H-2 (*eq–eq*) to be smaller than that between H-1*ax* and H-2 (*ax–eq*), this is, in fact, observed, although the difference between the two coupling constants is small (0 7 Hz)

In the subsequent study of the reactions of epimines of this type, the dibenzylidene derivative **12** was used exclusively It was found that *N*-acylation could be achieved only if acidic conditions or the presence of a nucleophilic anion were avoided

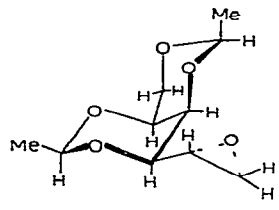
Thus, the epimine **12** was converted into the *N*-acetyl (**13**) and *N*-benzoyl derivatives (**14**) by treatment with the appropriate acid anhydride in ethanol or pyridine However, the use of acid chlorides in pyridine resulted in *N*-acylation and subsequent rupture of the epimine ring by chloride ion to give 5-acylamido-6-chloro derivatives Treatment of **12** with benzoyl chloride or acetyl chloride in pyridine at ~0° or ambient temperatures afforded high yields of the 5-benzamido-6-chloro- and 5-acetamido-6-chloro derivatives, **17** and **16**, respectively, assuming nucleophilic attack by chloride had occurred at the terminal position (see below). The lability of these epimines to ring opening in the presence of pyridinium chloride was shown by the fact that the free epimine **12** underwent ring opening in the presence of pyridinium chloride at room temperature to give the 5-amino-6-chloro derivative **18** in high yield, which was related to the products of acylation with acid chlorides by *N*-benzoylation and *N*-acetylation to give **17** and **16**, respectively. The amino-chloro derivative **18** was readily converted back into the epimine by the action of either sodium methoxide or lithium aluminium hydride It is of note that this method of epimine formation is similar to the original method used by Gabriel[21] for the preparation of "ethylenimines"

The structure of the 5-benzamido-6-chloro derivative **17**, and hence the structures of the amino and acetamido derivatives **18** and **16**, was proved in the following way The compound was converted into an oxazoline derivative (m p 181–183°, $[\alpha]_D$ −112°) by treatment with sodium acetate in boiling 2-methoxyethanol containing 5% of water If the benzamido-chloro derivative had been formed by attack at the secondary carbon atom, the oxazoline formed from this 5-chloro derivative would have the structure **30**, whereas the more plausible 6-chloro derivative would give a different oxazoline **29** An unequivocal synthesis of oxazoline **30** (m p 234–236°, $[\alpha]_D$ +75°) showed that it was not identical with the oxazoline obtained from the benzamido-chloro derivative, which must therefore, by implication, be the 6-chloro derivative The oxazoline **30** was synthesised from the 6-azido-5-sulphonate **9**, the immediate precursor of the epimine **12** The azide was hydrogenated and the product treated with benzoic anhydride to give the 6-benzamido-5-sulphonate **10**, which was then converted into the oxazoline **30** by the action of sodium methoxide in 95% 2-methoxyethanol The non-terminal nature of the benzamido group was also convincingly demonstrated by the p m r spectrum of **17**, in which the resonance due to the amide proton was observed as a doublet at τ 1 72 (methyl sulphoxide-d_6 at 75°) which collapsed to a singlet on irradiation of the complex multiplet due to H-5 at τ 5 50 If the amide group had been at a terminal position, it would have been coupled to two protons and would have appeared as a triplet For example, the n m r spectrum of the 6-benzamido-5-sulphonate **10** showed the amide proton as a triplet at τ 3 35 (chloroform-*d*)

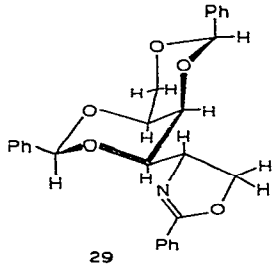
The terminal ring opening of the epimine **12** by chloride ion accords well with the observations that terminal epoxides undergo predominant attack by nucleophilic reagents at the terminal carbon atom[22]

Nucleophilic ring-opening of the epimine **12** and its *N*-acetyl derivative **13** with azide also afforded the terminal azido derivatives **19** and **20**, respectively, the

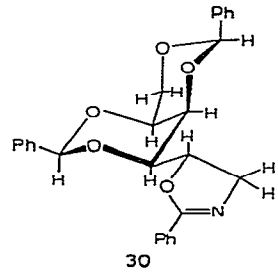
reagent in this case was a mixture of sodium azide and ammonium chloride in 2-methoxyethanol[2] The two products were inter-related by *N*-acetylation of the azido-amine **19** which gave the acetamido-azide **20** in high yield These products were also inter-related with the chloro-amines described above by the displacement of the terminal chloro substituent with azide Thus, the amino-azide **19** was formed in high yield when the chloro-amine **18** was treated with sodium azide in 2-methoxyethanol However, this latter reaction could not be relied upon as proof of the terminal nature of the azido group, since initial ring closure of the chloro-amine **18** to the epimine **12** could have occurred, followed by ring opening with azide When the 5-acetamido-6-chloro-derivative **16** was treated with sodium azide in the same way, an azide was obtained which was identical with that prepared by ring opening of the *N*-acetyl-epimine **13** with azide It is considered unlikely that initial ring closure to the *N*-acetyl-epimine could have occurred in this case, because we have shown above that the related benzamido-chloro derivative is ring closed to the oxazoline derivative rather than the epimine, and these oxazolines are resistent to nucleophilic ring opening under basic, or slightly basic conditions[2,3] The azido amine **19** was reduced to the diamine **21** which was characterised as the di-*N*-benzoyl derivative **22**



28



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Similarly, the epimine **12** underwent both mesylation and ring-opening by chloride ion when treated with mesyl chloride in pyridine, to give the 6-chloro-5-methanesulphonamido derivative **23**, the structure of which was proved by its formation from the 5-amino-6-chloro derivative **18** by mesylation The *N*-mesylepimine **15** was formed with particular ease when **23** was subjected to mildly basic conditions, but it was very sensitive to ring-opening by nucleophilic reagents This greater reactivity of *N*-mesylepimines has been noted before[8], and is undoubtedly due to the greater electron-withdrawing effect of the sulphonate group and the fact that, unlike *N*-acyl derivatives which are readily hydrolysed under mildly basic conditions[3], the *N*-sulphonyl group is resistant to removal and remains on the nitrogen to assist, by its inductive effect, the cleavage of the C–N bond The n m r spectrum of **15** was very similar to that of **12** (Table I)

The 6-chloro-5-methanesulphonamido derivative **23**, which is the precursor of the *N*-mesylepimine, underwent several replacement reactions under basic conditions which obviously proceeded by way of the *N*-mesylepimine **15** Thus, when the chloro derivative was treated with lithium aluminium hydride, the 6-deoxy-5-methanesulphon-

amido derivative **24** was obtained, the structure of which was shown by the presence, in its n m r spectrum, of a three-proton doublet at τ 8 67 due to the terminal methyl group Furthermore, when the chloro derivative was heated with sodium methoxide, a methoxy derivative was formed, almost certainly the 6-methoxy derivative **25** The action of sodium acetate in aqueous 2-methoxyethanol afforded the 6-hydroxy analogue **26** in 94% yield

EXPERIMENTAL

Concentrations were carried out under diminished pressure Melting points were determined on a Kofler microscope hot-stage and are uncorrected Optical rotations were measured to an accuracy of at least $\pm 0\,002°$ with a Bendix-NPL Automatic Polarimeter, using a 0 2-dm tube at $\sim 21°$, and unless otherwise stated, chloroform solutions were used Thin-layer chromatography (t l c) was performed on microscope slides coated with silica gel (Whatman Chromedia SG41) and detection was with ethanolic sulphuric acid Column chromatography was performed on Silica Gel MFC (Hopkin and Williams), 10-ml fractions being collected for examination by t l c. Light petroleum was the fraction of b p 40–60°, and pyridine was dried over sodium hydroxide pellets Infrared spectra were determined on a Unicam SP200 spectrometer, by using Nujol mulls for solids, and were in agreement with the structures proposed

5-O-Acetyl-6-azido-6-deoxy-1,3 2,4-di-O-ethylidene-D-glucitol (**3**) — A mixture of 5-*O*-acetyl-1,3 2,4-di-*O*-ethylidene-6-*O*-tosyl-D-glucitol[17] **2** (40 g) and sodium azide (15 g) was added to boiling *N,N*-dimethylformamide (80 ml), and the mixture was heated under reflux for 10 min, cooled, and treated with ether. After the removal of the precipitated inorganic material, the solution was evaporated to a yellow syrup, which was dissolved in a little ether and purified by chromatography on silica gel with 9 1 ether–light petroleum After the evaporation of the appropriate fractions, the crystalline residue was recrystallised from ether–light petroleum to give **3** (14 5 g, 52%) After a further recrystallisation from the same solvent mixture, the product had m.p. 83–84°, $[\alpha]_D$ −28° (*c* 0 6, water) (Found C, 47 65, H, 6 55, N, 13 85 $C_{12}H_{19}N_3O_6$ calc C, 47 85, H, 6 3, N, 13 9%)

6-Azido-6-deoxy-1,3 2,4-di-O-ethylidene-D-glucitol (**4**) — (*a*) Slightly impure **3** (14 g) was dissolved in methanol (100 ml) and treated with 3M methanolic sodium methoxide (17 ml) After 30 min, a small amount of crystalline product {m p 126–127°, $[\alpha]_D$ −34° (*c* 1 7)} was removed which was shown to be 6-azido-6-deoxy-1,3 2,4-di-*O*-ethylidene-5-*O*-tosyl-D-glucitol (**7**) by comparison with an authentic sample (see below) The filtrate was neutralised with Amberlite IR-120(H^+) resin and concentrated to a syrup which was extracted with ether (150 ml) Concentration of the extract afforded a crystalline solid which was recrystallised from ether–light petroleum to give the 6-azide **4** (8 25 g, 68%), m p 120–122°, $[\alpha]_D$ −9° (*c* 0 9, water) (Found C, 46 5, H, 6 7, N, 16 1 $C_{10}H_{17}N_3O_5$ calc C, 46 35, H, 6 55, N, 16 2%)

A further crop (1 5 g, 13%) was obtained by evaporation of the mother liquors, followed by recrystallisation

(*b*) 5,6-Anhydro-1,3 2,4-di-*O*-ethylidene-D-glucitol[17] **28** (0 3 g) was heated under reflux with sodium azide (0 2 g) and ammonium chloride (0 15 g) in 95% aqueous 2-methoxyethanol (2 ml) for 30 min The mixture was then diluted with water and extracted three times with methylene chloride, and the dried ($MgSO_4$) extracts were concentrated Two recrystallisations of the residue from ether–light petroleum gave the 6-azide **4** (0 18 g, 50%), m p 118–119°, $[\alpha]_D$ −8 1° (*c* 1, water), which was identical (i r) with the product from (*a*)

*6-Azido-6-deoxy-1,3 2,4-di-*O*-ethylidene-5-*O*-tosyl-*D*-glucitol* (**7**) — (*a*) The 5,6-ditoluene-*p*-sulphonate[17] **6** (30 g) was added to a mixture of boiling *N*,*N*-dimethylformamide (100 ml) and sodium azide (15 g) After being heated under reflux for 5 min, the mixture was cooled, and the addition of water afforded the crystalline azide **7** (19 4 g, 85%) which was washed well with water and methanol and dried, m p 126–127 5°, $[\alpha]_D$ −35° (*c* 2 4) (Found C, 49 5; H, 5 9, N, 10 3, S, 7 65 $C_{17}H_{23}N_3O_7S$ calc C, 49 4; H, 5 55; N, 10 15, S, 7 75%)

(*b*) Tosyl chloride (3 g) was added to a solution of the 6-azido-5-hydroxy derivative **4** (0 5 g) in pyridine (10 ml) and the resulting solution was kept overnight at room temperature The reaction mixture was then poured into water, and the oil which was first precipitated crystallised upon seeding and scratching. Recrystallisation from ethanol gave the azido-sulphonate 7 (0 74 g, 93%), m p 127 5–128°, $[\alpha]_D$ −32° (*c* 2), which was identical (i r) with the product from (*a*)

*5,6-Dideoxy-5,6-epimino-1,3 2,4-di-*O*-ethylidene-L-iditol* (**11**) — (*a*) Mesyl chloride (0 5 ml) was added to a solution of the 6-azido-5-hydroxy derivative **4** (0 33 g) in dry pyridine (1 ml), and the solution was kept for 3 h at room temperature and then decomposed by the addition of water The mixture was processed *via* chloroform extraction in the usual way to give the syrupy sulphonate **5**

A solution of this syrup in dry ether (25 ml) was heated under reflux with lithium aluminium hydride (0 4 g) for 20 min The excess of hydride was then decomposed by the addition of a saturated solution of Rochelle salt, the mixture was diluted with chloroform and filtered, and the residue was extracted three times with boiling chloroform The combined extracts and filtrate were concentrated to a crystalline solid, recrystallisation of which from acetone–ether–light petroleum afforded the epimine **11** (0 117 g, 43%), m p. 150–180°, $[\alpha]_D$ −25° (*c* 0 5, water) Further recrystallisation of the epimine failed to improve the m p

(*b*) A solution of the 6-azido-5-toluene-*p*-sulphonate 7 (8 g) in dry ether (200 ml) was heated under reflux with lithium aluminium hydride (4 g) for 15 min The reaction mixture was processed as in (*a*), and the resulting white solid was recrystallised from chloroform–ether–light petroleum to give the product **11** (1 78 g, 43%), m p 150–171°, $[\alpha]_D$ −25° (*c* 2, water), which was identical (i r) with the product from (*a*) (Found C, 55.75, H, 8 2, N, 6 7 $C_{10}H_{17}NO_4$ calc C, 55 8, H, 7 9, N, 6 5%). The wide range in m p could not be improved for this compound and appears to be a characteristic property

*6-Azido-1,3 2,4-di-*O*-benzylidene-6-deoxy-5-*O*-tosyl-*D*-glucitol* (**9**) — The 5,6-

ditoluene-*p*-sulphonate[18] **8** (5 g) was heated under reflux with sodium azide (3 g) in 2-methoxyethanol (23 ml) and water (2 ml) for 1 h The azide **9** (3 84 g, 95%) was precipitated by the addition of water and recrystallised from acetone–ether, m p 142–145°, $[\alpha]_D$ −2 9° (*c* 1 3) (Found C, 60 4, H, 5 25, N, 7 75, S, 5 85 $C_{27}H_{27}N_3O_7S$ calc · C, 60 35, H, 5 05, N, 7 8, S, 5 95%)

1,3.2,4-Di-O-benzylidene-5,6-dideoxy-5,6-epimino-L-iditol (**12**) — (*a*) The azidosulphonate **9** (10 g) was suspended in dry ether (250 ml) and heated under reflux with lithium aluminium hydride (5 g) for 20 min The reaction mixture was then processed in the usual way, and the product was recrystallised from chloroform–light petroleum to give the epimine **12** (1 93 g, 31%), m p 185–195° After two further recrystallisations, it had m p 204–207° and $[\alpha]_D$ +23° (*c* 1 7) (Found C, 70 75, H, 6 25, N, 4 25 $C_{20}H_{21}NO_4$ calc C, 70 8, H, 6 2, N, 4 1%)

A further crop (2 49 g, 39%) was obtained by evaporation of the mother liquors to dryness and recrystallisation of the residue

(*b*) A suspension of the 5-amino-6-chloro derivative **18** (0 1 g) in methanol (5 ml) was heated under reflux with sodium methoxide (0 1 g) for 1 h The addition of water to the mixture afforded the epimine (0 08 g, 89%) which was recrystallised from chloroform–ether, m p 190–195°, $[\alpha]_D$ +21° (*c* 1) After two more recrystallisations, it had m p 197–200° and was identical (i r) with the product from (*a*)

(*c*) The 5-amino-6-chloro derivative **18** (0 05 g) was suspended in dry ether (3 ml) and heated under reflux with lithium aluminium hydride (0 05 g) for 45 min The mixture was processed in the usual way to give the epimine **12** (0 02 g, 44%), m p 180–185° (from chloroform–light petroleum), $[\alpha]_D$ +21° (*c* 1) There was insufficient material to carry out extensive recrystallisations but, despite the poor m p , its i r spectrum was identical with the previous two samples

N-Acyl derivatives were prepared in the usual way by treatment with the appropriate acid anhydride in pyridine, followed by the addition of water The *N*-acetyl derivative **13** had m p 212–215° (from chloroform–ether) and $[\alpha]_D$ −8 6° (*c* 1) (Found C, 69 4, H, 6 35, N, 3 55 $C_{22}H_{23}NO_5$ calc C, 69 3, H, 6 05, N, 3 65%) The *N*-benzoyl derivative **14** had m p 219–222° (from chloroform–ether), $[\alpha]_D$ +36° (*c* 1) (Found C, 72 8, H, 5 55, N, 3 05 $C_{27}H_{25}NO_5$ calc C, 73 15; H, 5 65, N, 3 15%)

5-Amino-1,3 2,4-di-O-benzylidene-6-chloro-5,6-dideoxy-L-iditol (**18**) — The dibenzylidene-epimine **12** (1 g) was suspended in pyridine (5 ml) and treated with pyridinium chloride (1 g) for 3 h at room temperature and then treated with ice–water The precipitated chloro derivative was collected to give **18** (0 77 g, 69%), m p 196–197° (decomp), $[\alpha]_D$ +28° (*c* 1) (Found C, 63 85, H, 5 95, N, 3 7 $C_{20}H_{22}ClNO_4$ calc C, 63 9; H, 5 85, N, 3 75%) Recrystallisation (chloroform–ether) lowered the m p [191–193° (decomp)], and it is inadvisable to subject this compound to prolonged heating in solution because of its ease of ring closure to the epimine, with the consequent liberation of hydrogen chloride

5-Acetamido-1,3 2,4-di-O-benzylidene-6-chloro-5,6-dideoxy-L-iditol (**16**) — (*a*) To a stirred suspension of the epimine **12** (1 g) in pyridine (10 ml) was added acetyl chloride (0 26 ml) in a dropwise fashion The flask was cooled initially, and, after

1 h, the addition of water precipitated the *N*-acetyl derivative (1 06 g, 86%), m p 218–220° It was recrystallised from chloroform–light petroleum to give the analytical sample, m.p 220–221 5°, $[\alpha]_D$ +31° (*c* 0 7, *N,N*-dimethylformamide) (Found C, 62 9, H, 5 9; N, 3 25 $C_{22}H_{24}ClNO_5$ calc C, 63 3; H, 5 5, N, 3 35%)

(*b*) The same compound, prepared by acetylation, in the usual way, of the amine **18** had m p 223–224° (from *N,N*-dimethylformamide–ethanol), $[\alpha]_D$ +30° (*c* 0 7, *N,N*-dimethylformamide) and was identical (i r) with the product from (*a*)

*5-Benzamido-1,3 2,4-di-*O*-benzylidene-6-chloro-5,6-dideoxy-*L*-iditol* (**17**) — (*a*) The epimine **12** (0 5 g) was suspended in pyridine (10 ml) and treated with benzoyl chloride (0 35 ml) The solution was kept for 16 h at below −5° and then treated with water to precipitate the *N*-benzoyl derivative **17** (0 63 g, 89%) After recrystallisation from *N,N*-dimethylformamide–ethanol, it had m p 221–226°, $[\alpha]_D$ +73° (*c* 0 5, *N,N*-dimethylformamide) (Found C, 67 25, H, 5 6; N, 3 15 $C_{27}H_{26}ClNO_5$ calc C, 67 55, H, 5 4, N, 2 9%)

(*b*) The *N*-benzoyl derivative, prepared in the usual way from the amine **18**, had m p 215–220°, $[\alpha]_D$ +63° (*c* 0 3, *N,N*-dimethylformamide), and was identical (i r) with the product from (*a*)

*6-Benzamido-1,3 2,4-di-*O*-benzylidene-6-deoxy-5-*O*-tosyl-*D*-glucitol* (**10**) — The 6-azido-5-toluene-*p*-sulphonate **9** (5 g) was dissolved in a mixture of ethanol (100 ml) and acetone (100 ml) and hydrogenated over Raney nickel T4 catalyst[24] (*ca* 6 g) at *ca* 4 atmos After 4 h, the catalyst was filtered off, and the solution was treated with benzoic anhydride (2 5 g) for 2 5 h at room temperature before evaporation to dryness The crystalline mass was then recrystallised from ethanol–ether–light petroleum to give the *N*-benzoyl derivative **10** (4 3 g, 75%), m p 138–142°, $[\alpha]_D$ −2 1° (*c* 1 3) (Found C, 66 75, H, 5 4, N, 2 4 $C_{34}H_{33}NO_8S$ calc C, 66 35, H, 5 35, N, 2 3%)

Conversion of **10** *into 5-*R*-(2,4 3,5-di-*O*-benzylidene-*L*-xylo-tetrahydroxybutyl)-2-phenyloxazoline* (**30**) — The benzamido-sulphonate **10** (0 1 g) was heated under reflux with sodium methoxide (0 05 g) in 95% 2-methoxyethanol (4 ml) for 5 h. The addition of water gave a solid product which was recrystallised from ethanol to give the oxazoline (0 004 g, 6%), m p 216–219°, $[\alpha]_D$ +75° (*c* 1 6) A further recrystallisation from ethanol gave the analytical sample having m p. 234–236° (Found C, 73 7, H, 5 85, N, 3 2 $C_{27}H_{25}NO_5$ calc C, 73 15, H, 5 65; N, 3 15%).

Conversion of the 5-benzamido-6-chloro derivative **17** *into 4-*R*-(2,4 3,5-di-*O*-benzylidene-*L*-xylo-tetrahydroxybutyl)-2-phenyloxazoline* (**29**) — Compound **17** (0 1 g) was heated under reflux with sodium acetate (0 1 g) in 95% 2-methoxyethanol (6 ml) for 4 h, when t l c indicated the presence of two components The addition of water to the reaction mixture gave a solid which was purified on a column of silica gel by using 1.1 ether–light petroleum as solvent The faster-moving component was recrystallised from ethanol–ether to give a product (0 015 g), m p. 160–163°, which was not further characterised The slower-moving material was recrystallised from ether to give the oxazoline **29** (0 01 g, 11%), m p 177–178°, $[\alpha]_D$ −112° (*c* 0 5) A further recrystallisation from ethyl acetate–ether afforded the analytical sample, m p

182–185° (Found C, 72 95, H, 6 16, N, 2 9 $C_{27}H_{25}NO_5$ calc C, 73 15; H, 5 65, N, 3 15%)

5-Amino-6-azido-1,3 2,4-di-O-benzylidene-5,6-dideoxy-L-iditol (**19**) — (*a*) The epimine **12** (1 g) was heated for 5 min under reflux with sodium azide (1 g) and ammonium chloride (0 2 g) in 95% 2-methoxyethanol (10 ml) The addition of water to the reaction mixture was accompanied by the separation of a solid which was recrystallised from ethanol–water to give the azide **19** (0 85 g, 75%), m p 149–153°, $[\alpha]_D$ +5° (*c* 0 4) (Found C, 62 3, H, 5 75, N, 13 2 $C_{20}H_{22}N_4O_4$ calc C, 62 8; H, 5 75, N, 14 65%)

When the reaction time was increased to 15 min, an impure product was isolated which required purification by column chromatography

(*b*) The 5-amino-6-chloro derivative **18** (0 1 g) was heated for 3 h under reflux with sodium azide (0 1 g) in 95% 2-methoxyethanol (2 6 ml) The reaction mixture was processed as in (*a*) to give the azide **19** (0 95 g, 93%), m p 153–157°, $[\alpha]_D$ +5° (*c* 0 5), which was identical (i r) with the product from (*a*) Further recrystallisations from a variety of solvents did not improve the m p

The *N*-benzoyl derivative **27**, prepared in the usual way by using benzoic anhydride in ethanol, had m p 269–273°, $[\alpha]_D$ +32° (*c* 0 2, *N*,*N*-dimethylformamide) (Found C, 67 3, H, 5 3, N, 11 75 $C_{27}H_{26}N_4O_5$ calc C, 66 7, H, 5 35, N, 11 5%)

5-Acetamido-6-azido-1,3 2,4-di-O-benzylidene-5,6-dideoxy-L-iditol (**20**) — (*a*) The *N*-acetylepimine **13** (0 1 g) was heated for 20 min under reflux with sodium azide (0 1 g) and ammonium chloride (0 05 g) in 95% 2-methoxyethanol (4 ml) The addition of water afforded the azide **20** (0 11 g) which was purified on a column of silica gel by using chloroform as solvent This gave material having m p 230–234° (from methanol–ethyl acetate–ether) and $[\alpha]_D$ +12° (*c* 0 6)

(*b*) The 5-acetamido-6-chloro derivative **16** (0 2 g) was heated for 20 h under reflux with sodium azide (0 2 g) in 2-methoxyethanol (3 ml) T l c (chloroform–methanol, 14 1) indicated that the reaction was essentially complete after *ca* 1 5 h and that two components had been formed The major, fast-moving component was obtained pure after chromatography on silica gel and was found to be the required azide **20** (0 015 g, 7%), m p 230–232° (decomp), $[\alpha]_D$ +11° (*c* 0 5) (Found C, 61 8, H, 5 75, N, 12 7 $C_{22}H_{24}N_4O_5$ calc C, 62 25, H, 5 65, N, 13 2%)

The slower-moving component was not isolated, but it was suspected of being the corresponding 2-methyloxazoline

(*c*) *N*-Acetylation of the amine **19**, in the usual way with acetic anhydride in pyridine, afforded the *N*-acetyl derivative **20** in 80% yield, m p 235–237° (from chloroform–light petroleum), $[\alpha]_D$ +17° (*c* 1) All three products had identical i r spectra

5,6-Diamino-1,3 2,4-di-O-benzylidene-5,6-dideoxy-L-iditol (**21**) — A solution of the 6-azide **19** (0 2 g) in methanol (80 ml) was hydrogenated for 5 h at 4 atmos with Raney nickel T4 catalyst[24] (*ca* 2 g) The catalyst was then removed, and the filtrate was concentrated to dryness to give a syrup which crystallised in the presence of a little ethanol Recrystallisation from ethanol–ether–light petroleum afforded the

diamine **21** (0 047 g, 25%), m p 135–145°, which was best characterised as its di-*N*-benzoyl derivative **22**, prepared by the addition of benzoic anhydride to an ethanolic solution of the diamine. It had m p 305–308°, $[\alpha]_D$ +63° (*c* 0 8, *N*,*N*-dimethylformamide) (Found C, 71 6, H, 5 95, N, 4 9 $C_{34}H_{32}N_2O_6$ calc C, 72 3, H, 5 65, N, 4 95%)

*1,3 2,4-Di-O-benzylidene-6-chloro-5,6-dideoxy-5-methanesulphonamido-*L*-iditol* (**23**) — (*a*) A suspension of 0 3 g of the epimine **12** in pyridine (3 ml) was treated with mesyl chloride (0 1 ml) with cooling and then kept overnight at room temperature The addition of water to the reaction mixture afforded crystals of **23** (0 29 g, 72%), m p 194–204° Recrystallisation from chloroform–ether gave the analytical sample having m p 214–216°, $[\alpha]_D$ +38° (*c* 1 3, acetone) (Found C, 55 65, H, 5 4, N, 3 1 $C_{21}H_{24}ClNO_6S$ calc C, 55 5, H, 5 3, N, 3 1%)

When the reaction was repeated with a reaction time of only 2 5 h, a mixture of the *N*-mesylepimine **15** and the ring-opened chloro derivative **23** was obtained in a ratio of *ca* 1 4, based on the specific rotation of the product (+13°)

(*b*) A suspension of the *N*-mesylepimine **15** (1 06 g) in pyridine (10 ml) was allowed to stand at room temperature for 6 h in the presence of pyridinium chloride (1 g) The addition of water to the mixture afforded **23** (0 8 g, 69%), m p 202–205° After recrystallisation from chloroform–ether, the product had m p 213–215°, $[\alpha]_D$ +33° (*c* 1 1, acetone)

(*c*) A suspension of the 5-amino-6-chloro derivative **18** (0 1 g) in pyridine (3 ml) was treated with mesyl chloride (0 06 ml), and the mixture was kept overnight The slow addition of ice-cold water gave the *N*-mesyl derivative **23** (0 9 g, 74%) After recrystallisation twice from chloroform–ether, the product had m p 212–215°, $[\alpha]_D$ +36° (*c* 1 3, acetone)

The i r spectra of all three samples were identical

1,3 2,4-Di-O-benzylidene-5,6-dideoxy-5,6-mesylepimino L*-iditol* (**15**) — (*a*) The 6-chloro-5-methanesulphonamido derivative **23** (1 g) was suspended in M sodium methoxide (30 ml), and the mixture was stirred for 1 h at room temperature The *N*-mesylepimine **15** (0 92 g, 99%) was precipitated by the addition of water and then recrystallised twice from chloroform–ether to give the analytical sample, m p 230–232°, $[\alpha]_D$ +6° (*c* 1 9) (Found C, 60 1, H, 5 6, N, 3 3 $C_{21}H_{23}NO_6S$ calc C, 60 3, H, 5 5, N, 3 35%)

(*b*) Compound **23** (0 16 g) was heated for 40 min under reflux with sodium acetate (0 16 g) in methanol (2 ml) The *N*-mesylepimine **15** (0 14 g, 95%) was precipitated by the addition of water and recrystallised from chloroform, m p 231–235°, $[\alpha]_D$ +6° (*c* 1 2)

The i r spectra of the two samples were identical

*1,3 2,4-Di-O-benzylidene-5,6-dideoxy-5-methanesulphonamido-*L*-iditol* (**24**) — Compound **23** (0 1 g) was suspended in dry ether (*ca.* 30 ml), lithium aluminium hydride (0 1 g) was added, and the mixture heated under reflux for 1 h and processed in the usual way to give, after recrystallisation from chloroform–ether, **24** (0 06 g, 67%),

m p 220–221°, $[\alpha]_D$ +42° (*c* 0 2) (Found C, 60 0, H, 6 15; N, 3 25 $C_{21}H_{25}NO_6S$ calc C, 60 1; H, 5 95, N, 3 35%)

The n m.r spectrum contained, *inter alia*, a three-proton doublet at τ 8 67 (*J* 6 Hz) which confirmed the presence of the *C*-methyl group.

1,3 2,4-Di-O-benzylidene-5-deoxy-5-methanesulphonamido-6-O-methyl-L-iditol (**25**) — Compound **23** (0.1 g) was suspended in 0 5M sodium methoxide (9 ml) and heated under reflux for 30 min, when a clear solution had been obtained The reaction mixture was then concentrated to dryness and partitioned between chloroform and water. The dried ($MgSO_4$) organic layer was concentrated to yield a solid, recrystallisation of which from ethanol gave **25** (0 04 g, 40%), m p 181 5–182 5°, $[\alpha]_D$ +52° (*c* 0 3) (Found C, 59 0, H, 6 3, N, 3 05 $C_{22}H_{27}NO_7S$ calc C, 58 8, H, 6 0, N, 3 1%)

The n m r spectrum of the product showed a 3-proton singlet at τ 6 66, confirming the presence of a methoxyl group

1,3 2,4-Di-O-benzylidene-5-deoxy-5-methanesulphonamido-L-iditol (**26**) — The 6-chloro-5-sulphonamide **23** (0 1 g) was heated for 2 h under reflux with sodium acetate (0 1 g) in 90% aqueous 2-methoxyethanol (2 ml) The product **26** (0 09 g, 94%), which crystallised from the reaction mixture upon the addition of water, had m p 223–225°. Recrystallisation from ethanol gave the analytical sample, m p 225–228°, $[\alpha]_D$ +39° (*c* 0 3) (Found C, 57 85; H, 6 0, N, 3 1 $C_{21}H_{25}NO_7S$ calc C, 57 9, H, 5 75, N, 3 2%)

The i r. spectrum showed, *inter alia*, peaks at 3560 and 3320 cm^{-1} due to –NH and –OH stretching frequencies

ACKNOWLEDGMENTS

CIBA Ltd is thanked for providing generous financial assistance, and the Physical Chemical Measurements Unit at Harwell is thanked for the 100-MHz p m r. spectra

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Carbohydrate Research
Elsevier Publishing Company Amsterdam
Printed in Belgium

DERIVATIVES OF 3,4-DIDEOXY-3,4-EPIMINO-D-IDITOL

A D BARFORD* AND A C RICHARDSON
Department of Chemistry, Queen Elizabeth College (University of London), London W 8 (Great Britain)
(Received January 22nd 1970)

ABSTRACT

Selective monoesterification of 1,2 5,6-di-*O*-isopropylidene-D-mannitol with *p*-bromobenzenesulphonyl chloride afforded a crude monoester which was converted into 3-azido-3-deoxy-1,2 5,6-di-*O*-isopropylidene-D-altritol by replacement with azide anion Sequential methanesulphonylation and reduction with lithium aluminium hydride gave the corresponding 3,4-epimino-D-iditol derivative An alternative route involved reduction of the 3-azide (as the 4-benzoate) to the 3-amine, and this was converted into the sulphonamido-sulphonate which underwent ring closure to the *N*-mesylepimine when treated with sodium acetate in boiling methanol

INTRODUCTION

We[1,2] and others[3] have shown that the reduction of *vic-trans*-azido-sulphonates with lithium aluminium hydride provides a convenient route to epimines Furthermore, the ready ring closure of *vic-trans*-sulphonamido-sulphonates to the corresponding *N*-mesylepimine has been noted[4] We have now applied these methods to the synthesis of derivatives of 3,4-dideoxy-3,4-epimino-D-iditol

RESULTS AND DISCUSSION

As a result of its two-fold axis of symmetry, the two hydroxyl groups of 1,2 5,6-di-*O*-isopropylidene-D-mannitol (**1**) are chemically equivalent, so that selective modification of either would afford the same product This property made the mannitol derivative an attractive starting material for the synthesis of the 3,4-epimine **17**, since this must involve a selective reaction of one of the hydroxyl groups at some stage in the synthesis

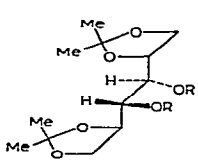
Initially, it was hoped that the required precursor of **17**, namely, 3-azido-3-deoxy-1,2 5,6-di-*O*-isopropylidene-4-*O*-toluene-*p*-sulphonyl-D-altritol (**9**), could be prepared by selective displacement of one of the sulphonate residues of the corresponding mannitol disulphonate **2** However, when **2** was treated with sodium azide

*Present address Chester Beatty Research Institute, Royal Cancer Hospital, Fulham Road, London S W 3, Great Britain

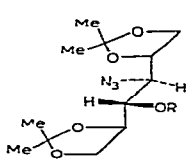
in *N,N*-dimethylformamide, the only product which could be isolated was the corresponding diazide **12**, together with unchanged starting material The syrupy diazide was characterised by reduction to the diamine **13**, followed by *N*-benzoylation to give the dibenzamido-D-iditol derivative **14** The diazide **12** was assigned the D-*ido* stereochemistry on the assumption that both sulphonate groups had been displaced by a direct S_N2 process (*i e* , inversion of configuration at both carbon atoms) The possibility that the second sulphonate group could have been displaced *via* participation of the newly introduced *vic*-azide substituent, which would afford the 3,4-diazido-D-altritol derivative, is considered unlikely Only one case of such participation has been recorded in carbohydrate chemistry[5], and other work has shown that the azide group is a very weak participating group[6]

The fact that the diazide was isolated whilst there was still unreacted starting material present suggested that the sulphonate group of the intermediary azido-sulphonate **9** was more readily replaced than those of the starting material, and so this means of synthesis was not further explored

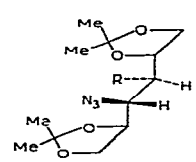
Attention was then turned to the possibility of synthesising the azido-sulphonate **10** by selective monosulphonylation, followed by azide displacement and mesylation of the remaining hydroxyl group The monosulphonate **3** has been reported in the literature[7] It was prepared by the treatment of **1** with one equivalent of tosyl chloride, but its purification was very difficult, and we were unable to obtain it crystalline even after chromatography The monomethanesulphonate has been obtained as the benzoate **7** by a three-stage procedure involving selective monobenzoylation[8], but this process is tedious for the preparation of large amounts of the azide **11** Because of this, we investigated the monoesterification of **1** with *p*-bromobenzenesulphonyl chloride Both the monoester **4** and the diester **5** were obtained as crystalline solids from this selective reaction, but it was not possible to obtain the monoester completely free of the diester However, the crude monoester **4** was suitable for carrying through to the epimine **17** The crude **4** underwent replacement with sodium azide in hexamethylphosphoric triamide, and the resulting 3-azide **8** was used without purification to prepare the syrupy azido-sulphonate **10** Reduction of **10** with lithium aluminium hydride gave the crystalline epimine **17** in an overall yield of 14% from the crude **4** The epimine was characterised as the *N*-acetyl (**18**) and *N*-mesyl (**19**) derivatives



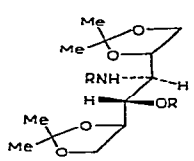
1 R = R = H
2 R = R = Ts
3 R = Ts R = H
4 R = Bs R = H
5 R = R = Bs
6 R = Bz, R = H
7 R = Bz R = Ms



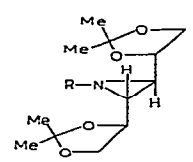
8 R=H
9 R=Ts
10 R=Ms
11 R=Bz



12 R=N_3
13 R=NH_2
14 R=NHBz



15 R=H
16 R=Ms



17 R=H
8 R=Ac
19 R=Ms

An alternative method of synthesising the *N*-mesylepimine **19** employed the 3-amino-3-deoxy-D-altritol derivative[8] **15** which was synthesised by selective mono-

benzoylation of **1**, followed by mesylation of the resulting benzoate **6** The derived monosulphonate **7** underwent replacement with azide in hexamethylphosphoric triamide to give the crystalline azido-benzoate **11** (75% yield) Reduction of **11** with lithium aluminium hydride gave the 3-amino-altritol derivative **15**, which was then converted into the sulphonamido-sulphonate **16** Treatment of compound **16** with sodium acetate in boiling methanol afforded the *N*-mesylepimine **19** identical with the product obtained above

Due to the symmetry of the epimine, ring opening of the compound with nucleophiles should give a single product In agreement with this, only one ring-opened product could be detected when the epimine **17** was treated with sodium azide in 2-methoxyethanol 3-Amino-4-azido-3,4-dideoxy-1,2 5,6-di-*O*-isopropylidene-D-altritol was isolated from this reaction and characterised as its *N*-benzoyl derivative

EXPERIMENTAL

For general procedures, see Ref 2

3,4-Diazido-3,4-dideoxy-1,2 5,6-di-O-*isopropylidene*-D-*iditol* (**12**) — 1,2 5,6-Di-*O*-isopropylidene-3,4-di-*O*-tosyl-D-mannitol[9] **2** (5 g) was heated under reflux with sodium azide (5 g) in *N,N*-dimethylformamide (20 ml) for 1 25 h The addition of water yielded a black syrup which was extracted thrice with ether The combined extracts were thoroughly washed with water, dried ($MgSO_4$), and concentrated to a syrup This was then extracted with 1 4 ether–light petroleum, and the extract was filtered to remove residual starting material The filtrate was concentrated, and purified by chromatography on silica gel with ether–light petroleum (1 4) After examination (ether–light petroleum, 1 1), fractions containing the major component were combined to yield the syrupy diazide **12** (250 mg, 9%) (Found C, 46 45; H, 6 8, N, 25 95 $C_{12}H_{20}N_6O_4$ calc C, 46 1, H, 6 4, N, 26 9%)

3,4-Benzamido-3,4-dideoxy-1,2 5,6-di-O-*isopropylidene*-D-*iditol* (**14**) — The 3,4-diazide **12** (0 29 g) was heated under reflux with lithium aluminium hydride (340 mg) in ether for 1 h The reaction mixture was processed in the usual way, and the resulting chloroform extract was concentrated to give the syrupy diamine (180 mg) which was dissolved in ethanol (3 ml) and treated with benzoic anhydride (180 mg) for 45 min at room temperature Concentration of the reaction mixture gave syrupy crystals, which were triturated with a little ethyl acetate and light petroleum to give **14** (160 mg, 25%), m p 182–183°, $[\alpha]_D$ +129° (*c* 0 3) (Found C, 66 8; H, 7 35, N, 5 7 $C_{26}H_{32}N_2O_6$ calc C, 66 6, H, 6 85, N, 6 0%)

3-O-p-*Bromobenzenesulphonyl-1,2 5,6-di*-O-*isopropylidene*-D-*mannitol* (**4**) — A solution of 1,2 5,6-di-*O*-isopropylidene-D-mannitol[10] **1** (20 g) in dry pyridine (100 ml) was treated with *p*-bromobenzenesulphonyl chloride (19.5 g, 1 mol) portionwise at −10° The reaction mixture was kept for 18 h at room temperature and then poured on to crushed ice (600 g) The crude product which separated out was recrystallised from ethanol (200 ml) to yield 3,4-di-*O*-*p*-bromobenzenesulphonyl-1,2 5,6-di-*O*-isopropylidene-D-mannitol (**5**) (3 9 g, 7%) Recrystallised three times from

ethanol–acetone, it had m p 120–128°, $[\alpha]_D$ +14° (*c* 1) (Found· C, 41 45; H, 3 95 $C_{24}H_{28}Br_2O_{10}S_2$ calc · C, 41 1; H, 4 0%) Concentration of the mother liquors from the first recrystallisation of the above product gave crystals of **4** (6 23 g, 17%), which could not be obtained free from diester by further recrystallisations

A second crop (11 6 g, 31%), m p 89–97°, was obtained by concentration of the mother liquors.

3,4-Dideoxy-3,4-epimino-1,2 5,6-di-O-*isopropylidene*-D-*iditol* (**17**) — The crude monosulphonate **4** (95 g) was heated for 3 days at 85° with sodium azide (60 g) in hexamethylphosphoric triamide (125 ml) Water was added, and the resulting mixture was extracted four times with ether The combined extracts were dried ($MgSO_4$), concentrated to a dark syrup, and fractionated on a silica gel column by using 1 1 ether–light petroleum as eluent The fractions containing the major product were combined and evaporated to yield the crude azide **8** as a black syrup (39 6 g, 70%), which was dried, dissolved in pyridine (250 ml), and treated with mesyl chloride (20 ml, 1 9 mol) After initial cooling, the solution was kept for 23 h at room temperature and then poured on to crushed ice (300 g) to yield a thick, dark syrup which was extracted into methylene chloride The extract was dried ($MgSO_4$) and concentrated to a dark syrup, which was thoroughly dried over conc sulphuric acid for 4 days and then purified on a column of silica gel with 1·1 ether–light petroleum Fractions containing the major component were combined and concentrated to yield syrupy 3-azido-3-deoxy-1,2 5,6-di-*O*-isopropylidene-4-*O*-mesyl-D-altritol (**10**) (23 5 g, 47%) which was dried *in vacuo* over conc sulphuric acid for 16 h*

Lithium aluminium hydride (2 5 g) was added portionwise to a solution of **10** in dry ether over a period of 1 5 h The mixture was processed in the usual way to give a syrup which crystallised on the addition of light petroleum Recrystallisation from light petroleum gave the epimine **17** (1 47 g, 9%), m p 80–81°, $[\alpha]_D$ −30° (*c* 1, water) (Found C, 58 85, H, 8 7, N, 5.65 $C_{12}H_{21}NO_4$ calc C, 59 2, H, 8 65, N, 5 75%) The mother liquors were concentrated and purified on an alumina (basic) column by using 1 1 ether–light petroleum as eluent Fractions containing the pure epimine were combined and concentrated to a crystalline residue Recrystallisation from light petroleum yielded a further amount (0 75 g, 5%) of the epimine, m p 79–80° The total overall yield from **4** was 14%

3,4-Acetylepimino-3,4-dideoxy-1,2 5,6-di-O-*isopropylidene*-D-*iditol* (**18**) — A solution of the epimine **17** (50 mg) in ethanol (1 ml) was treated with acetic anhydride (0 05 ml) and kept for 1.5 h at room temperature The solution was concentrated to a syrup and crystallised from 1 10 ethanol–water to yield the *N*-acetyl derivative (15 mg, 26%), m p 94 5–96°, $[\alpha]_D$ −31° (*c* 0 4) (Found C, 58 9, H, 8 6 N, 4 7 $C_{14}H_{23}NO_5$ calc. C, 58 9, H, 8 1, N, 4.9%).

*In one experiment, a crystalline compound was eluted from the column before the azide, and this was characterised as *p*-dibromobenzene by mixed m p , i r and n m r spectra, and analysis We are unable to account for the presence of this compound in the reaction mixture, but it may have been present as an impurity in the *p*-bromobenzenesulphonyl chloride used in this particular instance

3-Azido-4-O-benzoyl-3-deoxy-1,2 5,6-di-O-isopropylidene-D-altritol (**11**) — 3-*O*-Benzoyl-1,2 5,6-di-*O*-isopropylidene-4-*O*-mesyl-D-mannitol[8] **7** (5 45 g) was heated on a water bath for 19 h at 70–80° with sodium azide (5 g) in hexamethylphosphoric triamide (15 ml) Water was added, and the mixture was extracted three times with ether The combined extracts were dried ($MgSO_4$) and concentrated to a yellow syrup which slowly crystallised Recrystallisation from methanol–water afforded the azide **11** (3 62 g, 75%), m p. 74–77° After a further recrystallisation from methanol–water, it had m p 77–77 5°, $[\alpha]_D$ −36° (*c* 1 7) (Found 58 45; H, 6 6, N, 10 65 $C_{19}H_{25}N_3O_6$ calc C, 58 3, H, 6 4; N, 10 75%)

3-Amino-3-deoxy-1,2 5,6-di-O-isopropylidene-D-altritol (**15**) — A solution of 3-azido-4-*O*-benzoyl-3-deoxy-1,2 5,6-di-*O*-isopropylidene-D-altritol **11** (3 g) in dry ether (15 ml) was added dropwise to a solution of lithium aluminium hydride (1 g) in dry ether (20 ml), and the mixture was heated for 0 75 h under reflux The reaction mixture was processed in the usual way to give a syrup which crystallised. Recrystallisation from ethyl acetate–light petroleum gave the amine (1 4 g, 70%), m p 115–115 5°, $[\alpha]_D$ +1 5° (*c* 3), lit[8] m p 115–116°, $[\alpha]_D$ +2 5 ±0 7°

3-Deoxy-1,2 5,6-di-O-isopropylidene-4-O-mesyl-3-methanesulphonamido-D-altritol (**16**) — A solution of 3-amino-3-deoxy-1,2 5,6-di-*O*-isopropylidene-D-altritol (**15**) (500 mg) in pyridine (5 ml) was treated portionwise with mesyl chloride (0 6 ml, 4 1 mol), and, after initial cooling, the solution was kept for 3 h at room temperature Ice-cold water was then added, and a syrup separated out. The aqueous supernatant was decanted off, and a solution of the syrup in methylene chloride was dried ($MgSO_4$) and concentrated to small bulk Crystallisation of the residue from light petroleum and then from ethanol gave **16** (130 mg, 16%), m p 112–114°, $[\alpha]_D$ +24° (*c* 1) (Found C, 40 9, H, 6.6, N, 3 4. $C_{14}H_{27}NO_9S_2$ calc · C, 40 3, H, 6 45, N, 3 35%)

3,4-Dideoxy-1,2 5,6-di-O-isopropylidene-3,4-mesylepimino-D-iditol (**19**) — (*a*) A solution of **16** (50 mg) in methanol (1 ml) was heated under reflux with sodium acetate (50 mg) for 1 5 h Water was added, and the mixture was extracted three times with methylene chloride The dried ($MgSO_4$) extracts were concentrated, and a crystalline residue was recrystallised from ethanol–light petroleum to give the *N*-mesylepimine **19** (23 mg, 60%), m p 140–141°, which was identical with the product from (*b*)

(*b*) A solution of the epimine **17** (150 mg) in pyridine (3 ml) was treated with mesyl chloride (0 1 ml, 2 mol), and, after initial cooling, the solution was kept for 2 h at room temperature The addition of water gave **19** (150 mg, 76%), m p 139 5–140 5°, $[\alpha]_D$ −6 6° (*c* 1) (Found C, 48 2, H, 7 5, N, 4 4. $C_{13}H_{23}NO_6S$ calc C, 48 6, H, 7 2, N, 4 35%)

4-Azido-3-benzamido-3,4-dideoxy-1,2 5,6-di-O-isopropylidene-D-altritol — The epimine **17** (100 mg) was heated under reflux with sodium azide (100 mg) and ammonium chloride (50 mg) in 95% 2-methoxyethanol (3 ml) for 1 5 h Water was then added, and the resulting solution was extracted three times with chloroform The combined extracts were dried ($MgSO_4$) and concentrated to give a syrup which failed to crystallise A solution of this product in ethanol (2 ml) was treated with

benzoic anhydride (100 mg) and kept overnight at room temperature. The reaction mixture was then concentrated, and the crystalline residue recrystallised from ethanol to give the benzamido-azide (35 mg; 22%), m p 157–158°, $[\alpha]_D$ −27° (*c* 1) (Found· C, 58.9, H, 6 8; N, 14 35. $C_{19}H_{26}N_4O_5$ calc. C, 58.5; H, 6 65; N, 14 .35%).

ACKNOWLEDGMENT

The authors are indebted to CIBA Ltd , for generous financial assistance

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

DIISOPROPYLIDENE ACETALS OF D-ALLOSE† AND D-*ribo*-HEXULOSE (D-PSICOSE)

MASANOBU HAGA, MITSUKO TAKANO*, AND SETSUZO TEJIMA**
Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo (Japan)
(Received January 23rd, 1970)

ABSTRACT

Acetonation of D-allose in the presence of sulfuric acid gives 2,3 5,6-di-*O*-isopropylidene-β-D-allofuranose (**4**) in good yield Oxidation of **4** with methyl sulfoxide and acetic anhydride gave 2,3 5,6-di-*O*-isopropylidene-D-allono-1,4-lactone (**7**) Compound **4** was also obtained from 1,2 5,6-di-*O*-isopropylidene-α-D-allofuranose (**1**) in one step by an acetal-migration reaction The acetonation of D-*ribo*-hexulose (D-psicose) in the presence of anhydrous copper sulfate and sulfuric acid has been re-examined, and the main product has been shown to be 1,2 3,4-di-*O*-isopropylidene-D-psicofuranose (**12**), by conversion of **12** into 6-deoxy-D-psicose (**16**) through 1,2 3,4-di-*O*-isopropylidene-6-*O*-*p*-tolylsulfonyl-D-psicofuranose (**14**)

INTRODUCTION AND DISCUSSION

Recent advances with oxidants such as ruthenium tetraoxide[1] and methyl sulfoxide with acid anhydrides[2,3] have greatly facilitated studies on rare sugars[4] For example, convenient preparations through oxidative procedures of D-allose from D-glucose[3,5], and D-*ribo*-hexulose (D-psicose) from D-fructose[6,7], have been reported in recent years Nevertheless, only a few derivatives of these two sugars are known These sugars have stimulated the attention of biochemists since, although D-allose is still unreported in Nature, D-psicose has been shown to exist in molasses[8], *Itea* plants[9], human urine[10], antibiotics[11], and metabolites of *Pseudomonas methanica*[12]. In this paper, the diisopropylidene acetals of D-allose and D-psicose are described

Crystalline D-allose was prepared[3,5] from 1,2 5,6-di-*O*-isopropylidene-α-D-glucofuranose, and a crystalline acetate (**2**) and benzoate (**3**) of the intermediate 1,2 5,6-di-*O*-isopropylidene-α-D-allofuranose (**1**) were prepared.

Acetonation of D-allose in the presence of sulfuric acid yielded a crystalline diisopropylidene acetal (**4**) in good yield as the sole product (t l c). The diacetal **4** was apparently different from the known diacetal **1** and gave a crystalline monoacetate (**5**) and mono-benzoate (**6**) On the other hand, compound **4** was resistant

†After submission of this manuscript, a paper entitled "The acetonation of D-allose" by J M Ballard and B E Stacey appeared in this journal, 12 (1970) 37
*School of Medicine, Hokkaido University, Sapporo, Japan
**Faculty of Pharmaceutical Sciences, City University of Nagoya, Nagoya, Japan

to sulfonylation by *p*-toluenesulfonyl chloride in pyridine This fact implied that the anomeric hydroxyl group of **4** was free[14] Compound **4** did not reduce Fehling solution, but it is known that 2,3 5,6-di-*O*-isopropylidene-α-D-mannofuranose, which has a free hydroxyl group at the anomeric carbon atom, also resists the action of hot Fehling solution[15].

The structure of **4** was tentatively assigned as 2,3 5,6-di-*O*-isopropylidene-β-D-allofuranose because oxidation with methyl sulfoxide and acetic anhydride gave a lactone that was considered to be 2,3 5,6-di-*O*-isopropylidene-D-allono-1,4-lactone (**7**), since it had a characteristic i r absorption maximum at 1785 cm^{-1}, typical of a γ-lactone[16] The anomeric configuration of **4** was deduced by n m r spectroscopy (chloroform-*d*) The H-1 signal of **4** appeared at low field as a singlet at τ 4 65, and the small coupling ($J_{1,2}$ <0 5 Hz) signified a *trans*-arrangement[17] of the protons at C-1 and C-2

Further confirmation of the furanose structure was provided by mass spectroscopy As shown in Figs 1 and 2, a peak at *m/e* 101 was prominent in the mass spectra of **1** and **4**, indicating the well known fragmentation process of scission of the C-4–C-5 bond in the di-*O*-isopropylidenealdohexofuranose structure[18]. The mass spectra of **1** and **4** are closely similar Peaks at *m/e* 245 (M −15), 187, 159, 127, 73, 59, and 43 indicated that both **1** and **4** have a di-*O*-isopropylidenealdohexofuranose structure Compound **4** has an additional peak at *m/e* 243 (M −17), because of the location[18] of the hydroxyl group on the anomeric carbon atom rather than on C-3 These results are in good agreement with the data reported for the diacetal of D-talose[19] Treatment of 1,2 5,6-di-*O*-isopropylidene-α-D-allofuranose (**1**) with sulfuric acid in acetone gave 2,3 5,6-di-*O*-isopropylidene-β-D-allofuranose (**4**) in high yield The

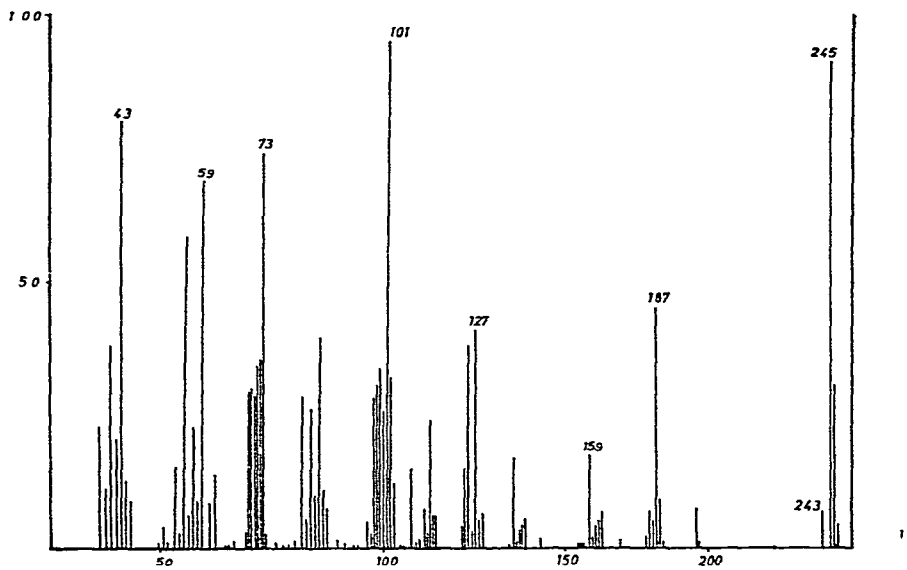


Fig 1 The mass spectrum of 2,3 5,6-di-*O*-isopropylidene-β-D-allofuranose (**4**)

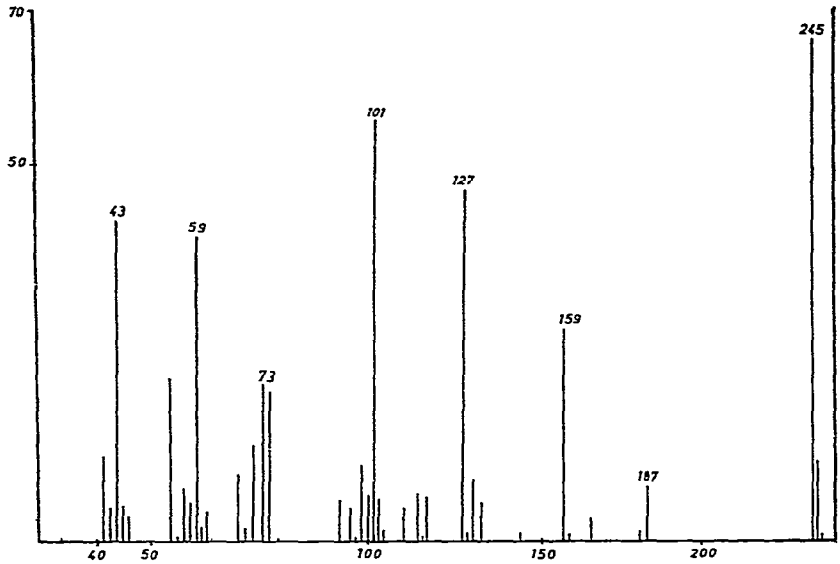
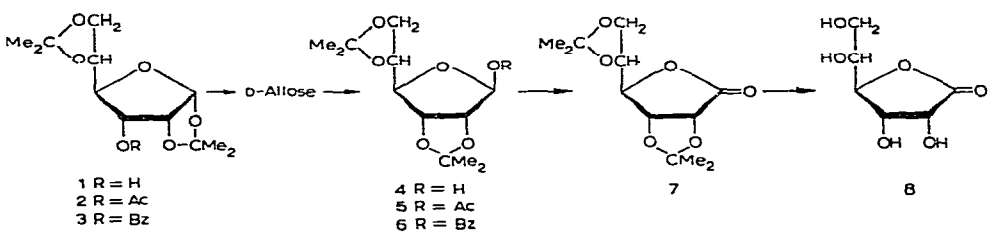
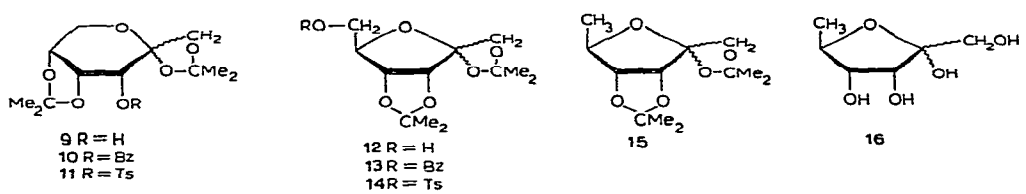


Fig 2 The mass spectrum of 1,2,5,6-di-*O*-isopropylidene-α-D-allofuranose (**1**)

facile migration of the 1,2-acetal group is consistent with recent observations on the rates of hydrolysis of 1,2-*O*-isopropylidenealdoses[13]

In 1935, Steiger and Reichstein[20] reported that acetonation of L-psicose in the presence of anhydrous copper sulfate and sulfuric acid gave a crystalline diacetal as the main product The structure of 1,2 3,4-di-*O*-isopropylidene-L-psicofuranose was assigned since oxidation with potassium permanganate gave a carboxylic acid (indicating a primary hydroxyl group) which, after the removal of the isopropylidene groups, was not converted into the so-called ascorbic acid homolog (that is, it was not a 2-ketohexanoic acid) Later on, the diacetal of the D-sugar was also reported by the same authors[21] The ready availability of D-psicose[6 7] facilitated a re-examination of the diacetal[21]. The intermediate, known[6 7] diacetal 1,2 4,5-di-*O*-isopropylidene-D-





psicopyranose (**9**), was further characterized through the preparation of a crystalline 3-benzoate (**10**) and a crystalline 3-*p*-toluenesulfonate (**11**)

Acetonation of D-psicose according to the direction of Steiger and Reichstein[21] in the presence of anhydrous copper sulfate and sulfuric acid yielded a crystalline diacetal (**12**) having physical constants as stated. The acetal migration procedure[6] from **9** also gave the same diacetal **12** in high yield Benzoylation or *p*-toluenesulfonylation of **12** in the usual manner gave the crystalline 6-benzoate (**13**) and 6-*p*-toluenesulfonate (**14**), respectively, in good yield

Although 6-*p*-tolylsulfonyloxy groups in aldohexose derivatives undergo reduction with lithium aluminum hydride to give 6-deoxy derivatives, 1-*p*-tolylsulfonyloxy groups in ketohexoses, on the other hand, are usually only desulfonylated[14]. Accordingly, it was expected that the sulfonate **14** would be desulfonylated to the original diacetal **12** by lithium aluminum hydride if the tosyl group were to be at C-1

However, treatment of the sulfonate **14** with lithium aluminum hydride in tetrahydrofuran yielded a desulfonyloxylated product, 6-deoxy-1,2 3,4-di-*O*-isopropylidene-D-psicofuranose (**15**) as a mobile syrup, indicating that the sulfonyloxy group had been at C-6 Definitive structural proof for **15** was provided by n m r spectroscopy, since the newly formed methyl group in the sugar moiety resonated as a doublet (J = 6 Hz) centered at τ 8 75 The appearance of this signal as a doublet indicated that it arose from a methyl group at C-6, since a methyl group at C-1 of a ketose would have resonated as a singlet

Removal of the isopropylidene groups from **15** gave syrupy 6-deoxy-D-psicose (**16**), which was characterized as its known phenylosazone[22]. The anomeric configuration of the D-psicose derivatives is still unknown

Furanose derivatives such as **4** and **14** are of interest, since they provide potential intermediates for the preparation of nucleosides containing these sugars[23].

EXPERIMENTAL

General. — Melting points are uncorrected Evaporations of solvents were conducted under diminished pressure below 40° Reactions were followed by t l c on Silica Gel G (E Merck, Darmstadt, Germany), with solvent systems (*A*) 4 1(v/v) benzene–ether, (*B*) 3 1 ethyl acetate–light petroleum (b p 30–70°), and (*C*) 19 1 benzene–methanol Detections were effected with sulfuric acid or iodine vapor Descending paper chromatography was conducted on Whatman No 1 paper with the following solvent systems (*A*) 3 1 1(v/v) propyl alcohol–28% ammonia solution–water and (*B*) 14 3 3 ethyl acetate–acetic acid–water. Spots were detected by periodate–benzidine. I r spectra were obtained on Nujol mulls with a Shimazu spectrometer. N m r spectra were obtained at 60 MHz with a Hitachi H-60 spectrometer with tetramethylsilane as internal reference and chloroform-*d* as solvent Mass spectra were measured with a Hitachi IMU-6E spectrometer (ionizing potential 80 eV), by using an indirect insertion technique.

3-O-Acetyl-1,2 5,6-di-O-isopropylidene-α-D-allofuranose (**2**) — 1,2 5,6-Di-*O*-

isopropylidene-α-D-allofuranose[5] (**1**, 1 0 g), m p 74–75°, $[\alpha]_D^{20}$ +37°, was acetylated with acetic anhydride (5 ml) and pyridine (5 ml) overnight at room temperature The reaction mixture was poured into ice–water (200 ml) and extracted with dichloromethane (3 × 20 ml) The extract was washed successively with 5% sulfuric acid, saturated aqueous sodium hydrogen carbonate, and water The dried (sodium sulfate) solution was evaporated, and the residue was crystallized from light petroleum, yield 1 0 g (86%), m p 75 5–76 5°, $[\alpha]_D^{20}$ +107 6° (*c* 1 0, chloroform)

Anal Calc for $C_{14}H_{22}O_7$. C, 55 62, H, 7 34 Found C, 55 77, H, 7 37.

3-O-Benzoyl-1,2 5,6-di-O-isopropylidene-α-D-allofuranose (**3**) — The diacetal **1** (1 0 g) was benzoylated with benzoyl chloride (2 ml) and pyridine (5 ml) overnight at room temperature After decomposition of the excess reagent with ice, the mixture was poured into ice–water (200 ml) The precipitate was collected by filtration and recrystallized from cyclohexane, yield 760 mg (56%), m p. 73–74.5°, $[\alpha]_D^{20}$ +125 9° (*c* 1 0, chloroform)

Anal Calc for $C_{19}H_{24}O_7$ C, 62 62, H, 6 64 Found C, 62 54, H, 6 54

2,3 5,6-Di-O-isopropylidene-β-D-allofuranose (**4**) — *A* β-D-Allose was prepared from 1,2 5,6-di-*O*-isopropylidene-α-D-allofuranose **1** as follows: compound **1** (20 0 g) was suspended in water (300 ml) and stirred with the H^+ form of Amberlite IR-120 (50 ml) for 4 h at 80° The resin was removed by filtration and washed with water (3 × 50 ml) The combined filtrate and washings were concentrated to give a crystalline mass, which was recrystallized from ethanol–water, yield 11 6 g (85%), m p 127–128 5°, $[\alpha]_D^{20}$ +15° (equil , *c* 2 3, water) [lit [24], m p 128–128 5°, $[\alpha]_D$ +14° (equil , water)] D-Allose (10 0 g) was shaken with anhydrous acetone (300 ml) containing sulfuric acid (7 ml) for 6 h The reaction mixture was neutralized with anhydrous sodium carbonate (100 g), filtered, and the filter washed with acetone (3 × 50 ml) The filtrate and washings were concentrated to a syrup that was dissolved in ether (100 ml) The solution was washed with water, dried (sodium sulfate), and evaporated to dryness Crystallization and recrystallization were effected with light petroleum, yield 9 8 g (68%), m p 66–67°, $[\alpha]_D^{20}$ −27 4° (*c* 3 4, chloroform) and −1 5° (*c* 1 9, water), n m r data (after deuteration) τ 4 65 (1-proton singlet, H-1), τ 5 18 (1-proton doublet, *J* 10 Hz, H-2), τ 5 46 (1-proton doublet, *J* 10 Hz, H-3), τ 5 60–6 20 (4-proton multiplet, H-4,5,6,6′), τ 8 45 (6-proton singlet, 2Me), τ 8 68 (3-proton singlet, Me), and τ 8 70 (3-proton singlet, Me)

Anal. Calc for $C_{12}H_{20}O_6$ C, 55 37, H, 7 75 Found C, 55 18, H, 7 65

B. 1,2 5,6-Di-*O*-isopropylidene-α-D-allofuranose **1** (10 0 g) was dissolved in anhydrous acetone (200 ml) containing sulfuric acid (2 ml) and kept overnight at room temperature A spot having R_F 0 23 (solvent *B*), corresponding to the starting material **1**, had completely disappeared and a spot having R_F 0.40, corresponding to the rearranged product **4**, was detected by t l c The solution was neutralized with anhydrous sodium carbonate (50 g), filtered, and the filter was washed with acetone (3 × 50 ml) The combined filtrate and washings were evaporated to dryness and the residue was recrystallized from light petroleum, yield 8 9 g (89%), m p and mixed m p. 66–67°.

1-O-Acetyl-2,3-5,6-di-O-isopropylidene-β-D-allofuranose (**5**). — 2,3 5,6-Di-*O*-isopropylidene-β-D-allofuranose (**4**, 1 0 g) was acetylated with acetic anhydride (5 ml) and pyridine (5 ml) as described for **2** and the pure acetate **5** was obtained after recrystallization from ligroin, yield 870 mg (75%), m p. 51–51 5°, $[\alpha]_D^{20}$ −46 2° (*c* 1 1, chloroform)

Anal Calc for $C_{14}H_{22}O_7$ C, 55 62; H, 7 34 Found C, 55 76; H, 7 30

1-O-Benzoyl-2,3 5,6-di-O-isopropylidene-β-D-allofuranose (**6**) — Benzoylation of **4** (1 0 g) with benzoyl chloride (2 ml) and pyridine (5 ml) as described for **3**, with recrystallization of the product from ethanol, gave the pure benzoate **6**, yield 890 mg (64%), m.p 109–110°, $[\alpha]_D^{20}$ −38 5° (*c* 1 0, chloroform)

Anal Calc for $C_{19}H_{24}O_7$ C, 62 62, H, 6 64 Found C, 62 77, H, 6 72

2,3 5,6-Di-O-isopropylidene-D-allono-1,4-lactone (**7**) — The diacetal **4** (3 0 g) was dissolved in a mixture of dimethyl sulfoxide (50 ml) and acetic anhydride (30 ml) and kept overnight at room temperature Evaporation of the solvent below 70° gave a slightly colored syrup Crystallization and recrystallization were effected with light petroleum, yield 2 3 g (78%), m p 38–39°, $[\alpha]_D^{20}$ −78 7° (*c* 3 2, chloroform)

Anal Calc for $C_{12}H_{18}O_6$ C, 55 80, H, 7 03 Found C, 55 68, H, 6 95

D-Allono-1,4-lactone (**8**) — 2,3 5,6-Di-*O*-isopropylidene-D-allono-1,4-lactone (**7**, 1 0 g) was heated with Amberlite IR-120 (H^+) resin (2 ml) in water (10 ml) for 6 h on steam-bath The resin was removed by filtration and washed with water (2 × 5 ml) The combined filtrate and washings were concentrated to give crystalline mass, which was recrystallized from ethanol; yield 600 mg (87%), m p 119–120°, $[\alpha]_D^{20}$ −6.6° (*c* 4 2, water), λ_{max} 1770 cm^{-1} (γ-lactone) [Lit [25], m p 120°, $[\alpha]_D$ −6 8° in water]

Anal Calc for $C_6H_{10}O_6$ C, 40 45, H, 5 56 Found C, 40 32, H, 5 66

3-O-Benzoyl-1,2 4,5-di-O-isopropylidene-D-psicopyranose (**10**) — 1,2 4,5-Di-*O*-isopropylidene-D-psicopyranose (**9**, 1 0 g), m p 64–66°, $[\alpha]_D^{20}$ −125 5° in chloroform[6,7], was benzoylated with benzoyl chloride (2 ml) and pyridine (5 ml), and 730 mg (58%) of the benzoate **10** was obtained; m p 143–144°, $[\alpha]_D^{20}$ −141 7° (*c* 3 0, chloroform).

Anal Calc for $C_{19}H_{24}O_7$ C, 62 62, H, 6 67 Found C, 62 75; H, 6 76

1,2 4,5-Di-O-isopropylidene-3-O-p-tolysulfonyl-D-psicopyranose (**11**). — The diacetal **9** (1 0 g) was treated with *p*-toluenesulfonyl chloride (2 0 g) in pyridine (5 ml) overnight at room temperature After conventional processing, the sulfonate **11** was recrystallized from ethanol, yield 1 0 g (64%), m p 108–109°, $[\alpha]_D^{20}$ −103 2° (*c* 0 93, chloroform)

Anal Calc for $C_{19}H_{26}O_8S$ C, 55 18, H, 6 32, S, 7 73 Found C, 55 15, H, 6 44, S, 7.67.

1,2 3,4-Di-O-isopropylidene-D-psicofuranose (**12**) — *4* D-Psicose (10 0 g), $[\alpha]_D^{20}$ +3 3 in water[21], R_{Fru} 1 15 in solvent *A* and 1.41 in solvent *B*, was acetonated according to Steiger and Reichstein[21] with anhydrous copper sulfate (30 g) and sulfuric acid (1 ml) in anhydrous acetone (700 ml) for 48 h at room temperature. The title compound was obtained in 48% yield; m p. 56–58°, $[\alpha]_D^{20}$ −94 6° (*c* 2 6, chloro-

form), lit , m p 57–57 5°, $[\alpha]_D$ −89 6° in chloroform[6], m.p 57–58 5°, $[\alpha]_D$ −98 1° in chloroform[21]

Anal Calc for $C_{12}H_{20}O_6$ C, 55 37, H, 7 75 Found C, 55 38, H, 7 55

B The diacetal **9** (20 0 g) was dissolved in anhydrous acetone (300 ml) containing sulfuric acid (4 ml) and the solution was kept overnight at room temperature The diacetal **12** (12 8 g, 64%) was obtained after treatment as described for **4**, m p and mixed m p 56–58°.

6-O-Benzoyl-1,2 3,4-di-O-isopropylidene-D-psicofuranose (**13**) — The diacetal **12** (1 5 g) was benzoylated with benzoyl chloride (2 ml) and pyridine (5 ml) as described for **3**, and the pure benzoate was obtained after recrystallization from ethanol, yield 1 5 g (75%), m p 70–71°, $[\alpha]_D^{20}$ −59 6° (*c* 2 9, chloroform)

Anal Calc for $C_{19}H_{24}O_7$. C, 62 62, H, 6 67 Found C, 62 70, H, 6 62

1,2 3,4-Di-O-isopropylidene-6-O-p-tolylsulfonyl-D-psicofuranose (**14**) — The diacetal **12** (6 0 g) was treated with *p*-toluenesulfonyl chloride (6 0 g) in pyridine (50 ml) overnight at room temperature The reaction mixture was poured into ice–water (1 liter) The resultant precipitate was recrystallized from ligroin, yield 7 7 g (74%), m p 98–99°, $[\alpha]_D^{20}$ −42 4° (*c* 3 4, chloroform)

Anal Calc for $C_{19}H_{26}O_8S$ C, 55 18, H, 6 32; S, 7 73 Found C, 55 26, H, 6 36, S, 7 69

6-Deoxy-1,2 3,4-di-O-isopropylidene-D-psicofuranose (**15**) — 1,2 3,4-Di-*O*-isopropylidene-6-*O*-*p*-tolylsulfonyl-D-psicofuranose (**14**, 5 0 g) was dissolved in anhydrous tetrahydrofuran (100 ml) and lithium aluminum hydride (4 0 g) was added The reaction mixture was gently refluxed for 8 h under protection from moisture. The excess of hydride was then decomposed by the addition of water and the mixture was filtered The filtrate was evaporated to dryness, and the residue was dissolved in ether (100 ml), washed with water, dried (sodium sulfate), and evaporated to a syrup Distillation of the product under a high vacuum gave a mobile syrup, yield 2 5 g (85%), b p 85–90°/10^{-3} torr, $[\alpha]_D^{20}$ −97 3° (*c* 1 1, chloroform), n m r data τ 5 45 (2-proton quartet, H-1,1'), τ 5 60–6 20 (3-proton multiplet, H-3,4,5), τ 8 60 (6-proton singlet, 2Me), τ 8 65 (3-proton singlet, Me), τ 8 70 (3-proton singlet, Me), and τ 8 75 (3-proton doublet, H-6)

Anal Calc for $C_{12}H_{20}O_5$ C, 59 00, H, 8 25. Found C, 58 97, H, 8 17

6-Deoxy-D-psicose (**16**) — 6-Deoxy-1,2 3,4-di-*O*-isopropylidene-D-psicofuranose (**15**, 650 mg) was dissolved in water (10 ml) and treated with Amberlite IR-120 (H^+) (2 ml) for 4 h at 60° After removal of the resin, water was evaporated off to give a thick syrup, yield 370 mg (85%), optical rotation in water was not observed

Anal Calc for $C_6H_{12}O_5$ C, 43 90, H, 7 37 Found C, 43 75, H, 7 30

6-Deoxy-D-*ribo*-hexulose phenylosazone, m p 178–180°, $[\alpha]_D^{20}$ −67 2 (*c* 0 9, pyridine) was obtained by treatment of the sugar **16** with phenylhydrazine in aqueous acetic acid [lit [22], m p 179–180°, $[\alpha]_D$ −70 4° in pyridine–ethanol]

ACKNOWLEDGMENTS

We are indebted to H Kakizaki, A Maeda, and M. Sato for the microanalysis, to H. Fujisawa for recording the n m r. spectra, and to Y Imai for obtaining the mass spectra

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Carbohydrate Research
Elsevier Publishing Company Amsterdam
Printed in Belgium

THE FORMATION OF *N*-ACYLGLYCOSYLAMINES OF SOME MONOSACCHARIDES D-MANNOSE AND L-RHAMNOSE DERIVATIVES

ALBERTO B ZANLUNGO, JORGE O DEFERRARI, AND RAÚL A CADENAS
Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Perú 222, Buenos Aires, (Argentina)

(Received January 6th, 1970, in revised form, February 16th, 1970)

ABSTRACT

The factors that determine the favored formation of furanoid rings in the *N*-acylglycosylamines obtained by ammonolysis of *O*-acyl derivatives of some monosaccharides are discussed The isolation and structure of *N*-acetyl-α- and -β-D-mannofuranosylamine, *N*-acetyl-α-D-mannopyranosylamine, *N*-acetyl-α-L-rhamnopyranosylamine, and *N*-acetyl-α-L-rhamnofuranosylamine are reported, and the conformation of the last two compounds is discussed on the basis of data from n m r spectroscopy

INTRODUCTION

The steric and conformational factors that are involved in the formation of bis(acylamido) derivatives at C-1 of certain sugars when the *O*-acyl derivatives are ammonolyzed were considered in a previous paper[1] Another feature of that reaction, namely, the favored formation of *N*-acetylglycofuranosylamines will now be considered.

In irreversible reactions in carbohydrate chemistry, a great tendency for the formation of furanose rings is observed[2]. This is thermodynamically justified, because the five-membered rings have lower entropy requirements in passage from the acyclic form[3], which shows the highest degree of freedom, or disorder, and has a great tendency to cyclize Apart from thermodynamic considerations, in the favored formation of a certain ring, the stereochemistry of the transition state is an important factor, and the configuration of the sugar exerts a strong influence on the high or low yield of furanoid products Examples are given by Capon and Thacker[4] in their quantitative study of the reaction of hydrochloric acid on the dimethyl acetals of D-glucose and D-galactose, and by Hudson and Barker[5] in work on the dehydration of alditols to give furanoid rings

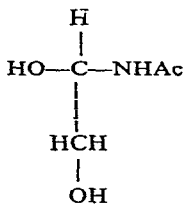
It is now accepted that the transition state for a cyclization shall have virtually the geometry of the final, cyclic molecule[6] The different yields of *N*-acylglycofuranosylamines obtained in the ammonolysis of acylated monosaccharides can be analyzed from this point of view

When acetylated sugars having the D-*xylo* configuration were ammonolyzed,

the corresponding *N*-acetyl-D-glycofuranosylamines were the principal products of the reaction, the products formed through concomitant, competitive reactions[1] being obtained in lower yields. For example, when penta-*O*-acetyl-D-glucopyranose is ammonolyzed in aqueous media, the principal product is *N*-acetyl-α-D-glucofuranosylamine, which is obtained in 41% yield[7]. On ammonolysis of tetra-*O*-acetyl-β-D-xylose[8], *N*-acetyl-α-D-xylofuranosylamine is the principal product (yield of 9.2%), and *N*-acetyl-β-D-xylopyranosylamine (3.1%) and D-xylose are also obtained.

With other acetylated aldoses, the opposite occurs, the 1,1-bis(acetamido) derivatives being formed in various proportions, but always preponderating over the *N*-acetyl-D-glycofuranosylamines. The two reactions (formation of mono- and bis-acylamides) are competitive, and their relative rates depend on configurational factors.

A reasonable hypothesis[1] for the formation of both types of compound involves formation of a common, acyclic intermediate (**1**), from which (*a*) through attack by ammonia and subsequent migration of the second acyl group, the



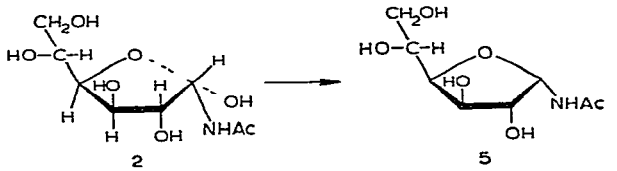
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bis(acylamido) sugar would be obtained, or (*b*) by cyclization with O-4 or O-5 through a nucleophilic displacement, the respective furanose or pyranose *N*-acylglycosylamines are formed. For cyclization to occur, it would not be necessary for all of the acyl groups to be ammonolyzed, in carbohydrate chemistry[9], examples are known that show the possibility of cyclization when the molecule has some of its hydroxyl groups esterified.

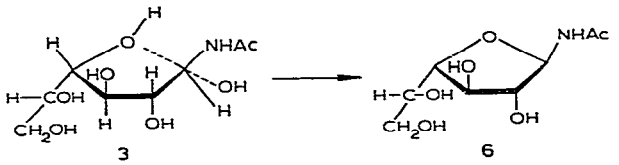
The transition states for the cyclization of the acyclic intermediates of D-glucose, D-galactose, and D-mannose may be represented by formulas **2**, **3**, and **4**, respectively. It is probable that the energy involved in the cyclization overcomes the energy barrier among the conformers, making irrelevant the emphasizing of any particular conformation of the quasi-cyclic intermediates (Curtin–Hammett principle)[10].

By ammonolysis in aqueous media, penta-*O*-acetyl-β-D-glucopyranose gives 41% of *N*-acetyl-α-D-glucofuranosylamine[7] (**5**), and penta-*O*-acetyl-β-D-galactopyranose gives 24% of *N*-acetyl-β-D-galactofuranosylamine[1] (**6**). Other cyclic compounds have not been isolated from such treatment of these acetates. The favored formation of these anomers may be explained by the *trans* relationship between the bulky groups on C-1 and C-4, as is shown in **5** and **6**, which, for each sugar, is less hindered than the opposite anomeric configuration. In addition, compound **6** has a *cis* relationship between the hydroxyl group on C-2 and the bulky substituent on C-4, a configurational feature that appears important in hindering the cyclization of some

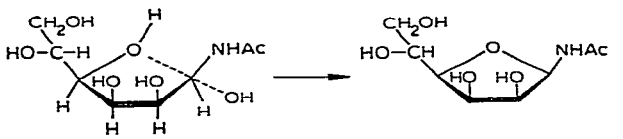
alditols to give furanoid rings[5] Probably, this factor and the high contribution of the acyl group on O-4 of the D-galactose pentaacetate [to give, by ammonolysis, the bis(acylamido) derivative[1]] would strongly diminish the yield of **6**, compared with that of **5**



2 5

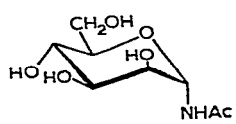


3 6



4 7

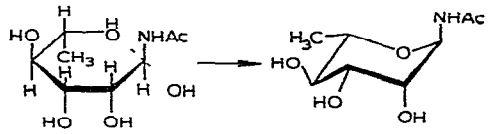
The strongest influence of the configurational factors is observed with D-mannose derivatives The formation of *N*-acetyl-*β*-D-mannofuranosylamine (7) involves great strain, as it has all of the bulky substituents in a *cis*-relationship, causing the yield to be only 3 9% For the same reason (except for the acetamido group), the yield of the α-D anomer is also low (8 4%) The total yield of furanoses was 12 3% Simultaneously, a good yield of the bis(acetamido) derivative (29–35%) is obtained, and a 3 9% yield of *N*-acetyl-α-D-mannopyranosylamine (**8**) is also formed The production of *N*-acetyl-D-glycopyranosylamines was not evident in the ammonolysis of D-glucose and D-galactose acetates On the basis that the acyl group on O-2 has a low contribution to the formation of the bis(acylamides)[1], this different behavior (compared with that of D-glucose) is noteworthy, and confirms the importance of the steric factors considered



8

Another feature of this reaction, namely, the importance of polar effects, can be observed in the ammonolysis of tetra-*O*-acetyl-L-rhamnose Here, along with 1,1-bis(acetamido)-1-deoxy-L-rhamnitol (37 5%), a 14 7% yield of *N*-acetyl-α-L-rhamnopyranosylamine (**10**) and a 4 9% yield of *N*-acetyl-α-L-rhamnofuranosylamine were obtained The observed inversion of yields (as regards the types of cyclic

products) from L-rhamnose tetraacetate (L-*lyxo* configuration) compared to D-mannose pentaacetate (D-*lyxo* configuration) could originate from the inductive effect of the methyl group on C-5 of L-rhamnose, which would enhance the nucleophilic character of the hydroxyl group on that carbon atom, making its attack proceed as in **9**

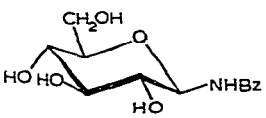


9 **10**

The favored formation of *alpha* anomers of the *N*-acetyl-L-rhamnosylamines (**10** and **12**) may be attributed to the *trans* relationship of the *N*-acetyl group on C-1 to the other groups of the molecule, with the exception of the equatorial hydroxyl group on C-4 in **10**

In the ammonolysis of the pentabenzoates of D-glucose, D-galactose, and D-mannose, the principal product is the corresponding bis(benzamido) derivative Only for D-mannose was the *N*-benzoylglycopyranosylamine crystallized (in 8% yield[11]) From the ammonolysis of the respective glycoheptonic nitriles[12], the *N*-benzoylglycopyranosylamines of D-glucose (8%), D-galactose (4 4%), and D-mannose (4 4%) were obtained The last-mentioned had already been obtained by Brigl *et al* [13] from hexa-*O*-benzoyl-D-*glycero*-D-*galacto*-heptononitrile All of these monobenzamido pyranose derivatives had the *beta* anomeric configuration

The low yields of cyclic forms that the benzoates afford can be explained by the size, as well as the low reactivity, of the benzoyl group The slowness of ammonolysis of these bulky groups makes the cyclization difficult, and the formation of bis-(benzamido) derivatives easier Consequently, pyranose rings would be favored, as they are less strained if the compound has the *C1* (D) conformation, because the benzoyl groups then have an equatorial orientation, as is shown in **11** for *N*-benzoyl-*β*-D-glucopyranosylamine



11

The anomeric configuration of the sugars obtained is postulated on the basis of the Hudson rules[14] The *N*-acetyl-α-D-mannopyranosylamine showed $[\alpha]_D$ +87 5°, whereas the anomer already isolated[15] had $[\alpha]_D$ −47 4° The n m r spectra of the two compounds at 60 MHz do not permit a distinction to be made between the anomers on the basis of the $J_{1,2}$ coupling constants, but, for the α-D anomer, a chemical shift at τ 4 54 ($J_{1\ 2}$ 1 5 Hz) is observed for H-1, and, for the *β*-D anomer, it appears at τ 4 77 ($J_{1,2}$ 0 5 Hz); this agrees with the values of chemical shifts at lower field that,

in general, show that the α-D-pyranose anomers exist in the *C1* (D) conformation For both anomers, the rest of the protons appear as a large multiplet at τ 6 50–6 00, and the protons of the acetamido methyl group at τ 7 94 At 60 or 100 MHz, the *N*-acetyl-D-mannofuranosylamines did not give spectra amenable to first-order analysis

For *N*-acetyl-α-L-rhamnopyranosylamine, conformation **10** was considered (instead of the alternative, having the *N*-acetyl group equatorial), on the basis of the fewer non-bonded interactions of **10** In the n m r spectrum of **10**, the anomeric proton appeared as a doublet at τ 4 63 ($J_{1,2}$ 2 4 Hz), which indicated an equatorial–equatorial relationship for H-1–H-2 However, the low values observed for $J_{3,4}$ and $J_{4\ 5}$, both corresponding to diaxial protons, suggests some contribution of the alternative conformation or the existence of **10** as a form slightly distorted from the chair depicted, probably by the influence of the bulky acetamido group in the axial position The chemical shifts and coupling constants are shown in Table I

TABLE I

CHEMICAL-SHIFT DATA FOR COMPOUNDS **10** AND **12** AT 20°

Compound	*No*	*Chemical shift of protons* (τ)						
		H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	N-*Ac*
N-Acetyl-α-L-rhamnofuranosylamine (in pyridine-d_5, at 100 MHz)	**12**	4 18	5 47	5 44	6 00	5 80	8 70	8 00
N-Acetyl-α-L-rhamnopyranosylamine (in D_2O, at 60 MHz)	**10**	4 63	6 08	6 23	6 44	6 48	8 77	7 98

In the n m.r spectrum of *N*-acetyl-α-L-rhamnofuranosylamine (**12**) at 100 MHz in pyridine-d_5 (see Fig 1), the anomeric proton (H-1) appears as a doublet that collapses to a singlet when H-2 and H-3 are irradiated, the signal for H-2 appears to be superimposed on that for H-3 By irradiation of H-1, it is possible to determine the signals pertaining to H-2 in the non-irradiated spectrum, and to deduce the coupling constant for H-2–H-3 The H-4 signal appears as an ill-defined pair of doublets which, by irradiation of H-1, become sharp, indicating the existence of long-range coupling between H-1 and H-4 By irradiation at the H-2–H-3 multiplet, the H-4 signal becomes a doublet from which $J_{4\ 5}$ is obtained Irradiation of H-4 changes the pattern of both H-3 and H-5, which appear as a multiplet centered at τ 5 80 The signal for the acetyl methyl group appears as an upfield singlet at τ 8 0, and for the methyl group on C-5, as a doublet at τ 8 70

Application of a modified Karplus equation[16] to the coupling constants of the ring protons, taking into account the configuration of the sugar, led to the dihedral angles $\varphi_{1\ 2}$ 144°, $\varphi_{2\ 3}$ 47°, and $\varphi_{3,4}$ 55° These values were compared with those tabulated by Abraham and McLauchlan for the puckering of a cyclopentane ring[17], and found to agree with an envelope conformation. Molecular models showed that a V_3 envelope conformation best fits the values obtained As with *N*-acetyl-β-D-galactofuranosylamine[1], full eclipsing of the σ (C-1–H) bond with a free *p*-electron

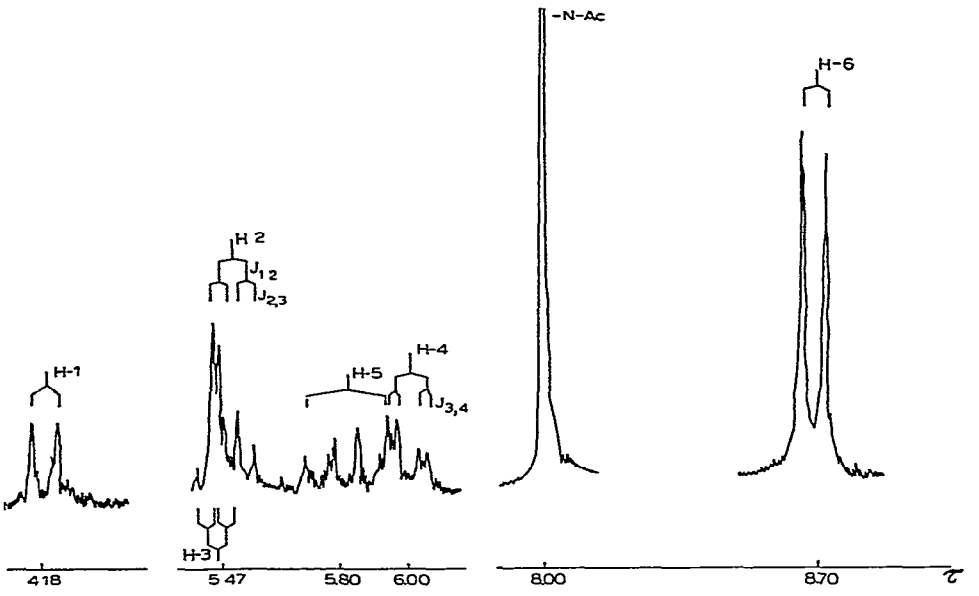
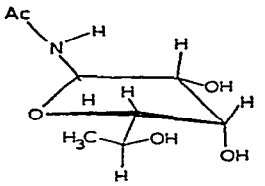


Fig 1 N m r spectrum of *N*-acetyl-α-L-rhamnofuranosylamine at 100 MHz in pyridine-d_5

orbital of the oxygen atom was observed in molecular models This would increase[18] the coupling constant $J_{1,2}$ to a value which would cancel the lowering effect of the electronegative acetamido group on the coupling constant[19], making the observed dihedral angle for H-1–H-2 almost the same as that tabulated[17] The $J_{4,5}$ coupling



12

(8 Hz) suggests that the preponderant conformer has an *anti* arrangement of H-4 and H-5, which also would be conformationally the best

EXPERIMENTAL

General procedures — Paper chromatography was conducted on Whatman No 1 paper with the following eluants, which were also used in cellulose-column chromatography (*A*) 2 1.1 (v/v) butyl alcohol–ethanol–water, (*B*) 50 7 (v/v) butyl alcohol–water, (*C*) 5 3 3 1 (v/v; top layer) ethyl acetate–pyridine–water–benzene The fractions from the chromatographic columns were evaporated under diminished pressure below 60°, and collected after comparison by paper chromatography Melting points are not corrected The structures of the *N*-acetylglycosylamines were

determined by metaperiodate oxidation by the following general technique to 3 ml of 100 mM sodium metaperiodate was added a solution of the glycosylamine (7 mg) in 3 ml of water The solution was made to 10 ml with water and kept at 20° during the oxidation The sodium metaperiodate uptake was measured[20] with a Beckman D U. spectrophotometer at 222 5 nm, and the formaldehyde[21] at 570 nm, and formic acid was titrated with 10 mM sodium hydroxide N m r spectra were recorded, at 20–25°, at 100 MHz with an HA-100 and, at 60 MHz with an A-60 Varian spectrometer Tetramethylsilane (τ 10 00) was used as the internal standard Coupling constants (see Table II) were read directly from the spectra Hydroxyl protons were interchanged by adding deuterium oxide The data on chemical shifts are given in Table I The rotatory powers were determined at 25°.

TABLE II

PROTON–PROTON COUPLING CONSTANTS (Hz) FOR COMPOUNDS **10** AND **12** AT 20°

Compound	*No*	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$
N-Acetyl-α-L-rhamnofuranosylamine (in pyridine-d_5 at 100 MHz)	**12**	6 5	4 2	2 5	8 0	6 0
N-Acetyl-α-L-rhamnopyranosylamine (in D_2O, at 60 MHz)	**10**	2 4	3 0	5 0	7 5	5 6

Ammonolysis of penta-O-acetyl-β-D-mannopyranose — (*A*) *Isolation of 1,1-bis-(acetamido)-1-deoxy-D-mannitol* Penta-*O*-acetyl-β-D-mannopyranose (13 5 g) was suspended in 25% aqueous ammonia (280 ml) and dissolved by shaking for 1 h After 24 h at room temperature, the solution was evaporated to 30 ml, and, on cooling, needles (2 10 g), m p 215–216°, were obtained which, after recrystallization from methanol, gave pure 1,1-bis(acetamido)-1-deoxy-D-mannitol, m p 216–217°, $[\alpha]_D$ −13 5° (*c* 1 1, water), lit[11] m p 218–219°, $[\alpha]_D$ −13 8° (water) The mother liquors were evaporated, and the resulting syrup was extracted with ethyl acetate (5 × 60 ml) to remove the acetamide The solid obtained was dissolved in 100 ml of boiling ethanol and, on cooling, a further 720 mg of 1,1-bis(acetamido)-1-deoxy-D-mannitol was obtained; total yield, 2 82 g (29%)

(*B*) *Isolation of* N-*acetyl-α-D-mannofuranosylamine* (**5**) The mother liquors from the 1,1-bis(acetamido)-1-deoxy-D-mannitol were evaporated, and the resulting syrup was chromatographed on a column of cellulose (70 × 3 cm), elution was performed with solvent *C*, and 130 fractions (50 ml each) were collected Evaporation of fractions 88–102 gave needles (500 mg), m p 157–159° After two recrystallizations from butyl alcohol, pure **5** was obtained, m p 162–163°, $[\alpha]_D$ +120 9° (*c* 1 0, water).

Anal Calc for $C_8H_{15}NO_6$ C, 43 44, H, 6 77, N, 6 33 Found C, 43 35, H, 6 98; N, 6 47

On metaperiodate oxidation, this compound produced 0 95 molar proportion of formaldehyde, indicating a furanose structure, it also showed great overoxidation, giving 2 33 moles of formic acid per mole, with the uptake of 5 22 moles of periodate after 24 h.

N-*Acetyl-tetra*-O-*acetyl*-α-D-*mannofuranosylamine* — Compound **5** (80 mg) was acetylated at room temperature with 1 1 pyridine–acetic anhydride (8 ml) The solution was kept for 24 h at room temperature and then evaporated over sulfuric acid in a vacuum desiccator The resulting solid was recrystallized by dissolving it in isopropyl alcohol (2 ml) and adding light petroleum (2 ml, b p 60–80°) After three recrystallizations from the same mixture, 130 mg of compound, m p 123–124°, $[\alpha]_D$ +43 5° (*c* 1 0, chloroform) was obtained

Anal Calc for $C_{16}H_{23}NO_{10}$ C, 49 35, H, 5 91; N, 3 59 Found C, 49 43, H, 6 00; N, 3 61

Isolation of N-*acetyl*-β-D-*mannofuranosylamine* (**7**) — Evaporation of fractions 104–121 (from the column described), gave needles (280 mg) of m p 143–147° After three recrystallizations from isopropyl alcohol, pure *N*-acetyl-β-D-mannofuranosylamine was obtained, m p 148–149°, $[\alpha]_D$ −113.2° (*c* 1 0, water).

Anal Calc for $C_8H_{15}NO_6$· C, 43 44, H, 6 77, N, 6 33 Found C, 43 75, H, 6 87; N, 6 51.

Metaperiodate oxidation of this compound showed high overoxidation, with an uptake, after 24 h, of 5 72 moles of periodate per mole and the formation of 2 0 moles of formic acid The production of 0 91 molar proportion of formaldehyde indicated a furanose structure

N-*Acetyl-tetra*-O-*acetyl*-β-D-*mannofuranosylamine* — Compound 7 (60 mg) was acetylated as described for **5** The resulting solid was recrystallized from 1 10 butyl alcohol–light petroleum, giving needles (70 mg) of m p 129–130° After three recrystallizations from the same mixture, it had m p 131–132°, $[\alpha]_D$ +29 0° (*c* 0 9, chloroform)

Anal Calc. for $C_{16}H_{23}NO_{10}$· C, 49 35, H, 5 91, N, 3 59 Found C, 49 43, H, 5 72, N, 3 82

Isolation of N-*acetyl*-α-D-*mannopyranosylamine* (**8**) — Evaporation of the mother liquors from fractions 109–121 gave needles (300 mg) of m p 180–182° After three recrystallizations from 1 1 isopropyl alcohol–light petroleum, compound **8**, m p 183–184°, $[\alpha]_D$ +87 5° (*c* 0 9, water) was obtained

Anal Calc for $C_8H_{15}NO_6$ C, 43 44, H, 6 77, N, 6 33 Found C, 43 70, H, 7 07, N, 6 53

By oxidation with sodium metaperiodate for 24 h, this compound gave 0 97 mole of formic acid per mole and consumed 2 27 moles of periodate, no formaldehyde was detected These data agree with a pyranose structure

The fractions that did not crystallize were combined with the mother liquors from the rest of the fractions from the cellulose-column chromatography described, and were evaporated to dryness The resulting syrup was chromatographed on a column of cellulose (65 × 3 cm) with solvent *C*, and 70 fractions (50 ml each) were collected From fractions 45–48, *N*-acetyl-α-D-mannofuranosylamine (140 mg) and, from fraction 49, its anomer (**7**) (30 mg) were isolated The total yield of the former compound was 640 mg (8 4%), and that of **7**, 300 mg (3 9%)

N-*Acetyl-tetra*-O-*acetyl*-α-D-*mannopyranosylamine* — Compound **8** (40 mg)

was acetylated in the usual way. The product was recrystallized from butyl alcohol–light petroleum (b p 60–80°), giving 45 mg of product, m p. 176–178° After three recrystallizations from the same mixture, it had m p 181–182°, $[\alpha]_D$ +139 6° (*c* 0 2, chloroform)

Anal Calc for $C_{16}H_{23}NO_{10}$ C 49 35, H, 5 91, N, 3 59 Found C, 49 68, H, 6 25, N, 3 51

*Ammonolysis of tetra-*O*-acetyl-*L*-rhamnopyranose Isolation of 1,1-bis(acetamido)-1-deoxy-*L*-rhamnitol* — A mixture of tetra-*O*-acetyl-α- and -β-L-rhamnose[22] (11 3 g) was dissolved in 25% aqueous ammonia (240 ml) by shaking After 24 h at room temperature, the solution was evaporated, and the residual syrup was extracted with six 40-ml portions of ethyl acetate (to remove the acetamide) The solid was then dissolved in boiling 2 1 ethanol–methanol (450 ml) On cooling, needles (2 9 g), m p 234–236° (dec), were obtained Recrystallization from 70% ethanol gave 1,1-bis-(acetamido)-1-deoxy-L-rhamnitol, m p 237–238° (dec), $[\alpha]_D$ +23 8° (*c* 1 1, water), lit [23] m p 239–240°, $[\alpha]_D$ +23 1° (in water) The mother liquors were evaporated, and the resulting solid was dissolved in boiling 3 1 isopropyl alcohol–ethanol (500 ml), on cooling, needles (600 mg) were obtained which, after recrystallization from 80% ethanol, had m p 233–235°, total yield 3 36 g (37 5%)

Isolation of N-*acetyl-α-L-rhamnopyranosylamine* (**10**) — The mother liquors from 1,1-bis(acetamido)-1-deoxy-L-rhamnitol were evaporated, and the resulting syrup was chromatographed on a column of cellulose (76 × 3 5 cm) Elution was conducted with solvent *A*, and 30 fractions (75 ml each) were collected, these were individually evaporated, and dissolved in boiling ethanol From fraction 12, plates (200 mg, m p 199–202°) were isolated which, after three recrystallizations from butyl alcohol, gave **10**, m p 206–207°, $[\alpha]_D$ −99 0° (*c* 1 0, water)

Anal Calc for $C_8H_{15}NO_5$ C, 46 82, H, 7 31, N, 6 87 Found C, 47 10, H, 7 36, N, 7 10

The structure of this compound was ascertained by oxidation with meta-periodate After 24 h, it had consumed 2 21 moles of periodate per mole and produced 0 95 mole of formic acid, results which agree with a pyranose structure

Fractions 36–42 gave a further 850 mg of **10**, m p 204–206°, total yield 1 05 g (14 7%) The rest of the fractions did not give crystalline products

N-*Acetyl-tri-*O-*acetyl-α-L-rhamnopyranosylamine* — Compound **10** (100 mg) was acetylated in the usual way, and a syrup was obtained which did not crystallize from the common solvents T l c on Silica Gel G with 1 49 methanol–benzene gave only one spot It had $[\alpha]_D$ −39 9° (*c* 1 1, chloroform)

Anal Calc for $C_{14}H_{21}NO_8$ C, 50 77, H, 6 39, N, 4 23 Found C, 50 58, H, 6 65, N, 4 00

Isolation of N-*acetyl-α-L-rhamnofuranosylamine* (**12**) — The fractions (that did not crystallize) from the column chromatography described for the isolation of **10** were evaporated, and the resulting syrup (2 5 g) was rechromatographed on a column (60 × 2 5 cm) of cellulose, elution was conducted with solvent *B*, and 50 fractions (50 ml each) were collected Fractions 29–35 gave needles (340 mg, 4 9%) of m p 134–

135°, which, after three recrystallizations from butyl alcohol, gave **12**, m p 135–136°, $[\alpha]_D$ −122.1° (*c* 1 0, water)

Anal Calc for $C_8H_{15}NO_5$ C, 46 82, H, 7 31; N, 6 87 Found. C, 46 88, H, 7 56, N, 6 62

On oxidation with metaperiodate, this compound consumed 0 90 mole of periodate per mole and did not produce formic acid after 24 h, indicating a furanose structure

ACKNOWLEDGMENTS

The authors are indebted to Dr G E McCasland (San Francisco University) for his assistance in the determination of the 100-MHz spectra

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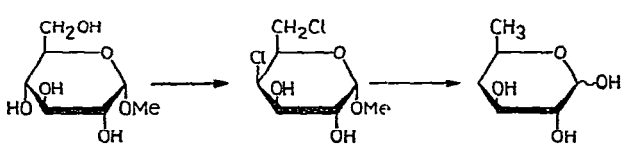
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Note

A facile synthesis of 4,6-dideoxy-D-*xylo*-hexose*

B T. LAWTON**, W A SZAREK, AND J K N JONES
Department of Chemistry, Queen's University, Kingston, Ontario (Canada)
(Received December 30th, 1969)

4,6-Dideoxy-D-*xylo*-hexose (**3**) is of some interest because of its occurrence as the 3-methyl ether in the antibiotics chalcomycin[2] and lankamycin[3] A number of syntheses of this dideoxy sugar have been described The first reported[4] synthesis was from methyl 2,3-di-*O*-benzoyl-4,6-di-*O*-mesyl-α-D-glucopyranoside by a replacement reaction with potassium thiocyanate in *N,N*-dimethylformamide to give the 4,6-dideoxy-4,6-dithiocyanato-D-galactoside, followed by desulfurization with Raney nickel and removal of the substituents, the free dideoxy sugar was obtained but only in 11% overall yield Another synthesis in low yield was achieved[5] by a multi-step route, which involved the preparation of methyl 4-deoxy-α-D-*xylo*-hexopyranoside by the cleavage of the epoxide ring in methyl 3,4-anhydro-α-D-galactoside with hydrogen in the presence of Raney nickel More recently, Čapek *et al* [6] have used methyl 3,4-anhydro-6-deoxy-α-D-galactopyranoside to prepare methyl 4,6-dideoxy-α-D-*xylo*-hexopyranoside Siewert and Westphal[7] have obtained a higher yield of compound **3** from methyl 2,3-di-*O*-benzoyl-4,6-di-*O*-*p*-tolylsulfonyl-α-D-glucoside by sequential treatment with sodium iodide in boiling *N,N*-dimethylformamide, catalytic hydrogenation, de-*O*-esterification, and acid hydrolysis The present report describes a synthesis of 4,6-dideoxy-D-*xylo*-hexose, in an overall yield of 65% from the commercially available methyl α-D-glucopyranoside (**1**), by a route that is considerably more facile than those employed by other workers This work demonstrates further the importance of chlorodeoxy sugars as valuable intermediates in the synthesis of other sugar derivatives[1,8]



1 2 3

*Part II in the series "Synthesis and Reactions of Chlorodeoxy Sugars" For Part I, see ref 1
**Present address Department of Chemistry, The University of Edinburgh, Edinburgh, Great Britain

Treatment of methyl α-D-glucopyranoside (1) with sulfuryl chloride and pyridine in chloroform solution by the method of Jennings and Jones[9,10] gave methyl 4,6-dichloro-4,6-dideoxy-α-D-galactopyranoside 2,3-dichlorosulfate, which was dechlorosulfated using sodium iodide to afford a high yield of methyl 4,6-dichloro-4,6-dideoxy-α-D-galactopyranoside[10,11] (2) Hydrogenation of compound 2, in the presence of potassium hydroxide, over a W-4 Raney nickel catalyst, prepared by the procedure described by Pavlic and Adkins[1,12], gave a chromatographically homogeneous syrup, which crystallized after distillation* Recrystallization of the product from ethyl acetate–petroleum ether gave long needles, the specific rotation (+172° in methanol) was close to the values recorded for methyl 4,6-dideoxy-α-D-*xylo*-hexopyranoside [+171° (ref 5), +176° (ref 6), +182° (ref 7) in methanol]. but the melting point (75°) was considerably lower [103–104° (ref 5), 108 5–109 5° (ref 6), 104–106° (ref 7)] A different crystalline form (prisms), however, was obtained by recrystallization from ethyl acetate. the melting point (107–109°) was now close to the published values Acid-catalyzed hydrolysis of the two types of crystals gave in each case the same product, 4,6-dideoxy-D-*xylo*-hexose (3), whose physical constants were in agreement with those reported by other workers for the sugar

In the present work a second high-yielding, but less convenient, synthesis of methyl 4,6-dideoxy-α-D-*xylo*-hexopyranoside by way of a chlorodeoxy sugar was also achieved The method involves an application of the reaction of alcohols with triphenylphosphine in carbon tetrachloride at reflux temperature to give the corresponding chloro derivatives[13] Thus, treatment of methyl 2 3-di-*O*-benzyl-α-D-glucopyranoside[14] under these conditions gave a syrupy product, which was presumably methyl 2,3-di-*O*-benzyl-4,6-dichloro-4,6-dideoxy-α-D-galactopyranoside** Reduction of this material with the active W-4 Raney nickel catalyst[1,12] afforded methyl 4,6-dideoxy-α-D-*xylo*-hexopyranoside

EXPERIMENTAL

Melting points were determined with a Fisher–Johns melting point apparatus and are uncorrected Optical rotations were measured with a Perkin–Elmer Model 141 automatic polarimeter at 23 ±2° Thin-layer chromatography (t l c) was performed with Silica Gel G as the adsorbent, and 9 1 (v/v) chloroform–methanol as the developing solvent The developed plates were air-dried, sprayed with 5% ethanolic sulfuric acid, and heated at about 150°

Methyl 4,6-dichloro-4,6-dideoxy-α-D-galactopyranoside (2) — Methyl 4,6-dichloro-4,6-dideoxy-α-D-galactopyranoside 2,3-dichlorosulfate (78 g) was prepared

*Occasionally crystallization was difficult because of the formation of side-products, when the reduction was performed on a scale much larger than that described in the Experimental section

**It has been shown[15] that, at least in one case, the attachment of the chloro group onto a secondary carbon atom occurs with inversion of configuration without any effect on the neighboring carbon atoms, methyl 2,3-di-*O*-methyl-α-D-glucopyranoside gave methyl 4,6-dichloro-4,6-dideoxy-2,3-di-*O*-methyl-α-D-galactopyranoside in almost quantitative yield

from methyl α-D-glucopyranoside (**1**, 40 g) by the method of Jennings and Jones[9, 10], and dechlorosulfated by using sodium iodide essentially according to their method, except that sodium hydrogen carbonate was used for neutralization and the filtered solution was then concentrated to dryness The residue was extracted with hot chloroform (3 × 200 ml), and the dried ($MgSO_4$) extracts were concentrated to give a crystalline product that was recrystallized from chloroform–petroleum ether (b p 40–60°); yield 36 g (76% based on **1**). The physical constants were in agreement with those reported previously[10, 11] for compound **2**

4,6-Dideoxy-D-xylo-*hexose* (**3**) — A solution of compound **2** (4 62 g) in absolute ethanol (50 ml) containing potassium hydroxide (4 4 g) and W-4 Raney nickel catalyst[1, 12] (10 g) was subjected to a hydrogen pressure of 45 lb in^{-2} for 4 h T l c showed that the starting material had all reacted The solution was filtered free of catalyst, and the filtrate was neutralized with M hydrochloric acid and concentrated to dryness The residue was extracted with hot chloroform (4 × 50 ml), and the dried ($MgSO_4$) extracts were concentrated to give a syrup [yield 3 g (91%)], which was distilled at 120° (bath)/0 1 torr, the distillate crystallized on standing Recrystallization from ethyl acetate–petroleum ether (b p 60–80°) gave methyl 4,6-dideoxy-α-D-*xylo*-hexopyranoside as long needles, m p 70–75°, $[\alpha]_D$ +172° (*c* 1 0, methanol), recrystallization from ethyl acetate gave the compound as prisms, m p 107–109°, $[\alpha]_D$ +171° (*c* 1 0, methanol) Lit , m p 103–104° (ref 5), 108 5–109 5° (ref 6), 104–106° (ref 7), $[\alpha]_D$ +171° (ref 5), +176° (ref 6), +182° (ref 7) (methanol)

Methyl 4,6-dideoxy-α-D-*xylo*-hexopyranoside (1 76 g) in water (20 ml) containing Rexyn-101 resin (H^+) (1 g) was heated for 4 h at reflux temperature T l c showed the presence of only one component The resin was removed and the solution was concentrated to a syrup, yield 1 6 g (99%) Crystallization from ethanol–petroleum ether (b p 60–80°) gave compound **3** as needles, m p 137–138°. $[\alpha]_D$ + 78° (3 min)→32 5 (30 min, constant value) (*c* 1 03, water) The physical constants are in agreement with those published for 4,6-dideoxy-D-*xylo*-hexose by other workers[4, 7]

Reaction of methyl 2,3-di-O-*benzyl*-α-D-*glucopyranoside with triphenylphosphine in carbon tetrachloride* — To a solution of methyl 2,3-di-*O*-benzyl-α-D-glucopyranoside[14] (20 mmoles) in carbon tetrachloride (250 ml) was added triphenylphosphine (40 mmoles), and the mixture was heated for 36 h at reflux temperature The reaction mixture was cooled and filtered, and the filtrate was concentrated to dryness The residue was extracted with ligroin, and the extracts were concentrated to leave a yellow syrup that could not be induced to crystallize. Hydrogenation over W-4 Raney nickel catalyst in abs ethanol containing potassium hydroxide for 16 h at a pressure of 45 lb in^{-2} gave methyl 4,6-dideoxy-α-D-*xylo*-hexopyranoside, having the same physical constants as those obtained above for this compound, yield 85%

ACKNOWLEDGMENT

The authors thank the National Research Council of Canada for financial support of this work

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Note

Infrared absorption spectra of aldobiouronic acids obtained from natural plant gums and mucilages

K S BAJPAI, V CHANDRASEKHARAN, S MUKHERJEE, AND A N SHRIVASTAVA
Department of Organic Chemistry, National Sugar Institute, Kanpur (India)
(Received November 13th, 1969, in revised form, January 12th, 1970)

Certain studies of the i r spectra of carbohydrates[1,2] have been mainly confined to the frequency range 960–730 cm^{-1}, and these studies are of value in establishing the anomeric configuration In this frequency range, three types of absorption (1, 2, and 3) may be noted Type 1 and 3 absorptions arise from the ring-stretching and ring-breathing vibrations, respectively Type 2 absorptions (2a and 2b) are due to the anomeric C—H deformation mode The α and β anomers of D-sugars differ in that, for some of them, the α-D-anomeric hydrogen atom is equatorially attached, whereas the β-D-anomeric hydrogen atom is axially attached (based on the assumption that the particular chair conformation adopted is that in which as many non-hydrogen substituents as possible are equatorial[3]) The type 2a absorption, characteristic of some α anomers (glucose 843 ± 4 cm^{-1}, galactose 825 ± 11 cm^{-1}) is observed in their monosaccharide derivatives as well as in those disaccharides where at least one of these two sugar moieties has the α configuration[4] Type 2b absorption of certain β anomers occurs at ~ 890 cm^{-1}. The presence of such a band is not conclusive evidence for a β anomer as some of the type 1 absorptions of α sugars also lie in this range Type 2c absorption has been observed for α and β-D-galactopyranose and α- and β-D-mannopyranose at about 870 cm^{-1}, this band has been assigned to the deformations of the C–H bonds at C-4 and C-2 in galactopyranose and mannopyranose, respectively An investigation has now been made of the i r spectra of aldobiouronic acids, in order to find out whether any band assignment could be made for the α or β linkage between the uronic acid residue and the glycosyl group

The aldobiouronic acids were isolated from natural, plant gums and mucilages by hydrolysing the pure polysaccharide with M sulphuric acid, the conditions of hydrolysis being so adjusted as to afford the maximum yield of the aldobiouronic acid[5] The mixture of sugar acids was isolated from the hydrolyzate as the barium salts, and was resolved by cellulose-column chromatography[5] into pure, single ingredients The aldobiouronic acid fractions, identified by paper chromatography, were mixed and evaporated under diminished pressure at 40–45° After removal of traces of acetic acid over potassium hydroxide pellets, the residue was dissolved in water, and the solution was made neutral (barium hydroxide), and evaporated to a

TABLE I

I.R. BANDS OF BARIUM SALTS OF URONIC AND ALDOBIOURONIC ACIDS IN THE REGIONS 600–1400 AND 960–730 cm^{-1}

Barium salt of uronic acid	*Frequency* (cm^{-1})[a]						
2-*O*-(α-D-Galactopyranosyluronic acid) L rhamnose	1588 s	1450 s	950 vw	918 vw		810 m	
4-*O*-(α-D-Galactopyranosyluronic acid) D galactose	1589 vs	1415 s	955 vw		885 w	820 m	
4-*O*-(β-D-Glucopyranosyluronic acid)-D-galactose	1585 s	1455 vs	940 vw				
6-*O*-(β-D-Glucopyranosyluronic acid)-D-galactose	1588 s	1455 s	940 w				
D-Galacturonic acid	1590 vs	1415 s		915 w	885 w		773 w
D-Glucuronic acid	1578 vs	1430 s		937 w			790–775 vw

[a]The relative intensities of peaks are indicated by m, medium, s, strong, v, very, w, weak

thin syrup This was poured into boiling methanol to precipitate the barium salt of the aldobiouronic acid The latter was isolated in an amorphous form by refluxing with dry methanol, and was found to be chromatographically pure The i.r. spectra of the barium salts of the aldobiouronic acids were recorded with a Perkin–Elmer model 521 Infracord i r spectrophotometer. The spectra of the barium salts of the corresponding uronic acids were also recorded for comparison

All of the spectra (see Table I) showed two distinct bands at 1590–1575 and 1460–1410 cm^{-1}, corresponding to the anti-symmetrical and symmetrical stretching vibrations of the carboxylate ion[6], the absorption bands reported for the symmetrical vibrations of organic acid salts are generally in a lower frequency range (1400–1300 cm^{-1}) In the spectra of aldobiouronic acids in the region 960–730 cm^{-1}, many of the bands characteristic of monosaccharides and dissaccharides are either absent or quite weak An aldobiouronic acid can be considered to be a disaccharide in which one of the moieties is a uronic acid, and the linkage can be either α or β The band at 871 cm^{-1}, characteristic of the 2c absorption in α- and β-(D and L)-galactose and their derivatives, was not observed in the spectra of any of the aldobiouronic acids having the *galacto* configuration either for the uronic acid or the sugar moiety

However, a distinct band of type 2a was observed in the spectrum of 2-*O*-(α-D-galactopyranosyluronic acid)-L-rhamnose and 4-*O*-(α-D-galactopyranosyluronic acid)-D-galactose at 810 and 820 cm^{-1}, respectively Both the α-acids have D-galacturonic acid as the uronic acid part Although this absorption is very close to the type 2a absorption for α-D-galactose and its derivatives (825 ± 11 cm^{-1}), assignment as α-D may not be correct, as many of the characteristic absorptions of the sugars are not clearly seen in the spectra of the aldobiouronic acids. It is possible that this absorption may arise from an α-linkage between the uronic acid and the sugar moieties The absence of the 2c absorption characteristic of the equatorial C-4–H bond of galactopyranose can be explained by assuming hydrogen-bond formation between the axial hydroxyl group on C-4 and the carboxyl group of α-D-galactopyranosyluronic acid. Hydrogen bonds of this type are likely to be very strong, and could affect the other bonds responsible for characteristic vibrations Thus, the replacement of the $-CH_2OH$ group by the $-CO_2H$ group brings about large changes in the spectra of the sugars.

ACKNOWLEDGMENT

The authors express their sincere thanks to Professor C N R Rao, of the Indian Institute of Technology, Kanpur, for valuable discussions, and to Shri S C Gupta, Director of the Institute, for his kind interest in this investigation. This work was supported by the U. S Department of Agriculture from P L 480 funds, Grant No FG-In-262

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Note

Acetylated aldosyl chlorides by reaction of aldose peracetates with zinc chloride–thionyl chloride *

L P EGAN, T G SQUIRES, AND J R VERCELLOTTI**
Department of Chemistry, University of Tennessee, Knoxville, Tennessee 37916 (U S A)
(Received November 24th, 1969, in revised form, January 23rd, 1970)

We report a new procedure for the synthesis of aldosyl chlorides and aldosyl chlorides of oligosaccharides from the corresponding peracetylated sugars by the use of zinc chloride–thionyl chloride Previously 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl chloride was prepared by use of this reagent[1].

The reactions proceeded smoothly in all cases, usually reaching completion in about 2 h The crude yields of crystalline product were generally 60–85% and by t l c were adequately pure for most purposes without further recrystallization (see Table I)

2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl chloride proved difficult to purify, requiring at least three careful recrystallizations from ethyl ether–petroleum ether to afford a product having physical properties similar to those in the literature[2]. Microanalysis of this compound gave the calculated values, and the m p agreed with the value (74–75°) given (see Table I) by Fischer and Armstrong[3], but the data did not agree with either the melting point (82°) nor the rotation given by Skraup and Kremann[4]. For this chloride Korytnyk and Mills[7] reported m p 78–79° and $[\alpha]^{25}$ +175 1° The optical rotation of +195 5° reported in Table I is the value obtained for several preparations after repeated recrystallizations

Chemical-shift data for the first crop of crystalline product from reaction mixtures showed in each case that only the α-D anomer was formed during chlorination, and the peracetylated starting material disappeared completely All structural assignments in the present investigation were confirmed by n m r spectroscopy. Chemical shifts and coupling constants for the acetylated glycosyl chlorides were in accord with values previously reported

Table I compares results from the chlorination of 1,2,3,4,6-penta-*O*-acetyl-α-D-glucopyranose and 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose A similar yield of the corresponding α-D chloride was formed from both anomeric acetates The

*This study was supported by the Agricultural Research Service, U S Department of Agriculture, Grant 12-14-100-9208 (71), administered by the Northern Utilization Research and Development Division, Peoria, Illinois 61640, U S A
**To whom inquiries should be directed

TABLE I

ACETYLATED GLYCOSYL CHLORIDES PREPARED BY REACTION WITH ZINC CHLORIDE–THIONYL CHLORIDE

Anomeric configuration of starting peracetate	*Product*[a]	*Crude yield*[b] (%)	T l c *solvent*[c]	*M p*[b] *(degrees)*		$[\alpha]^{20-25}$ *(degrees, $CHCl_3$)*		*Ref*
				Found	*Lit*	*Found*	*Lit*	
α-D	2,3,4,6-Tetra-*O*-acetyl-α D-glucopyranosyl chloride	85	B	72–73 5	76	+164	+166	10
β-D	2,3,4,6-Tetra-*O*-acetyl-α D-glucopyranosyl chloride	89	B	70 5–72	76	+165	+166	10
β-D	2,3,4,6-Tetra-*O*-acetyl α D-mannopyranosyl chloride	80	B	77–78	81	+89	+90 6	11
β-D	2,3,4,6-Tetra-*O*-acetyl-α D-galactopyranosyl chloride	89	A	75–76	76	+195 5	+212.3 +177 5	4 5
β-D	2,3,4-Tri-*O*-acetyl-α-D-xylopyranosyl chloride	81	B	100–101	105	+169 8 +205 (C_6H_6)	+171 2	10
β-D	Methyl 2,3,4-tri-*O*-acetyl-1-chloro-1-deoxy-α-D glucuronate	66	B	100 5–101 5	99–100 98 5–100	+160 7 +200 3 (C_3H_3)	+168 7 +162	12 13
α-D	Hepta-*O*-acetyl-α-lactosyl chloride	78 ($CHCl_3$–Et_2O)	C	118–120 ($CHCl_3$–Et_2O)	120–121	+84 2	+83 9	11

[a]Elementary analyses for C, H, and Cl gave values within ±0 2% of calculated values [b]Crystallized from ethyl ether–petroleum ether (b p 30–60°) unless otherwise noted [c]See introduction to experimental section

anomeric configurations of the sugar peracetates used (Table I) did not affect the yield of α-D glycosyl chlorides produced This observation differs from the experience of other investigators[2,5] who used titanium tetrachloride.

An attempt was made to prepare 2,3 5,6-di-*O*-isopropylidene-α-D-mannofuranosyl chloride from 2,3 5,6-di-*O*-isopropylidene-α-D-mannofuranose[6] by using zinc chloride–thionyl chloride N m r spectroscopy indicated the loss of one isopropylidene group and the formation of the glycosyl halide[7] 1,2,3,4,6-Penta-*O*-benzoyl-β-D-glucopyranose, when treated with zinc chloride–thionyl chloride, gave only crystalline starting material Other halogenating agents are better suited for the formation of perbenzoylated glycosyl chlorides[8]

These reactions of zinc chloride–thionyl chloride apparently proceed by a mechanism similar to the action of other Lewis acids and halogenating agents with sugar peracetates[2,5,9]

EXPERIMENTAL

General — Ascending t.l c was performed on Silica Gel G (E Merck Co , Darmstadt, Germany, activated for 1 h at 110°) Location of zones was achieved by charring after spraying with a 35% solution of ammonium sulfate. Solvents for t l c were 3 1 petroleum ether (b p 30–60°)–ethyl acetate (solvent *A*); 2 1 petroleum ether (b p 30–60°)–ethyl acetate (solvent *B*), and 1 1 petroleum ether (b p 30–60°)–ethyl acetate (solvent *C*) Microanalyses were performed by Galbraith Laboratories, Knoxville, Tennessee 37921, U S A

The crude, crystalline products (Table I) showed only traces of starting material and inorganic salts by t l c After one recrystallization from ether–petroleum ether (b p 30–60°) all products were chromatographically homogeneous Physical data recorded in Table I are for substances recrystallized several times until constant values were obtained.

General procedure for preparation of acetylated glycosyl chlorides — Preparations of all acetylated glycosyl chlorides were carried out in the same manner as here described for 2,3,4-tri-*O*-acetyl-α-D-xylopyranosyl chloride To a solution of tetra-*O*-acetyl-β-D-xylose (5 00 g, 15 8 mmoles) in benzene (40 ml) was added thionyl chloride (2 3 ml, 32 mmoles) followed by freshly fused zinc chloride (0 80 g, 5 9 mmoles) The mixture was stirred at room temperature T l c (solvent *B*) showed conversion of tetra-*O*-acetyl-β-D-xylose (R_F 0 32) into 2,3,4-tri-*O*-acetyl-α-D-xylopyranosyl chloride (R_F 0 59) within 2 h, whereupon the mixture was filtered by suction through Celite (2 cm) on a sintered-glass funnel The filtrate and benzene washings were evaporated to an oil, and then toluene (70 ml total) was evaporated three times at 65° from the oil to remove the last traces of thionyl chloride The resulting syrup was crystallized from ethyl ether–petroleum ether (b p 30–60°) to give 3 75 g (81%) of 2,3,4-tri-*O*-acetyl-α-D-xylopyranosyl chloride, m.p 89–96°, raised to 100–101° after 3 recrystallizations

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Carbohyd Res, 14 (1970) 263–266

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Note

Sugars with reactive substituents · Preparation of several epoxypropyl-derivatized sugars

R E WING, W M DOANE, AND C E RIST

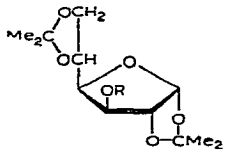
Northern Regional Research Laboratory, Northern Utilization Research and Development Division, Agricultural Research Service, U S Department of Agriculture, Peoria, Illinois 61604 (U S A)

(Received January 2nd, 1970)

Recently we described the preparation[1] and some reactions[2] of 3-*O*-(2,3 epoxypropyl)-1,2 5,6-di-*O*-isopropylidene-α-D-glucofuranose Another sugar derivative having pendent oxirane groups, 1,6-anhydro-2,3,4-tri-*O*-(2,3-epoxypropyl)-β-D-glucopyranose, was patented by Carlberg and Shafizadeh[3] Now we have made and characterized several new sugar derivatives, which contain one, two, three, or four epoxypropyl ether groups The multifunctional epoxide derivatives were prepared to assess their potential for use as reactive intermediates in producing crosslinked polysaccharides.

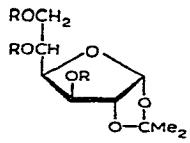
The reaction of 3-*O*-allyl-1,2 5,6-di-*O*-isopropylidene-α-D-glucofuranose[4] (**1**) with *m*-chloroperoxybenzoic acid gave 3-*O*-(2,3-epoxypropyl)-1,2 5,6-di-*O*-isopropylidene-α-D-glucofuranose[1] (**2**) in quantitative yield When **1** was treated with dilute hydrochloric acid at room temperature, 3-*O*-allyl-1,2-*O*-isopropylidene-α-D-glucofuranose[5] (**3**) was obtained in quantitative yield Epoxidation of **3** with *m*-chloroperoxybenzoic acid or benzonitrile–50% hydrogen peroxide resulted in 3-*O*-(2,3-epoxypropyl)-1,2-*O*-isopropylidene-α-D-glucofuranose (**4**). Allylation of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (**5**) gave the corresponding 2,3-di-*O*-allyl derivative[6] (**6**), which was epoxidized to yield methyl 4,6-*O*-benzylidene-2,3-di-*O*-(2,3-epoxypropyl)-α-D-glucopyranoside (**7**) as a solid Treatment of 1,2-*O*-isopropylidene-α-D-glucofuranose[7] (**8**) with sodium hydroxide–allyl bromide in *p*-dioxane gave the 3,5,6-tri-*O*-allyl derivative[8] (**9**), which was epoxidized to 3,5,6-tri-*O*-(2,3-epoxypropyl)-1,2-*O*-isopropylidene-α-D-glucofurnose (**10**) When methyl 2,3,4,6-tetra-*O*-allyl-α-D-glucopyranoside[6] (**11**) was treated with *m*-chloroperoxybenzoic acid, methyl 2,3,4,6-tetra-*O*-(2,3-epoxypropyl)-α-D-glucopyranoside (**12**) was obtained as a syrup

The epoxidation of a vinyl ether of a carbohydrate was attempted with little success Treatment of 1,2 5,6-di-*O*-isopropylidene-3-*O*-vinyl-α-D-glucofuranose[9] (**13**) with *m*-chloroperoxybenzoic acid gave 1,2 5,6-di-*O*-isopropylidene-α-D-glucofuranose (**14**) as the only sugar product. When benzonitrile–50% hydrogen peroxide was used as oxidant, a minor component was detected together with **14** The R_F value of the



1 R = $CH_2CH{=}CH_2$ 13 R = $CH_2{=}CH_2$

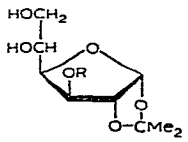
2 R = $CH_2CH\overset{O}{\frown}CH_2$ 14 R = H



8 R = H

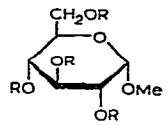
9 R = $CH_2CH{=}CH_2$

10 R = $CH_2CH\overset{O}{\frown}CH_2$



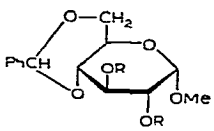
3 R = $CH_2CH{=}CH_2$

4 R = $CH_2CH\overset{O}{\frown}CH_2$



11 R = $CH_2CH{=}CH_2$

12 R = $CH_2CH\overset{O}{\frown}CH_2$



5 R = H

6 R = $CH_2CH{=}CH_2$

7 R = $CH_2CH\overset{O}{\frown}CH_2$

minor component when compared with **13** and **14** suggested that it was the corresponding oxirane The i r. spectrum of the syrupy product showed two weak bands for oxirane Based on the work of Stevens and coworkers[10–13] it might be expected that epoxidation of **13** under our conditions would give the oxirane, which would decompose into **14.**

Compounds **2, 4, 10,** and **12** were recovered as syrups, which failed to crystallize Compound **7** was obtained as a crystalline product Each was homogeneous by t l c and gave elemental analysis and spectral data consistent with their proposed structures The epoxypropyl derivatives were soluble and stable in most organic solvents

EXPERIMENTAL

Optical rotations were determined with a Model 80 Rudolph* Polarimeter in 1-dm tubes Melting points were recorded on a Fisher–Johns melting-point apparatus 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, New York) with a 2 1 hexane–acetone irrigant[1 2, 14] The plates were developed by spraying with a solution of 20% sulfuric acid in ethanol and

*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

heating then for 20 min at 140°. Colored solutions were decolorized with Darco G-60 carbon

3-O-(2,3-Epoxypropyl)-1,2-O-isopropylidene-α-D-glucofuranose (4) — 3-*O*-Allyl-1,2-*O*-isopropylidene-α-D-glucofuranose[5] (**3**) (1 5 g, R_F 0 16) was dissolved in benzene (50 ml) and *m*-chloroperoxybenzoic acid (1 02 g, 85% pure) was added After the reaction mixture had been stirred for 6 h at room temperature, t l c. showed approximately 60% conversion of **3** to **4**. An additional 1 0 g of *m*-chloroperoxybenzoic acid was added and after a total of 24 h the reaction was complete The mixture was evaporated to 10 ml under diminished pressure at room temperature and then placed on a silicic acid column to remove the acid. The product was eluted from the column with acetone and yielded **4** as a pure syrup (1 59 g, 100%)

Compound **4** was also formed by treating **3** (1 5 g, R_F 0 16) with benzonitrile (0 80 ml), 50% hydrogen peroxide (0 51 ml), and potassium hydrogen carbonate (0 15 g) in absolute methanol (5 ml) After 24 h, t l c showed a 95% conversion of **3** to **4** The addition of more reagents failed to increase the yield Evaporation of the reaction mixture under diminished pressure at room temperature, followed by the addition of benzene (10 ml) and separation by column chromatography as described above, yielded **4** as a pure syrup (1 41 g, 89%)

Analysis of **4** The i r spectrum showed absorption at 3550 cm^{-1} (OH) and at 840, 915, and 1250 cm^{-1} (oxirane) $[\alpha]_D^{20}$ −32° (*c* 1, $CHCl_3$), R_F 0 06

Anal Calc for $C_{12}H_{20}O_7$ C, 52 17, H, 7 24 Found C, 52 31, H, 7 42

Methyl 4,6-O-benzylidene-2,3-di-O-(2,3-epoxypropyl)-α-D-glucopyranoside (7) — Methyl 2,3-di-*O*-allyl-4,6-*O*-benzylidene-α-D-glucopyranoside[6] (**6**) (7 7 g, R_F 0 61) was dissolved in benzene (200 ml) and *m*-chloroperoxybenzoic acid (9 0 g, 85% pure) was added After the reaction mixture had been stirred for 24 h at room temperature, t l c showed about a 90% conversion of **6** to **7** An additional 2 0 g of *m*-chloroperoxybenzoic acid was added, and after a total of 48 h the reaction was complete The reaction mixture was filtered and the filtrate was washed with several 200-ml portions of a saturated sodium hydrogen carbonate solution The benzene solution was dried with sodium sulfate and evaporated under diminished pressure The remaining traces of the acid were removed by column chromatography to yield **7** The syrup was crystallized and recrystallized from hexane to afford **7** (8 0 g, 95%)

Analysis of **7** The i r. spectrum of **7** showed no absorption at either 3500 cm^{-1} (OH) or at 1650 cm^{-1} (C=C), but several absorptions for oxirane were present at 825, 858, 910, and 1250 cm^{-1} M p 91–92°, $[\alpha]_D^{20}$ +57° (*c* 1, $CHCl_3$), R_F 0 26

Anal Calc for $C_{20}H_{26}O_8$ C, 60 91, H, 6.60 Found C, 61 16, H, 6 60.

3,5,6-Tri-O-(2,3-epoxypropyl)-1,2-O isopropylidene-α-D-glucofuranose (10). — 3,5,6-Tri-*O*-allyl-1,2-*O*-isopropylidene-α-D-glucofuranose[8] (**9**) (1 8 g, R_F 0 75) was dissolved in benzene (200 ml) and *m*-chloroperoxybenzoic acid (6 0 g, 85% pure) was added After the reaction mixture had been stirred for 24 h at room temperature, t l c showed complete conversion of **9** to **10** Workup was completed as described in the preceding section to yield **10** as a pure syrup (2 09 g, 100%)

Analysis of **10**. The i r spectrum of **10** showed no absorption at either 3550 cm^{-1}

(OH) or at 1650 cm^{-1} (C=C), but several absorptions for oxirane were present at 805, 855, 910, and 1250 cm^{-1} $[\alpha]_D^{20}$ −16° (*c* 1, $CHCl_3$), R_F 0 23.

Anal Calc for C_{18} H_{28} O_9 · C, 55 67; H, 7 22 Found C, 55 62; H, 7 54

Methyl 2,3,4,6 tetra-O-(*2,3-epoxypropyl*) α-D-*glucopyranoside* (**12**). — Methyl 2,3,4,6-tetra-*O*-allyl-α-D-glucopyranoside[6] (**11**) (2.3 g, R_F 0 75) was dissolved in benzene (200 ml) and *m*-chloroperoxybenzoic acid (7 5 g, 85% pure) was added After the reaction mixture had been stirred for 24 h at room temperature, t l c showed about a 60% conversion of **11** to **12** Several 2 0-g portions of *m*-chloroperoxybenzoic acid were added on subsequent days, but only an 80% conversion could be obtained. Workup was completed as previously described to yield **12** as a pure syrup (2.18 g, 80%).

Analysis of **12** The i r spectrum of **12** showed no absorption at either 3550 cm^{-1} (OH) or at 1650 cm^{-1} (C=C), but several absorptions for oxirane were present at 810, 860, 910, and 1255 cm^{-1}. $[\alpha]_D^{20}$ +84° (*c* 1, $CHCl_3$), R_F 0.09

Anal. Calc for C_{19} $H_{30}O_{10}$ C, 54 54; H, 7 18 Found C, 54.43; H, 7 30

Epoxidation of 1,2 5,6-di-O-*isopropylidene-3*-O-*vinyl*-α-D-*glucofuranose*[9] (**13**) — Compound **13** (1.6 g, R_F 0 70) was dissolved in benzene (100 ml) and *m*-chloroperoxybenzoic acid (1.1 g, 85% pure) was added After the reaction mixture had been stirred overnight at room temperature, t l c. showed complete reaction Workup was completed as previously described to yield a solid, m p. and mixed m p with **14** 108–109°, R_F 0.41.

The experiment was repeated with a different oxidant Compound **13** (0 48 g, R_F 0.70) was dissolved in methanol (3 ml) and potassium hydrogen carbonate (0.1 g), benzonitrile (0 5 ml), and 50% hydrogen peroxide (0 34 ml) were added After the reaction mixture had been stirred overnight at room temperature, t l c showed complete reaction Workup was completed as previously described for **4** to yield a syrup T l c analysis showed **14** (80%, R_F 0 41) and another component (20%, R_F 0 37) which was assumed to be the oxirane The i r spectrum showed no absorption at 1655 and 1645 cm^{-1} (vinyl), but weak absorption for oxirane was present at 925 and 795 cm^{-1}.

ACKNOWLEDGMENTS

We are grateful to Mrs Bonita Heaton and Mrs Karen Jones for the microanalyses.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

Note

The isolation and characterization of hyaluronic acid from *Pasteurella multocida*

J A CIFONELLI,

Departments of Pediatrics and Biochemistry, La Rabida-University of Chicago Institute, and the Joseph P Kennedy, Jr Mental Retardation Center, University of Chicago, Chicago, Illinois 60637 (U S A)

P A REBERS,

Chemical and Physical Investigations, National Animal Disease Laboratory A D P, United States Department of Agriculture Ames, Iowa 50201 (U S A)

AND K H HEDDLESTON

Bacterial and Mycotic Investigations, National Animal Disease Laboratory A D P, United States Department of Agriculture, Ames, Iowa 50201 (U S A)

(Received February 6th, 1970)

The pathogenicity and destructiveness of *Pasteurella multocida* in various disease conditions of domestic animals and fowl has been of economic concern for many years and has led to extensive studies aimed at the elucidation of substances related to this pathogenicity[1–5] Four types of *P multocida* have been designated by Carter[1]; of these only one, type A, has been reported to possess capsules which possibly contain hyaluronic acid Evidence for this suggestion was based primarily on the observation that staphylococcal hyaluronidase removed capsules from growing cells[1], an action cited by Carter and Annau[2] to be similar to that occurring with the capsules of streptococcal cells

Soluble capsular products were isolated which also were affected by hyaluronidase, as indicated by turbidity reduction with acid albumen[6] It was noted that, after hydrolysis with acid, the soluble material produced reducing substances However, analytical data establishing the presence of hyaluronic acid were not given, and the relation of the soluble capsular products to hyaluronic acid from other sources was uncertain.

In the present study, a highly purified, electrophoretically homogeneous polysaccharide preparation was isolated from *P. multocida* (type A, Strain X-73, ATCC 11039), a highly virulent strain associated with fowl cholera[3] Chemical characterisation of this product indicated its identity with hyaluronic acid of streptococcal origin (Group A, type 18, Strain A III)[7] *P multocida* cells were grown in Adams–Roe medium[8], with or without the extra addition of sodium hydrogen carbonate Seed cultures were obtained by incubating (24 h, 37°) lyophilized cells on plates containing dextrose–starch agar (Difco) Subsequently isolated colonies were grown in tubes containing Adams–Roe media[7], except that thioglycollate and sodium

hydrogen carbonate were omitted After 24 h the content of a single tube was added to 3 liters of Adams–Roe medium, and this was incubated under the same conditions. After addition of 9 ml of formalin (37%), the cells were recovered by centrifugation (RC-2 Servall) at 9000 r p m for 1 h The cells were suspended in 0 85% sodium chloride containing 0 3% formalin, and the suspension was kept in a refrigerator. The dry weight of cells produced from 3 liters of medium was 2 5 g for cultures containing sodium hydrogen carbonate and 1 6 g for those grown without this addition

Isolation of the polysaccharide was accomplished by the following method The cells from a 3-liter culture were suspended in 50 ml of a buffer solution (pH 5 0) containing 0.15M sodium chloride and 0 10M sodium acetate Crystalline papain (20 mg), Versene (30 mg), and cysteine hydrochloride (10 mg) were added, and the mixture was incubated with stirring for 18 h at 60–65° Centrifugation at 10,000 *g* for 30 min provided a clear, yellow supernatant solution The sediment was found to contain an additional amount of hyaluronic acid which was recovered as indicated later

The supernatant solution was concentrated to 10 ml *in vacuo*, and 2 volumes of ethanol were added After separation, this crude preparation weighed approximately 200 mg No significant differences between the yields of polysaccharide obtained from cells grown with or without sodium hydrogen carbonate were noted

The crude material was heavily contaminated with protein (Table I) and with nucleic acid, as indicated by the u.v. spectrum, and it was fractionated by precipitation with cetylpyridinium chloride in 0 1M sodium chloride[9]. Final purification was performed by precipitation of the mucopolysaccharide with an alkaline copper solution, as described for the isolation of dermatan sulfate[10]. The yield of purified material obtained after this step was 60–70 mg An additional 20–30 mg of copper-precipitated polysaccharide was recovered after alkaline extraction of the cellular sediment. The total yield of hyaluronic acid was up to 4–5% of the dry weight of *P multocida* On some occasions, much smaller yields than those reported here were obtained, and in such instances varying amounts of polysaccharides containing neutral sugars were isolated, in addition to hyaluronic acid No hyaluronic acid was detected in extracts from *P. multocida*, type B, Strain 1404

The use of Dowex-1 for fractionation of the crude polysaccharide gave a relatively pure product, but it was difficult to obtain reasonable yields, and only one-half or less was generally recovered after elution under the usual conditions The data in Table I illustrate the similarity in composition between the polysaccharide from *P. multocida* and the hyaluronic acid from a streptococcal source[11]. The fraction obtained as the copper complex was not contaminated with compounds containing 2-amino-2-deoxy-D-galactose. Furthermore, u v. absorption spectra showed that only the copper-precipitated sample was free of nucleic acid The difficulty experienced in obtaining reasonably pure hyaluronic acid from *P multocida* contrasts with the relative ease in isolating this polysaccharide from streptococcal extracts, although the purity of preparations obtained as the copper complexes are comparable, as judged by their compositions

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TABLE I

COMPOSITION[a] OF HYALURONIC ACID PREPARATIONS FROM *Pasteurella Multocida* AND GROUP A STREPTOCOCCUS

Source and conditions of preparation	*N*, %	*Uronic acid*, %	*2-Amino-2 deoxy-D-glucose*, %	$[\alpha]_D^{25}$ (*in water*), *degrees*	*Amino Acid Autoanalyses*[b]								
					GalN	*Asp*	*Thr*	*Ser*	*Glu*	*Pro*	*Gly*	*Ala*	*Total of others amino acids*
P Multocida													
Ethanol	8 6	11 9	10 6		0 052	0 109	0 060	0 069	0 107	0 073	0 195	0 092	0 282
Cetylpyridinium chloride	3 9	37 3	28 3										
Alkaline-copper reagent	2 8	40 8	30 6	−63	0 001	0 006	0 004	0 009	0 007	traces	0 011	0 007	0 026
Streptococcus Alkaline-copper reagent	3 2	44 4	35 1	−70	0 001	0 006	0.007	0 010	0 013	traces	0.007	0 008	0 010

[a]Analytical methods described in Ref 17, the results have been corrected for moisture content, and the hexosamine values were taken from the amino acid autoanalysis data and were not corrected for destruction occurring under the conditions used [b]Expressed as ratios to 2-amino-2 deoxyglucose

The identity of the *P multocida* polysaccharide with hyaluronic acid was confirmed by the similarity of its electrophoretic mobility[12] with that of streptococcal hyaluronic acid (Fig 1) and by the disappearance of turbidity with acid albumen[6], as well as by the formation of products having an absorption maximum at 232 nm[13] after incubation with streptococcal hyaluronidase An ion-exchange chromatographic method developed for the detection of uronic acids[14] showed the presence of glucuronic acid only The purified hyaluronic acid emerged with the void volume after gel filtration through Sephadex G-200, as described by Wasteson[15], resembling in this respect the hyaluronic acid from other sources Treatment with alkali, therefore, appears not to have greatly affected the molecular size of the hyaluronic acid, in agreement with the findings of Swann[16] that alkali treatment of hyaluronic acid from rooster combs produced insignificant changes in molecular size

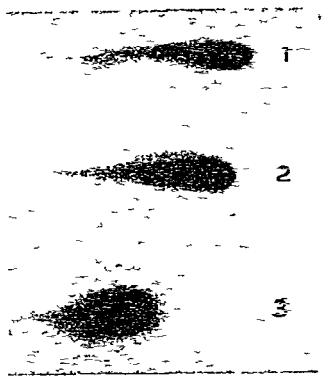


Fig 1 Electrophoretic mobilities of hyaluronic acid preparations The polysaccharides were applied in a 0 5% solution to Sepraphore III cellulose acetate strips, run for 45 min at 170 V in a pyridine–formate buffer, pH 3 0, as described by Mathews[12], and stained with Acridine Orange, (1) purified hyaluronic acid from *P multocida*, (2) streptococcal hyaluronic acid (3) purified hyaluronic acid from vitreous humor

In contrast to the similarity of the properties of hyaluronic acids obtained from Pasteurella and Streptococcus, the stability of the capsules of these bacteria appears to differ greatly Thus, hyaluronic acid is readily released from streptococcal cells into the culture medium, whereas the capsules of Pasteurella are removed with difficulty and, even after digestion with papain for 18 h, significant amounts of capsular material are attached to the cells.

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Carbohyd Res, 14 (1970) 272–276

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

SELECTIVE CLEAVAGE OF GLYCURONOSIDIC LINKAGES

N. K. KOCHETKOV, O. S. CHIZHOV, AND A. F. SVIRIDOV

N. D. Zelinsky Institute of Organic Chemistry, Academy of Sciences of U. S. S. R., Leninsky Prospect 47, Moscow (U. S. S. R.)

(Received December 16th, 1969)

ABSTRACT

A method for the selective cleavage of glycuronosidic linkages in polysaccharides has been evaluated with model compounds. The method involves Hofmann degradation of hexopyranuronamide residues, followed by mild hydrolysis with acid of the resulting 5-aminopentopyranose residues. The pentodialdoses produced by this process from several model hexopyranosiduronic acids have been characterised as pentitol acetates, following their reduction with borohydride and acetylation. Application of the method to birch xylan resulted in removal of *ca.* 94% of the (terminal) uronic acid residues, without effective hydrolysis of other glycosidic linkages.

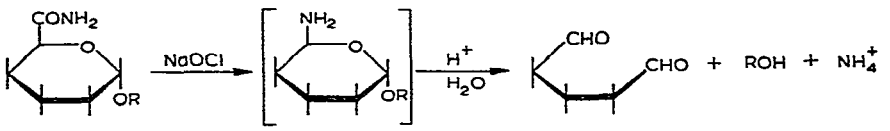
INTRODUCTION

There have been several reports[1–3] of methods for the selective fragmentation of polysaccharides. Such methods are of interest and importance, since they constitute a new stage in studies of the structure of polysaccharides and conjugated biopolymers.

We now present the detailed results of a method which has been reported in a preliminary form[4].

RESULTS AND DISCUSSION

The method involves selective cleavage of glycuronosidic linkages and is based on the conversion of the uronic acid residues into amides followed by their transformation into 5-aminopentopyranoses by the Hofmann reaction. These moieties would be cleaved by acids under mild conditions, as are glycosylamines[5], with concomitant release of the aglycone:



Only glycuronosidic linkages would be cleaved in this reaction sequence which has been studied on the model compound (−)-menthyl α-D-glucopyranosiduronamide[4,6] (**1**). The Hofmann rearrangement of **1** was effected with alcoholic sodium hypochlorite (0°, 1 h), and, without isolation of the intermediate, the mixture was

acidified and kept overnight. A pH of 12–13 is optimal for the rearrangement, and the use of a pH of 2–6 for the acid hydrolysis caused no significant side-reactions. Thus, an 80% yield of (−)-menthol has been obtained[6]. Control experiments have shown that, under these conditions, non-glycuronosidic linkages are not affected.

Experiments have now been undertaken to clarify the chemistry of the process. After the Hofmann rearrangement, followed by hydrolysis, the uronic acid amide should be converted into a pentodialdose. The reaction was investigated by using the amides of (−)-menthyl 2,3,4-tri-*O*-methyl-α-D-glucosiduronic acid (**2**), methyl 2,3,4-tri-*O*-methyl-α-D-glucosiduronic acid (**3**), methyl α-D-glucopyranosiduronic acid (**4**), methyl α-D-galactopyranosiduronic acid (**5**), and methyl α-D-mannopyranosiduronic acid (**6**). The formation of pentodialdoses was proved in several ways. Firstly, borohydride reduction of the reaction products followed by acetylation converted the pentodialdoses into pentitol acetates. The yields and the constants of the compounds formed are given in Table I. The structure of the pentitol derivatives was confirmed by their mass spectra; the fragmentation patterns are in full agreement with literature data[7,10].

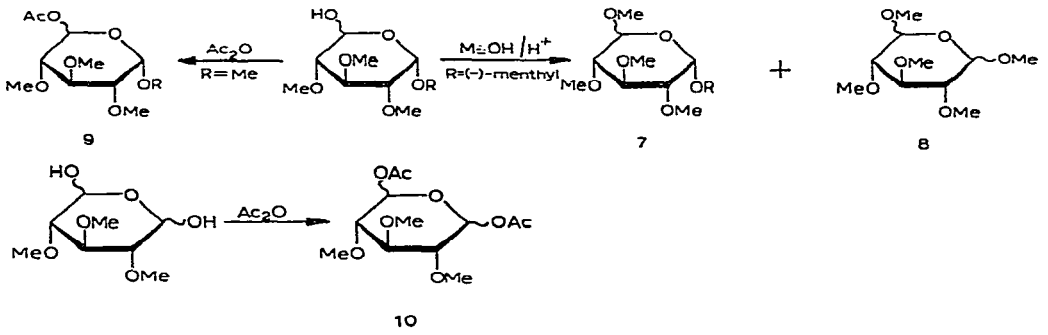
TABLE I

DEGRADATION PRODUCTS OF URONIC ACID AMIDES

Amide	*Reaction product*	*Yield* (%)	*M.p.* (*degrees*)	$[\alpha]_D$ ($CHCl_3$) (*degrees*)	*Literature data*[7–9] (*degrees*)
2	(*a*) 1,5-Di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol[7]	89			
	(*b*) (−)-Menthol	90	42.5		42.5
3	1,5-Di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol[7]	87			
4	Xylitol pentaacetate	67	60–61		60–61.5
5	L-Arabinitol pentaacetate	65	73–75	−31	76; −35
6	D-Arabinitol pentaacetate	70	74–75	+31.2	76; +35

The data presented in Table I show that the Hofmann rearrangement of the uronic acid amides proceeds in high yield and is not significantly accompanied by side reactions, especially when the resulting dialdose is not capable of further transformations as in the case of the methylated derivatives. Evidently, the reaction is of general character and is not accompanied by changes of configuration at C-2, C-3, and C-4 of the pyranoid ring. Thus, the structure of the original uronic acids can be inferred from the structure of the degradation products. From these experiments, a convenient procedure has been found for isolating the fragments resulting from the Hofmann rearrangement and involves reduction of the initial reaction product to the corresponding pentitol.

Secondly, the *O*-methyldialdose derivatives could be extracted from the reaction mixture with chloroform following treatment with sodium hypochlorite, and stabilised by methyl glycosidation or acetylation. In this manner, the products **7**–**10**



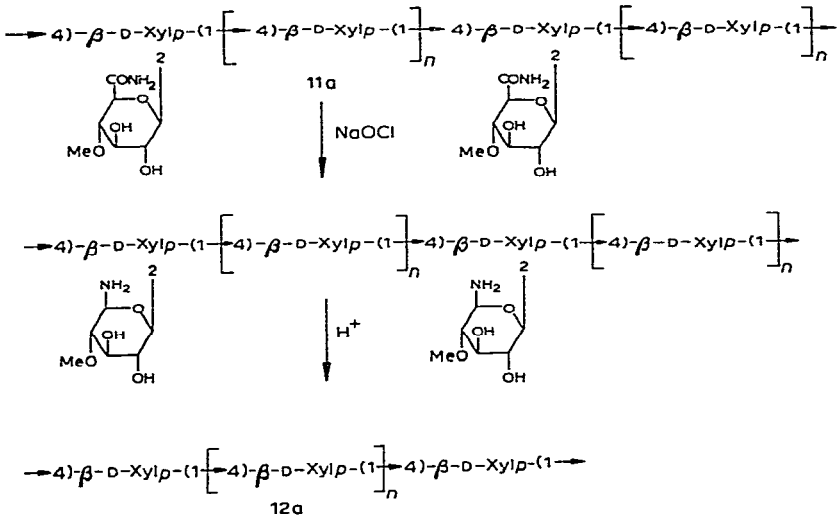
were isolated. The structures are supported by the mass-spectral data (*cf.* Ref. 11) in Table II, and the degradation mechanism is thereby confirmed.

TABLE II

MASS SPECTRA OF PENTODIALDOSE DERIVATIVES

m/e	*Intensity* (%)				m/e	*Intensity* (%)			
	7	8	9	10		7	8	9	10
251	0.40				145	2.0	2.1	3.3	2.0
250	0.25				141	5.7	3.0	3.0	1.9
249	0.30				139	13.0			
248	1.0				138	8.0			
244	0.70				135			2.1	2.4
243	0.90				131	1.4	2.0	2.3	2.4
242	0.30				129	18.7	1.9	4.1	2.0
241	0.25				127	1.7	2.0	2.1	2.4
239	0.20				123	3.3		1.0	5.5
233	0.30				119			1.5	5.5
223	0.60				117	1.9	1.8	7.8	2.4
221	0.35				116	1.2		5.0	1.9
219	0.50				115	1.1		1.2	2.4
218	0.40				113	1.5	1.5	2.0	2.4
217	3.0				111	3.0	2.0	2.6	5.2
215	0.40		1.3		103			6.2	4.3
212	1.0				102	6.1	6.5	6.3	2.4
211	0.40				101	100.0	100.0	100.0	34.1
205	0.75	1.2	1.1		99			3.0	6.6
201	13.2	3.3	3.0	37.0	97	7.8		2.7	8.5
199	8.5				96	2.9		4.0	6.0
197	0.30				95	9.0		3.0	12.0
191	2.0				88	16.0	83.0	53.4	64.0
189	8.5				87	4.2	3.1	5.0	2.4
173	1.5	1.3	1.5	0.80	85	10.4	16.0	5.0	11.6
169	2.0		1.3	1.0	83	32.6	2.0	3.5	21.7
161	10.4		1.8		81	11.6	2.1	4.0	12.8
159	3.5		1.4	1.0	75	8.0	31.6	31.5	10.0
157	16.0				73	6.1	26.0	6.1	17.1
155	1.0		1.3	1.7	71	6.4	4.7	5.0	14.5
149				9.1	43	10.9	8.0	18.0	100.0

To prove the validity of the Hofmann rearrangement for studying the structure of polysaccharides containing uronic acid residues, its application to birch xylan, one of the simplest polysaccharides of this type, was studied[12]. Birch xylan is known[13] to posess a linear chain of β-(1→4)-linked D-xylopyranose residues. On the average, 4-*O*-methyl-D-glucuronic acid residues are linked glycosidically to C-2 of every tenth D-xylose unit, approximately 70% of the carboxylic groups being esterified[14].



The birch xylan amide **11a** was isolated by extracting birch sawdust with hot water after ammonolysis[15]. To effect quantitative amidation, the birch xylan amide **11a** was decationised, acetylated, then esterified with diazomethane in chloroform, and finally treated with liquid ammonia. I.r. spectroscopy of the resulting polysaccharide **11b** did not show any free carboxylic acid or ester groups.

The amides **11a** and **11b** were treated with 0.5M sodium hypochlorite in nitrogen for 1 h at pH 13.5 and 0°. The mixture was then acidified with acetic acid (to pH 5) and kept for 24 h. The modified polysaccharides (**12a,b**) were isolated by a standard procedure, purified by dialysis, and precipitated from water with methanol–butyl alcohol. Provided the cleavage of glycosiduronic acid linkages proceeds to completion, the modified polysaccharides (**12a,b**) should be β-(1→4)-linked D-xylans.

A comparative study of the properties of polysaccharides **11a** and **12a** allowed an evaluation of the extent of cleavage of glycuronosidic linkages and of non-specific degradation of the other glycosidic linkages. Treatment of **11a** and **12a** with M sulphuric acid (100°, 7 h) was followed by determination of the monosaccharides in the hydrolysate. The original xylan **11a** contained 11.7% of uronic acid, whereas, after the treatment under the conditions described above, the content was 1.6%. These data were confirmed by periodate oxidation. In the original xylan **11a**, *ca.* 9% of the D-xylose units survived periodate oxidation because they were located at the branch points, whereas, for **12a**, the survival was only 0.5%. Thus, Hofmann degradation of **11a** results in a loss of not less than 80–85% of the uronic acid residues. After similar

treatment of the xylan **11b**, the content of uronic acid residues in the modified polysaccharide **12b** was 0.7%, the degradation being 94%.

As indicated by gel-filtration on Sephadex G-50, polysaccharides **11a** and **12a** were each eluted as a single peak at the solvent front. No other peaks were detected. Thus, no polysaccharide fragment having a molecular weight of less than 10,000 is formed in the Hofmann degradation of **11a** and **11b**. The possible occurrence of single cleavages of the main xylan chain cannot be ruled out. However, it is considered that glycosidic linkages other than of the glycuronosidic type are virtually unaffected.

The results reported herein indicate that organochemical approaches to the complex problem of selective polysaccharide degradation are possible. There are other potential approaches of a chemical nature for the selective hydrolysis of polysaccharide chains (see, for example, Ref. 16).

EXPERIMENTAL

Reactions were monitored by thin-layer chromatography (t.l.c.) on silica gel, i.r. and n.m.r. spectroscopy, and mass spectrometry. I.r. spectra were recorded for KBr pellets by using a UR-10 spectrometer; mass spectra were measured with an MX-1303 instrument at 70 eV and 170°.

(−)-*Menthyl 2,3,4-tri-*O*-methyl-*α*-D-glucopyranosiduronamide* (**2**). — (−)-Menthyl α-D-glucopyranoside[17] (10 g, 30 mmoles) was treated with chlorotriphenylmethane (10 g, 36 mmoles) in pyridine by the usual method. Without further purification, the resulting product was methylated[18] in freshly distilled *N,N*-dimethylformamide (30 ml) by treatment with silver oxide (90 g) and methyl iodide (55 ml) for 48 h. The reaction product, isolated by the standard procedure, was treated[19] with 80% acetic acid, and chromatographed on a column of silica gel (benzene–chloroform gradient). The fractions containing (−)-menthyl 2,3,4-tri-*O*-methyl-α-D-glucopyranoside (**13**) were evaporated, and the residue was crystallised from light petroleum to give material (7.29 g, 67%), m.p. 85–86°, $[\alpha]_D^{18}$ +83.8° (*c* 10.0, chloroform) (Found: OMe, 26.01, 25.87. $C_{19}H_{33}O_6$ calc.: OMe, 25.82%). The i.r. spectrum had bands at 3515 (strong, hydroxyl), 910, 842, and 770 cm^{-1} (α-D-hexopyranose ring[20]).

Compound **13** (6 g, 16.5 mmoles) was oxidized[21] with potassium permanganate in 100:100:10 ml acetone–acetic acid–water. The resulting mixture was dissolved in 2M potassium hydroxide (100 ml) and extracted with chloroform. The aqueous layer was acidified with M sulphuric acid and re-extracted with chloroform. The extract was washed with water, dried (Na_2SO_4), evaporated, and chromatographed on silica gel to give (−)-menthyl 2,3,4-tri-*O*-methyl-α-D-glucopyranosiduronic acid (**14**, 5 g, 80%), $[\alpha]_D^{18}$ +32.2° (*c* 7, chloroform). The i.r. spectrum contained bands at 1773 (strong, carboxyl), 915, 853, and 770 cm^{-1}.

Compound **14** (4.5 g, 12 mmoles) was treated with ethereal diazomethane and converted quantitatively into the methyl ester **15**. The product, which crystallised from light petroleum, had m.p. 43–46°, $[\alpha]_D^{18}$ +41.4° (*c* 14.5, chloroform). The i.r.

spectrum contained bands at 1750, 913, 853, and 764 cm^{-1}. The n.m.r. spectrum confirmed the α-D configuration (H-1 doublet, δ 4.6, $J_{1,2}$ 4 Hz), and also showed the presence of the menthyl residue (Me signals at δ 1.6–0.7) as well as four methoxyl groups (δ 3.9, 3.4, 3.3, 3.26).

A solution of the methyl ester **15** (4 g, 10 mmoles) in dry methanol (100 ml) was saturated at 0° with dry ammonia. After storage for 24 h at room temperature, the mixture was evaporated, and the residue was crystallised from benzene–light petroleum to give the title compound **2** (3.4 g, 89.4%), m.p. 181–182°, $[\alpha]_D^{18}$ +65.8° (*c* 1.14, chloroform) (Found: C, 60.92; H, 9.28; N, 3.99; OMe 23.88. $C_{19}H_{35}O_6$ calc.: C, 61.09; H, 9.45; N, 3.74; OMe, 24.92%). The i.r. spectrum contained bands at 3451, 1700, and 1615 (amide group), 915, 855, and 765 cm^{-1} (α-D-hexopyranose ring). After complete hydrolysis of the amide in 0.25M sulphuric acid (100°, 3 h), t.l.c. (silica gel) revealed the presence of (−)-menthol and 2,3,4-tri-*O*-methyl-D-glucuronic acid. Hydrolysis in 50mM potassium carbonate (50°, 10 h) afforded **14**. $[\alpha]_D^{18}$ +31.1° (*c* 2.3, chloroform).

The amide of methyl 2,3,4-tri-*O*-methyl-α-D-glucopyranosiduronic acid[22] (**3**) was synthesized by a route similar to that described above and had m.p. 179–180°, $[\alpha]_D^{18}$ +144.3° (*c* 0.58, water). The amides of methyl α-D-glucopyranosiduronic acid (**4**), methyl α-D-galactopyranosiduronic acid (**5**), and methyl α-D-mannopyranosiduronic acid (**6**) were synthesized by the literature procedure[23–25].

Cleavage of the amides **4**, **5**, *and* **6**. — The samples of amides (50 mmoles) were dissolved in water (10 ml), cooled to 0°, and 0.5M sodium hypochlorite (10 ml) was added (pH of the mixture was 13.1). After 1 h, potassium borohydride was added gradually until the hypochlorite disappeared. The mixture was acidified with acetic acid (to pH 5) and left for 10–12 h at room temperature. It was then basified with saturated, aqueous sodium carbonate, potassium borohydride (0.5 g) was added, and the mixture was stirred for 10–15 h. The mixture was evaporated, the dry residue was acetylated with acetic anhydride–pyridine, and the pentitol acetates were isolated in the usual way and crystallized from 80% aqueous methanol after purification by silica gel chromatography. The data are given in Table I. The structures were confirmed on the basis of mixed m.p.s with the authentic samples and comparison of i.r. spectra. The mass spectrum of each pentitol derivative contained the set of peaks at *m/e* 303, 289, 243, 187, 175, 158, 145, 140, 127, 115, 103, 98, 85, 81, 73, and 43. These peaks and the ratio of their intensities are in good agreement with the literature data[7].

Cleavage of methyl and (−)*-menthyl 2,3,4-tri-*O*-methyl-α-D-glucopyranosiduronamides.* — The degradation of the amides was carried out as described above. After borohydride reduction, the mixture was extracted with chloroform, the extract was evaporated, and the residue was chromatographed on silica gel. The fractions containing 2,3,4-tri-*O*-methylxylitol were evaporated, and the residue was acetylated with acetic anhydride in pyridine. The constants and yields of the resulting compounds are given in Table I. The i.r. spectra of authentic 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol and of the samples resulting from the reaction were identical. Their mass spectra contained peaks at *m/e* 219 (0.2), 205 (0.25), 173 (1.1), 161 (21), 149 (1.0),

145 (1.2), 131 (3.1), 130 (16.8), 129 (5.0), 127 (1.0), 119 (1.2), 118 (5.5), 117 (84.2), 113 (1.2), 102 (5.5), 101 (76.3), 99 (3.3), 89 (5.0), 88 (5.1), 87 (12.0), 85 (4.0), 83 (1.8), 81 (1.5), 75 (5.0), 73 (4.0), 71 (8.0), 59 (4.6), 45 (11.6), and 43 (100%). The above set of peaks and the ratio of their intensities are in good agreement with the structure of the partially methylated xylitol and correspond to the literature data[10].

Cleavage of methyl and (−)-menthyl 2,3,4-tri-O-methyl-α-D-glucopyranosiduronamides. — The title menthyl amide (108.6 mg, 0.31 mmole) was dissolved in ethanol (10 ml), cooled to 0°, and treated with 0.5M sodium hypochlorite (10 ml). The mixture was stirred for 1 h and then extracted with chloroform. The extract was washed with water, dried (Na_2SO_4), filtered, and evaporated. The residue was treated with 10 mM methanolic hydrogen chloride (64°, 4 h), and then neutralised with Amberlite IRA-400 (HCO_3^-) resin. The filtered mixture was evaporated, and the residue was fractionated by t.l.c. on silica gel to give the pentodialdoses **7** (15 mg) and **8** (28 mg). The mass-spectral data are given in Table II.

The treatment of the title methyl amide (124.5 mg, 0.5 mmole) was carried out under similar conditions. After evaporation of the chloroform extract, the mixture was acetylated with acetic anhydride in pyridine, and the products were separated by t.l.c. on silica gel. In this way, the pentodialdose derivatives **9** (30 mg) and **10** (17 mg) were isolated. The mass-spectral data are given in Table II.

Cleavage of white-birch xylan amide. — White-birch xylan amide (**11a**) was isolated[15] from sawdust as a grey powder, $[\alpha]_D^{18}$ −92.1° (*c* 1.14, M potassium hydroxide) (Found: C, 37.84; H, 6.54; N, 1.30; ash 3.76; D-glucuronic acid, 11.7%).

A solution of the xylan amide **11a** (0.5 g) in water (15 ml) was cooled to 0°, and 0.5M sodium hypochlorite (15 ml) was added with stirring in nitrogen at pH 13.5. After 1 h, the mixture was placed in a boiling-water bath for 5 min. The cooled solution was acidified with acetic acid (to pH 5.0) and left overnight at room temperature. After the addition of alcohol (200 ml), the mixture was left for 24 h at 4°, the precipitate was collected and dissolved in water (50 ml), and the solution was dialysed for 6 days against distilled water. 1:4 Methanol–butyl alcohol (300 ml) was added to the dialysate, and, after 24 h, the precipitate was collected, washed with dry ethanol and ether, and dried *in vacuo* to give the modified polysaccharide **12a** (0.43 g, 86%), $[\alpha]_D^{18}$ −113.7° (*c* 1.4, 2M potassium hydroxide) (Found: C, 39.87; 39.75; H, 6.20; 6.21; ash, 2.44; 2.62%; N, trace). The i.r. spectrum showed weak bands at 1600–1500 and 1400 cm^{-1} (carboxylate ion), but did not show an amide absorption band at 1700–1600 cm^{-1}.

Determination of the uronic acid content of the modified polysaccharide **12a**. — The polysaccharide **11a** was hydrolysed with 0.5M sulphuric acid (100°, 7 h). The hydrolysate was neutralised with aqueous ammonia and evaporated to 10 ml, and methanol (50 ml) was added. The precipitate was collected and washed with methanol, and the combined filtrate and washings were evaporated. The dry residue was dissolved in water (5 ml) and transferred to a column of Amberlite IRA-400 (AcO^-) resin. The column was washed with water to remove completely the neutral products of hydrolysis (detected by the phenol–sulphuric acid reaction[26], and then with 2M

hydrochloric acid (1-ml fractions), and the uronic acid content was determined spectrophotometrically[26]. The modified polysaccharide **12a** was treated in a similar way. The relative decrease of uronic acid content was 86%.

Periodate oxidation of the polysaccharides. — The polysaccharide samples **11a** and **12a** (59.4 and 27.7 mg, respectively) were dissolved in 40mM sodium periodate (50 and 25 ml, respectively). Oxidation was carried out at 4° in the dark. Periodate consumption was determined periodically on 1-ml aliquots by titration with sodium arsenite[27]. The results were as follows:

Time (h)	*Consumption of periodate in moles per mole of anhydroxylose residue*	
	Polysaccharide **11a**	*Polysaccharide* **12a**
3.5	0.29	0.84
8.5	0.74	1.09
33	0.82	—
49	1.07	—

When 1 mole of periodate per mole had been consumed, ethylene glycol (1 ml) was added to each solution, and, after 2 h, the solutions were dialyzed against distilled water (24 h) and evaporated. The dry residues were dissolved in 0.5M sulphuric acid, and, after hydrolysis (100°, 7 h), the amount of D-xylose surviving was determined by reaction with L-cysteine[28]. For the starting polysaccharide **11a**, the quantity of D-xylose surviving (determined from the calibration curve constructed from the products of complete hydrolysis of the non-oxidized polysaccharide **11a**) was 8.3%, that of the modified polysaccharide (**12a**) being 1.5%. Thus, the relative decrease of the D-xylose content and, likewise, that of uronic acid in the modified polysaccharide **12a** is 83%.

Degradation of the white-birch xylan amide **11b**. — The birch xylan amide **11a** was converted into the acidic form by treatment with KU-2 (H^+) resin, and then acetylated with acetic anhydride in pyridine. The acetate was treated in succession with chloroformic diazomethane, and liquid ammonia in a sealed tube for 72 h at room temperature. The i.r. spectrum of the polysaccharide **11b** showed strong bands at 1690–1640 cm^{-1} and a weak absorption band at 1740 cm^{-1}. The cleavage of polysaccharide **11b** and detection of the relative decrease of uronic acid content were carried out as described above. The content of the uronic acid surviving was 0.7%. Thus, the relative decrease was 94%.

Gel-filtration of polysaccharides **11a** *and* **12a**. — The samples of polysaccharide (1–2 mg) were transferred to a previously washed column of Sephadex G-50 with M potassium chloride and eluted with the same solution (1-ml fractions). The polysaccharide content of the fractions was determined by the phenol–sulphuric acid method[26]. The polysaccharides emerged from the column as a single peak with the solvent front.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

CROSS-LINKED POLYACRYLAMIDE DERIVATIVES (ENZACRYLS) AS WATER-INSOLUBLE CARRIERS OF AMYLOLYTIC ENZYMES

S. A. BARKER, P. J. SOMERS,
Department of Chemistry, The University, Birmingham 15 (Great Britain)
R. EPTON, AND J. V. MCLAREN
Department of Chemistry and Biology, The Polytechnic, Wolverhampton (Great Britain)

(Received December 2nd, 1969; in revised form, December 31st, 1969)

ABSTRACT

Methods are described for the water-insolubilisation of alpha- and beta-amylase by chemical coupling to two cross-linked copolymers of acrylamide. For one copolymer, coupling was effected either by diazo or isothiocyanato groups, and for the other, acid azide groups were employed. Active derivatives of alpha-amylase were prepared by diazo, isothiocyanato, and acid azide coupling, whereas active derivatives of beta-amylase could be prepared only by diazo and isothiocyanato coupling. The stabilities of the active, copolyacrylamide-bound preparations of amylase were comparable to those of the corresponding, cellulose-bound enzymes. In the case of alpha-amylase, the copolyacrylamide derivatives were more stable than the free enzyme in solution.

INTRODUCTION

We have previously reported[1,2] the water-insolubilisation of amylolytic enzymes by chemical coupling to microcrystalline cellulose. Cellulose derivatives have been used as enzyme carriers by other workers[3–6], but these carriers often lead to strong, physical adsorption of enzymes which must be detached by rigorous washing with buffer and sodium chloride solution[5,7]. Such is the case with amylolytic enzymes. Furthermore, we have shown that prolonged washing with salt may lead to denaturation. Since it is also possible that polysaccharide carriers may undergo unique interactions with glycoside hydrolases, we have considered alternative types of carrier.

Other workers[8–10] have prepared water-insoluble derivatives of alpha-amylase by chemical coupling to cross-linked derivatives of polymethacrylic acid. Although this polymer is hydrophilic overall, hydrophobic interactions are also important, as evidenced by conformational studies in both gel[11] and free solution[12]. It is well known that hydrophilic features of the support may confer stability to the bound enzyme, whereas hydrophobic features have a marked, destabilising effect. Thus, derivatives of polymethacrylic acid were inferior to cellulose derivatives as carriers of

alpha-amylase, both with respect to enzyme activity retained on coupling and also to storage[1]. A further factor against the use of derivatives of polymethacrylic acid in the present studies was their polyionic nature which could have led to electrostatic adsorption of the enzymes.

Most other polymers used for enzyme coupling have either been hydrophobic[13–15] or, like polymethacrylic acid, polyionic[16].

An alternative method for the water-insolubilisation of enzymes is by trapping them in a polyacrylamide gel[17,18]. Water-insoluble preparations of D-glucose oxidase and lactate dehydrogenase were obtained, which had good stability to storage and lyophilisation. It thus seems that polyacrylamide could be a useful carrier for glycoside hydrolases.

Since macromolecules are unable to penetrate the gel matrix, the "trapping" method is unsuitable for such enzymes as amylases which are active against macromolecular substrates. In the present studies, therefore, we have sought to insolubilise alpha- and beta-amylase by chemical coupling to the surface of particles of cross-linked polyacrylamide gel.

EXPERIMENTAL

Crystalline alpha-amylase (*B. subtilis*) and crystalline beta-amylase (sweet potato) were purchased from the Sigma Chemical Company.

4-Nitroacrylanilide. — A solution of 4-nitroaniline (27.6 g, 0.2 mole) in warm, dry ether (1 litre) was stirred continuously for 2 h with acryloyl chloride (9.05 g, 0.1 mole). A further portion of acryloyl chloride (1 ml) was then added. After stirring for a further 2 h, the crystals which separated were collected, and the ether was evaporated to give more solid. The combined crystals and solid were triturated with water (5 × 500 ml) at 80–85°. Recrystallisation of the residue from acetone, followed by ethyl acetate, yielded 4-nitroacrylanilide (10.5 g, 54%), m.p. 239–240° (Found: C, 56.5; H, 4.1; N, 14.7. $C_9H_8N_2O_3$ calc.: C, 56.3; H, 4.2; N, 14.6%).

Preparation and selective reduction of Enzacryl AN. — A solution of acrylamide (3.55 g, 0.05 mole), 4-nitroacrylanilide (0.38 g, 0.002 mole), and *N,N'*-methylenediacrylamide (0.616 g, 0.004 mole) in ethanol (25 ml) at 75° was purged gently with nitrogen for 20 min, and a solution of benzoyl peroxide (100 mg) in chloroform (1 ml) was added. After storage for 12 h at 75°, the resulting copolymer was broken up under ethanol and passed through a coarse, syringe needle. After being washed with nearly boiling ethanol (3 × 500 ml), the copolymer (Enzacryl AN) was allowed to dry in air.

Selective reduction of the nitro groups in Enzacryl AN was achieved with titanous chloride. The copolymer (2 g) was suspended in a solution of titanous chloride (5–6%) in hydrochloric acid (6M, 100 ml), and the temperature was quickly raised to 100°. After 5 min, the mixture was rapidly cooled (ice bath), most of the solution was decanted off, and the polymer was repeatedly washed with distilled water. Finally, the reduced polymer (Enzacryl AA) was ground with distilled water,

passed through a coarse, syringe needle, washed with acetone, and allowed to dry in air.

Radiochemical determination of aromatic amine groups in Enzacryl AA as the hydrochlorides. — Samples of Enzacryl AN and AA were allowed to swell in aqueous sodium hydroxide (2M), exhaustively washed with distilled water, shrunk by drop-wise addition of acetone, and allowed to dry in air.

The prepared samples (0.25 g) of Enzacryl AN and Enzacryl AA were stirred magnetically for 1 h in centrifuge tubes with aliquots (15 ml) of 98mM hydrochloric acid containing radioactive chloride (^{36}Cl, 10 ncuries/ml). After centrifugation, the radioactivities of aliquots (10 ml) of each supernatant, and also of the original hydrochloric acid, were determined by means of a Geiger–Müller counter. After counting, each aliquot was transferred quantitatively to a conical flask, heated on a steam bath to expel carbon dioxide, and titrated with aqueous sodium hydroxide (99 mM).

Treatment of Enzacryl AA with hydrochloric acid leads to insolubilisation of a proportion as aromatic amine hydrochloride. By comparison of the radioactivity of the supernatant with that of the original hydrochloric acid, the total amount of aromatic amine hydrochloride in the polymer was determined. From the control experiment with Enzacryl AN, compensation was made for non-specifically adsorbed chloride (<7% of the total). Enzacryl AA contained 0.27 mequiv. of aromatic amine hydrochloride per gram (59% of the theoretical).

The total amount of hydrochloric acid neutralised by the polymer was determined by titration of the supernatant from the radiochemical determination of Enzacryl AA. This value was equivalent to the insolubilised acid plus an amount equivalent to the sodium carboxylate residues in the polymer (carboxylate residues are produced by hydrolysis of amide residues during acid reduction of Enzacryl AN). The total carboxylate in Enzacryl AA was 1.3–1.4 mequiv./g (corresponding to <10% hydrolysis of total amide).

tert-*Butyl 3-acryloylcarbazate.* — A solution of *tert*-butyl carbazate (2.6 g, 0.02 mole) in distilled water (15 ml) was stirred vigorously at room temperature with sodium hydrogen carbonate (6 g), and acryloyl chloride (1.6 g, 0.02 mole) was added during 5 min.

After 30 min, a further aliquot (1 ml) of acryloyl chloride was added. Stirring was continued for a further 30 min, and the resulting white solid was removed by filtration and washed well with water. Recrystallisation from ethyl acetate–light petroleum (b.p. 40–60°) yielded the title compound (1.8 g, 50%), m.p. 159–160° (Found: C, 51.8; H, 7.6; N, 15.3, $C_8H_{14}N_2O_3$ calc.: C, 51.6; H, 7.6; N, 15.1%).

Preparation of Enzacryl AH. — Acrylamide (3.55 g, 0.85 mole), *tert*-butyl 3-acryloylcarbazate (0.372 g, 0.002 mole), and *N,N'*-methylenediacrylamide (0.616 g 0.004 mole) were dissolved in ethylene glycol (25 ml) and irradiated for 20 h with a Hanovia u.v. lamp (80 watts). The resulting copolymer was broken up, ground thoroughly in a mortar, and washed exhaustively with water. Finally, the copolymer was shrunk by dropwise addition of acetone and allowed to dry in air.

Removal of protecting groups was achieved by shaking with an excess of 2M hydrochloric acid for 4 days at 18–20°. The resulting copolymer (Enzacryl AH) was washed exhaustively with distilled water, shrunk by dropwise addition of acetone, and allowed to dry in air.

Determination of acylhydrazide in Enzacryl AH as acylhydrazide hydrochloride. — The determination was performed as described for the aromatic amine residues in Enzacryl AA. Enzacryl AH contained 0.33 mequiv. of acylhydrazide hydrochloride per g (40% of the theoretical).

Total carboxylate (produced on shaking with dilute hydrochloric acid in the deprotection step) in Enzacryl AH was 0.1–0.3 mequiv./g (corresponding to 2% hydrolysis of total amide).

Preparation of water-insoluble alpha-amylase derivatives. — (*a*) *By coupling alpha-amylase to Enzacryl AA activated with nitrous acid.* Enzacryl AA (100 mg) was stirred with dilute hydrochloric acid (2M, 5 ml) at 0°. Ice-cold, aqueous sodium nitrite (2%, 4 ml) was added, stirring was continued for 15 min, and the diazo derivative of Enzacryl AA was washed four times with phosphate buffer (75mM, pH 7.6–7.7, 15 ml). After decantation of the final washings, a solution of alpha-amylase (2.5 mg) in phosphate buffer (75mM, pH 7.6–7.7, 0.5 ml) was added. Coupling was allowed to proceed for 48 h at 0–5° with stirring. A solution of phenol (0.01%, 10 ml) in aqueous acetate was then added, and, after a further 15 min, the water-insoluble alpha-amylase derivative was recovered by centrifugation. A control experiment was performed in which Enzacryl AH, preswollen in phosphate buffer (75mM, pH 7.6–7.7) but not treated with nitrous acid, was put through the above coupling procedure.

Both the water-insoluble alpha-amylase derivative and control were subjected to alternate washing with phosphate buffer (20mM, pH 6.9, 15 ml) and a solution of sodium chloride (0.5M, 15 ml) in the same buffer. Each washing was of 20-min duration with vigorous stirring. Five of the above washing cycles were necessary to wash the control to marginal amylolytic activity (<5% of that in the diazo-coupled derivative). Finally, the diazo-coupled alpha-amylase derivative was washed twice with phosphate buffer (15 ml) and, after decantation, resuspended in the same buffer (10 ml).

(*b*) *By coupling alpha-amylase to Enzacryl AA activated with thiophosgene.* Enzacryl AA (100 mg) was stirred with phosphate buffer (3.5M, pH 6.8–7.0, 1 ml) until a thick suspension was obtained. Stirring was then made very vigorous, and a solution of thiophosgene (10%, 0.2 ml) in carbon tetrachloride was added. After 20 min, a further aliquot (0.2 ml) of thiophosgene solution was added; 20 min later, the isothiocyanato derivative of the Enzacryl AA was washed once with acetone (15 ml), twice with 0.5M sodium hydrogen carbonate (15 ml), and twice with 50mM borate buffer (pH 8.6, 15 ml). After decantation of the final washings, a solution of alpha-amylase (2.5 mg) in 50mM borate buffer (pH 8.6, 0.5 ml) was added. Coupling was allowed to proceed for 48 h at 0–5° with stirring. The water-insoluble alpha-amylase derivative was then subjected to the washing procedure with phosphate buffer and buffered sodium chloride solution described in (*a*). Finally, the isothio-

cyanato-coupled alpha-amylase derivative was resuspended in phosphate buffer (10 ml).

(*c*) *By coupling alpha-amylase to Enzacryl AH activated with nitrous acid.* Enzacryl AH (100 mg) was treated with nitrous acid as described for Enzacryl AA. After the acid azide derivative had been washed four times with borate buffer (50 mM, pH 8.6, 15 ml), a solution of alpha-amylase (2.5 mg) in borate buffer (50 mM, pH 8.6, 0.5 ml) was added, and coupling was allowed to proceed for 48 h at 0–5° with stirring. As previously, a control experiment was performed in which Enzacryl AH, not activated with nitrous acid, was put through the coupling procedure. Three cycles of washing with phosphate buffer and buffered sodium chloride solution were sufficient to wash the control to zero amylolytic activity. Finally, the acid azide-coupled alpha-amylase derivative was resuspended in phosphate buffer (10 ml).

Preparation of water-insoluble beta-amylase derivatives. — (*a*) *By coupling beta-amylase to Enzacryl AA activated with nitrous acid.* Beta-amylase (5 mg) in phosphate buffer (75 mM, pH 7.6–7.7, 1 ml) was coupled with the diazo derivative of Enzacryl AA, following the procedures described for alpha-amylase. The washing procedure was also similar to that described for alpha-amylase except that acetate buffer (20 mM, pH 4.8) was used. Five washing cycles were necessary to decrease the amylolytic activity in the control to zero. The diazo-coupled beta-amylase derivative was finally suspended in acetate buffer (20 mM, pH 4.8, 10 ml).

(*b*) *By coupling beta-amylase to Enzacryl AA activated with thiophosgene.* Beta-amylase (5 mg) in borate buffer (50 mM, pH 8.6, 1 ml) was coupled with the isothiocyanato derivative of Enzacryl AA, as described for the corresponding derivative of alpha-amylase. The washing procedure and preparation of the final suspension of isothiocyanato-coupled beta-amylase was as described for diazo-coupled beta-amylase.

(*c*) *By coupling beta-amylase to Enzacryl AH activated with nitrous acid.* Beta-amylase (2.5 mg) in borate buffer (50 mM, pH 8.6, 0.5 ml) was coupled with the acid azide derivative of Enzacryl AH, as described for the corresponding alpha-amylase derivative. The washing procedure was similar to that employed for the other beta-amylase derivatives, except that only three washing cycles were necessary to decrease the amylolytic activity in the control to zero. The preparation of the final suspension of azide-coupled beta-amylase was as described for diazo-coupled beta-amylase.

Determination of amylolytic activity. — Alpha-amylase activity was determined against 1% soluble starch in phosphate buffer (20 mM, pH 6.9), and beta-amylase activity against 1% soluble starch in acetate buffer (20 mM, pH 4.8). Steady stirring was maintained for all determinations. As in previous studies[1,2], the initial rate of starch hydrolysis was determined by following the rate of formation of reducing sugar by assay of aliquots with the dinitrosalicylate reagent[19]. Absorbance changes (520 nm) thus recorded were related to reducing sugar by means of a standard graph relating absorbance to known amounts of maltose.

Determination of bound enzymes. — Samples of the water-insoluble enzyme derivatives and the non-coupled carriers were hydrolysed with 6M hydrochloric acid

for 18 h at 110°. The pH of aliquots (0.5 ml) of the hydrolysates was then adjusted to 14 by addition of the minimal quantity of 6M sodium hydroxide, and the solutions were lyophilised. The residues were then allowed to stand for 5 days over conc. sulphuric acid, after which they were re-constituted to a final volume of 1 ml, simultaneously being neutralised with concentrated hydrochloric acid. This procedure was necessary to ensure removal of ammonia and, in the case of hydrolysates of Enzacryl AH, hydrazine. The colour yield (570 nm) produced on ninhydrin assay[20] of the neutralised hydrolysates was then measured, and the colour yield produced on assay of hydrolysates of the non-coupled carriers was subtracted. The original enzyme concentration in the water-insoluble enzyme hydrolysates was calculated from standard graphs relating colour yield (570 nm) to known amounts of a hydrolysate of the free enzyme.

Stability of water-insoluble alpha- and beta-amylase to storage in buffer suspension. — The activity of the various preparations was redetermined after storage at 0–5° for 3 months, and the percentage of the original activity was calculated.

Stability of water-insoluble alpha-amylase to lyophilisation. — Aliquots (0.5 ml) of the three water-insoluble alpha-amylase suspensions were centrifuged and, after removal of most of the supernatant, lyophilised. After storage overnight at 0–5°, the samples were reconstituted with the appropriate buffer (0.5 ml) and their activities re-determined.

Stability of water-insoluble alpha- and beta-amylase to heat denaturation at 45°. — Suspensions of each enzymically active alpha-amylase derivative in phosphate buffer (20mM, pH 6.9) were incubated for 4 days at 45°, and their activity was determined at intervals (Fig. 1) against a solution of soluble starch. A control incubation was performed with a solution of free alpha-amylase in the same buffer.

A similar experiment was performed with suspensions of the enzymically active beta-amylase derivatives and a control solution of free beta-amylase in acetate buffer (20mM, pH 4.8).

DISCUSSION

In the preparation of water-insoluble enzyme derivatives, chemical coupling has most commonly been achieved by means of aromatic amine[1,2,13–15] or acid hydrazide[3–6] residues incorporated in the carrier. Activation with nitrous acid leads to diazonium and acid azide residues, respectively, which are effective in covalent bonding with enzymes. The simplicity of these methods probably accounts for their popularity. On superficial consideration, it might seem that these methods are not applicable to carriers based on polyacrylamide, since nitrous acid might be expected to react with amide residues in the copolymer matrix. However, polyacrylamide, in contrast to monomer amides, is refractory to nitrous acid.

By copolymerisation of acrylamide, 4-nitroacrylanilide, and *N,N'*-methylenediacrylamide, a cross-linked polymer, Enzacryl AN, was obtained which contained aromatic nitro groups. Reaction with titanous chloride in hydrochloric acid led to

Enzacryl AA, which contained aromatic amino groups (0.27 mequiv./g). Activation of the latter was achieved both by diazotisation and by conversion into the isothiocyanato group with thiophosgene. Subsequently, coupling was carried out with both alpha- and beta-amylase.

Diazo groups couple predominantly with the aromatic residues of the enzyme[21], whereas isothiocyanato groups couple with the amino groups of lysine and the terminal amino groups of the protein chains[22]. Such versatility is most desirable in the preparation of water-insoluble enzymes, since one or the other modes of coupling may either destroy, or lead to serious steric hindrance of, the enzymically active site. Thus, in previous work[2], we have shown that gamma-amylase may be coupled to the diazotised 3-(*p*-aminophenoxy)-2-hydroxypropyl ether of cellulose with retention of enzyme activity, whereas, on coupling to the corresponding 2-hydroxy-3-(*p*-isothiocyanatophenoxy)propyl ether of cellulose, inactivation resulted.

Copolymerisation of acrylamide, *tert*-butyl 3-acryloylcarbazate, and *N,N'*-methylenediacrylamide led to a copolymer which, on shaking with dilute hydrochloric acid, gave a copolymer, Enzacryl AH. Enzacryl AH thus contained acid hydrazide residues (0.33 mequiv./g). Nitrous acid treatment of the latter copolymer led to the generation of the acid azide group which, like the isothiocyanato group, is effective in coupling at the free amino residues of the enzymes[4]. Water-insoluble enzymes based on Enzacryl AH were of interest, since the absence of aromatic amino groups in this polymer meant it was relatively more hydrophilic than Enzacryl AA.

Active derivatives of alpha-amylase were obtained by all three coupling procedures (see Table I). The activities retained on coupling compared favourably with that of alpha-amylase coupled to other carriers[1,8–10]. We have previously demonstrated that the more an enzyme is dispersed over a carrier surface the greater is the activity retained on coupling. The present results confirm this hypothesis; the activity retained on coupling is greater for the water-insoluble preparations containing least alpha-amylase.

TABLE I

PROTEIN COUPLED AND ENZYME ACTIVITY IN THE WATER-INSOLUBLE ALPHA- AND BETA-AMYLASE PREPARATIONS

Enzyme	*Enzyme units[a]/mg of free protein*	*Carrier activated*	*Functional site active in protein binding*	*Bound protein (mg/100 mg of carrier)*	*Enzyme units/mg of bound protein*	*Enzyme activity (%) retained on coupling*
Alpha-amylase	1118	Enzacryl AA	Diazo	1.06	68.7	6.1
			Isothiocyanato	0.62	106.8	9.5
		Enzacryl AH	Acid azide	0.22	178.3	16.0
Beta-amylase	431.2	Enzacryl AA	Diazo	3.2	6.4	1.5
			Isothiocyanato	2.6	3.4	0.8
		Enzacryl AH	Acid azide	0.9	Inactive	Inactive

[a]One amylase unit is that which liberates reducing sugar equivalent to 1 μmole of maltose at 20° at optimum pH.

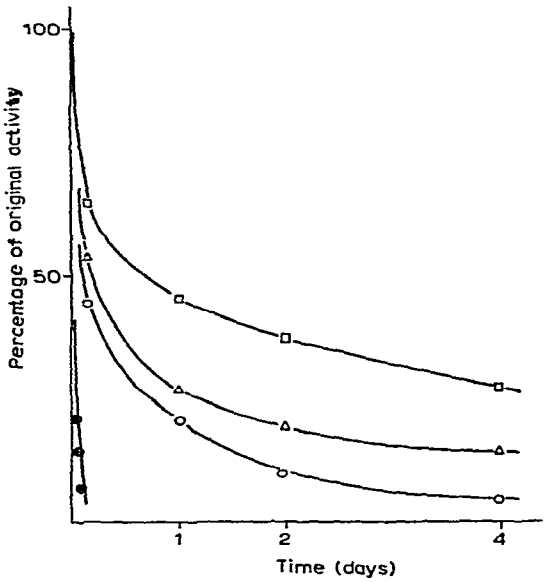


Fig. 1. Heat denaturation of soluble and water-insoluble forms of alpha-amylase on incubation in phosphate buffer at 45°. ●———●, Soluble alpha-amylase; ○———○, water-insoluble, diazo-coupled alpha-amylase; △———△, water-insoluble, isothiocyanato-coupled alpha-amylase; □———□, water-insoluble, acid azide-coupled alpha-amylase.

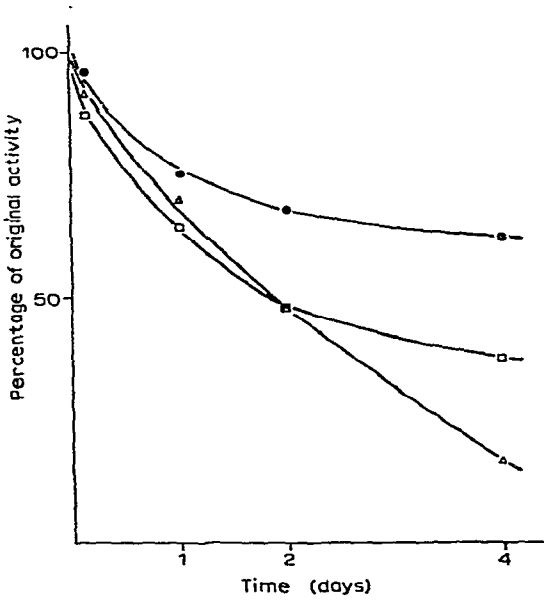


Fig. 2. Heat denaturation of soluble and water-insoluble forms of beta-amylase on incubation in acetate buffer at 45°. ●———●, Soluble beta-amylase; △———△, water-insoluble, diazo-coupled beta-amylase; □———□, water-insoluble, isothiocyanato-coupled beta-amylase.

Although beta-amylase was readily coupled by all procedures, the acid azide-coupled derivative was inactive, and the activity retained on diazo and isothiocyanato coupling to Enzacryl AA was lower than for the corresponding cellulose derivatives[2]. A possible explanation may be that the beta-amylase molecules tend to couple such that their active site is easily hindered. Previously, beta-amylase was coupled *via* activated 3-(*p*-aminophenoxy)-2-hydroxypropyl ethers of cellulose and would have been further away from the carrier matrix than in the present work. In the inactive, acid azide-coupled beta-amylase derivative based on Enzacryl AH, the enzyme must be particularly close to the polymer matrix, and this may account for the lack of activity. That steric effects are more important with Enzacryl AH is evident from the relatively smaller amounts of both alpha- and beta-amylase coupled to this carrier.

A feature of the water-insoluble polyacrylamide derivatives is that they were more easily washed free of physically attached enzyme than the corresponding cellulose derivatives. This was particularly noticeable with enzyme derivatives based on Enzacryl AH.

On storage for 3 months in buffer suspension, the activity retained by the acid azide-coupled alpha-amylase (85%) was rather better than diazo-coupled alpha-amylase (73%), isothiocyanato-coupled alpha-amylase (67%), diazo-coupled beta-amylase (73%), and isothiocyanato-coupled beta-amylase (51%). Similarly, the activity retained after lyophilisation and reconstitution of suspensions of acid azide coupled alpha-amylase (84%) was better than diazo-coupled alpha-amylase (28%) and isothiocyanato-coupled alpha-amylase (44%). The enhanced stability of acid azide-coupled alpha-amylase probably arises because it is attached to the more hydrophilic copolymer.

The stabilities of the water-insoluble alpha- and beta-amylase derivatives to heat denaturation were similar to those of the corresponding cellulose-based derivatives of these enzymes. The water-insoluble alpha-amylase preparations (see Fig. 1) exhibited a very large increase in stability over the free enzyme in solution. It is interesting to note that the most stable alpha-amylase derivative is again that involving Enzacryl AH, the more hydrophilic carrier. The difference in stability between the bound and soluble forms of beta-amylase (see Fig. 2) was much less marked, free beta-amylase being the more stable. An unusual point observed in this experiment was that the beta-amylase used was more stable in solution than samples of the same enzyme from batches supplied for previous work[2].

Our results seem to indicate that glycoside hydrolases may be usefully coupled to water-insoluble copolymers of acrylamide. Further work is projected, both to improve the carriers and to extend our studies to other enzymes.

ACKNOWLEDGMENTS

We thank Mr. D. H. Servant, M. A., for advice on the radiochemical assays. Dr. P. B. Koch and Koch–Light Ltd. are thanked for their interest in this work.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

PREPARATION OF NUCLEOSIDES *via* ISOPROPYLIDENE SUGAR DERIVATIVES

PART V. COUPLING REACTIONS USING THE TITANIUM TETRACHLORIDE METHOD

LEON M. LERNER AND YING YING CHENG

Department of Biochemistry, State University of New York, Downstate Medical Center, Brooklyn, New York 11203 (U. S. A.)

(Received January 10th, 1970)

ABSTRACT

Sugar derivatives that are blocked with isopropylidene groups can be converted directly to adenine nucleosides, if they are first acylated and then coupled with 6-benzamidochloromercuripurine by the titanium tetrachloride procedure. The preparation of 9-(2,3-*O*-isopropylidene-α-L-rhamnofuranosyl)adenine, 9-α-L-rhamnofuranosyladenine, 9-(2,3-*O*-isopropylidene-α-D-mannofuranosyl)adenine, 9-α-D-mannofuranosyladenine, and 9-β-D-mannofuranosyladenine are described. 9-β-D-Mannofuranosyladenine was also produced in extremely small yield by treatment of 2,3:5,6-di-*O*-isopropylidene-α-D-mannofuranosyl chloride with 6-benzamidochloromercuripurine in xylene at reflux.

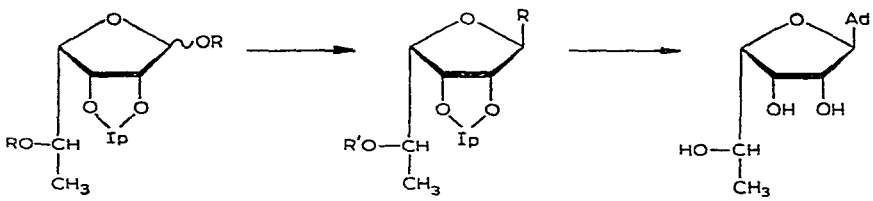
INTRODUCTION

Hexofuranosyl nucleosides could be prepared directly from sugars whose vicinal, *cis* hydroxyls were blocked with isopropylidene groups[1, 2]. The glycosyl chloride treated with 6-benzamidochloromercuripurine[3] gave good yields of nucleosides, and the reactions proceeded primarily with retention of configuration[2, 4, 5]. It is not always possible to introduce a chloride selectively at C-1 of an aldose, for example, when the anomeric hydroxyl is already blocked by an acyl group, necessitating its selective removal before a chloride could be substituted for it. This is required because isopropylidene sugars are generally unstable under the usual conditions of glycosyl halide synthesis[6]. Another instance is one in which another hydroxyl, in addition to the anomeric hydroxyl, is not blocked, making selective chlorination of the anomeric site impossible. This paper presents the results of the coupling of isopropylidene sugar derivatives with 6-benzamidochloromercuripurine using the titanium tetrachloride method[7].

RESULTS

The 1,5-di-*O*-acyl-2,3-*O*-isopropylidene-L-rhamnofuranoses and the 1-*O*-acyl-2,3:5,6-di-*O*-isopropylidene-D-mannofuranoses (acyl = benzoyl or acetyl), were

selected to facilitate the identification of the nucleoside products[1,8]. Both 1,5-di-*O*-acetyl-2,3-*O*-isopropylidene-L-rhamnofuranose (**1**) or 1,5-di-*O*-benzoyl-2,3-*O*-isopropylidene-L-rhamnofuranose[8] (**2**) were coupled with 6-benzamidochloromercuripurine. Several procedures for the isolation of intermediates were employed and two new compounds, 9-(5-*O*-acetyl-2,3-*O*-isopropylidene-α-L-rhamnofuranosyl)adenine picrate (**3**) and 9-(2,3-*O*-isopropylidene-α-L-rhamnofuranosyl)adenine (**4**), were obtained in crystalline form. The structure of **4** was verified by mild acid hydrolysis to be 9-α-L-rhamnofuranosyladenine (**5**), a compound previously prepared by another route[8].



1 R = Ac
2 R = Bz

3 R = AdH⁺ picrate ; R' = Ac
4 R = Ad ; R' = H
Ad = adeninyl

5

Removal of isopropylidene groups from purine nucleosides without concomitant hydrolysis of the glycosylic bond is difficult, and satisfactory conditions have to be worked out in the case of each nucleoside derivative. A new method[9], using trifluoroacetic acid, gave only a small yield of **5**, the main product being adenine. Treatment of **4** with 0.05M sulfuric acid at room temperature, which was successful with 9-(2,3-*O*-isopropylidene-D-gulofuranosyl)adenine[2], yielded mainly **4** and little of **5**. A promising procedure involving hydrolysis of the isopropylidene nucleoside picrate in boiling water, which worked well for 9-(2,3-*O*-isopropylidene-L-*erythro*-furanosyl)adenine picrate[5], completely degraded the nucleoside to adenine and L-rhamnose.

Although the titanium tetrachloride method proceeded satisfactorily for the L-rhamnose derivatives, the yield of coupled products was very poor for 1-*O*-acetyl-2,3:5,6-di-*O*-isopropylidene-D-mannofuranose (**6**), and 1-*O*-benzoyl-2,3:5,6-di-*O*-isopropylidene-D-mannofuranose (**7**). The low reactivity of some sugar derivatives in the titanium tetrachloride method has been noted previously[10]. Nevertheless, small yields of 9-(2,3-*O*-isopropylidene-α-D-mannofuranosyl)adenine (**8**) and 9-α-D-mannofuranosyladenine (**9**) were isolated after acid hydrolysis.

In an earlier preparation of **9**, no nucleoside possessing the β-D configuration was detected by paper chromatography or t.l.c.[1]. Application of the chromatographic procedure of Dekker[11] on the mother liquors of recent experiments resulted in the isolation of a very small amount of 9-β-D-mannofuranosyladenine (**10**). Similarly, a small yield of **10** resulted from the titanium tetrachloride procedure. Presumably, a small amount of the β-D anomer of **5** was present as the result of this type of synthesis, but no attempt was made to isolate or identify such a product. The titanium tetra-

chloride method often yields both anomers, even in the cases where the blocking group is an acetyl, the *trans* anomer being the product obtained in greater yield[12].

The physical properties of the known mannosides of adenine are reported in Table I. The elementary analysis of **10** was as expected for an adenine mannoside and the specific rotation (−35°) indicated a β-D configuration (Hudson's rules of isorotation). The molecular rotation also compared favorably with that of other known hexofuranosyl glycosides of β-D configuration. Compound **10** was homogeneous when chromatographed on t.l.c. plates, and its mobility was identical with that of **9**. The maximum at 259 nm suggested that the adenine bond to mannose was at N-9. The comparison of these properties with those of the anomeric mannopyranosyladenines demonstrates that the furanose ring was retained during the condensation and that no rearrangement to a pyranose ring had occurred.

TABLE I

PHYSICAL PROPERTIES OF 9-D-MANNOSYLADENINE COMPOUNDS

Compound	*M.p. (degrees)*	$[\alpha]_D$ *in water (degrees)*	λ_{max} *(nm)*
β-Furanosyl (**10**)	amorphous	−35	259
α-Furanosyl (**9**)[a]	237 (rods)	+75	259
β-Pyranosyl[b]	175 (needles)	+35	—
α-Pyranosyl[c]	146 (plates)	+73.5	260

[a]Ref. 1. [b]B. LYTHGOE, H. SMITH, AND A. R. TODD, *J. Chem. Soc.*, (1947) 355. [c]L. M. LERNER AND P. KOHN, *J. Med. Chem.*, 7 (1964) 655.

EXPERIMENTAL

Melting points were determined on a Kofler hot stage and correspond to corrected values. Elementary analyses were made by the Baron Consulting Co., Orange, Connecticut, or by the Spang Microanalytical Laboratory, Ann Arbor, Michigan. Evaporations were performed *in vacuo* in a rotary evaporator at a bath temperature of 40–45°. T.l.c. was performed on Silica Gel HF plates (E. Merck A.G., Darmstadt) of 0.25-mm thickness, or on precoated F_{254} plates where noted. Spots were located with an ultraviolet lamp or with chromic acid. Solvent systems (v/v): *A*, 86:14 butyl alcohol–water; *B*, 5% aqueous disodium hydrogen phosphate; *C*, 9:1 ethyl acetate–methanol.

1-O-Benzoyl-2,3:5,6-di-O-isopropylidene-D-mannofuranose (**7**). — Benzoyl chloride (1.4 ml) was added to a stirred mixture of 2,3:5,6-di-*O*-isopropylidene-D-mannofuranose (2 g) and dry pyridine (25 ml), which was chilled in an ice bath. After 1 h of stirring, the solution was kept for 24 h at room temperature and then poured into a vigorously stirred mixture of ice–water (250 ml). The mixture was stirred for 2 h and the crystals were isolated by filtration. The product was washed well with water and recrystallized from ethanol to give two crops of needles, 2.05 g (78% yield);

m.p. 133°; $[\alpha]_D^{25}$ +35° (*c* 3.23, chloroform); i.r. data: ν_{max}^{KBr} 1722 (C=O of benzoate), 1375 (*gem*-dimethyl), and 712 cm^{-1} (monosubstituted phenyl).

Anal. Calc. for $C_{19}H_{24}O_7$: C, 62.62; H, 6.64. Found: C, 62.78; H, 6.65.

1,5-Di-O-acetyl-2,3-O-isopropylidene-L-rhamnofuranose (**1**). — A solution of 2,3-isopropylidene-L-rhamnofuranose[8] (5 g) in pyridine (50 ml) was chilled in an ice bath, and acetic anhydride (20 ml) was added. The mixture was kept for 24 h at room temperature and then poured on ice. The product was extracted with chloroform, and the chloroform solution was washed with saturated sodium hydrogen carbonate and water, and dried. Evaporation of the solvent yielded a clear syrup (6.1 g), which was satisfactory for the next step. The i.r. spectrum showed no OH absorption, but distinctive peaks at 1741 (C=O of acetate) and 1369 cm^{-1} (*gem*-dimethyl).

9-(5-O-Acetyl-2,3-O-isopropylidene-α-L-rhamnofuranosyl)adenine picrate (**3**). — The coupling reaction and workup were performed according to previously described methods[7]. The reaction mixture consisted of **1** (6.9 g), 6-benzamidochloromercuripurine[3] (14.3 g), Celite-545 (14.3 g), titanium tetrachloride (3.3 ml), and 1,2-dichloroethane (1 liter). From this mixture was obtained 8.8 g of a dark syrup. A portion of this (4.8 g) was dissolved in hot abs. ethanol (40 ml), and 10% ethanolic picric acid (60 ml) was added[13]. The mixture was heated at reflux for 5 min and then kept for 24 h at room temperature. The yellow crystals (3.0 g) were filtered off and recrystallized from methanol–ethyl acetate (2.4 g in two crops). The analytical sample was obtained by recrystallization of a small amount from methanol, m.p. 226–228° (dec.) with a wet appearance at 210°; i.r. data: ν_{max}^{KBr} 3090 (NH), 1735 (C=O of acetate), 1692 (protonated adenine ring), 1546 (NO_2), 1361 (*gem*-dimethyl), and 1316 cm^{-1} (NO_2).

Anal. Calc. for $C_{22}H_{24}N_8O_{12}$: C, 44.58; H, 4.08; N, 18.91. Found: C, 44.15; H, 4.07; N, 18.24.

9-(2,3-O-Isopropylidene-α-L-rhamnofuranosyl)adenine (**4**). — *Method A.* Compound **3** (2.32 g) was dissolved in 80% aqueous acetone (150 ml), and the yellow color of the solution was removed[14] with Bio-Rad (AG1-X8, CO_3). After filtration, the filtrate was evaporated to dryness (with the aid of abs. ethanol), and the acetyl group was removed in 0.1M methanolic sodium methoxide (50 ml, for 16 h at room temperature). After neutralization with Dowex-50 (H^+), filtration, and evaporation, the product (193 mg) was crystallized from methanol–water as clusters of rosettes, m.p. 222–227° with prior wetting. Recrystallization gave the analytical sample, m.p. 225–228°, R_{Ade} 1.90 (solvent *A*), R_{Ade} 0.67 (solvent *B*); i.r. data: ν_{max}^{KBr} 3240, 3080 (OH, NH), 1680 ($NH_2C=N$), 1610, 1572 (purine ring), 1381 (*gem*-dimethyl), 1110, 1096, 1085, and 1068 cm^{-1} (C–O).

Anal. Calc. for $C_{14}H_{19}N_5O_4$: C, 52.32; H, 5.97; N, 21.80. Found: C, 52.40; H, 5.59; N, 21.92.

Method B. The reaction mixture consisted of 1,5-di-*O*-benzoyl-2,3-*O*-isopropylidene-L-rhamnofuranose[8] (**2**, 6.41 g), 6-benzamidochloromercuripurine (9.1 g), Celite-545 (9.1 g), and 1,2-dichloroethane (680 ml). The procedure followed the methods described earlier, and a dark syrup (6.2 g) was obtained. A portion of this

syrup (4.1 g) was dissolved in 0.1M methanolic sodium methoxide and heated for 1 h at reflux. After being cooled to room temperature, the solution was neutralized with Dowex-50 (H^+). The resin was removed by filtration, and the solution was concentrated to a volume of about 40 ml. To this solution was added 10% methanolic picric acid (75 ml), and the flask was chilled in an ice bath for 1.5 h. The yellow crystals (2.65 g) were removed by filtration and washed well with ice-cold methanol. The picrate ion was removed in an aqueous acetone solution as described in Method A. The syrup was dissolved in methanol, and some brown impurities were removed with Darco G-60. Evaporation gave a white foam (1.18 g) which was purified by chromatography on a column of silicic acid (Maelinckrodt, 100 mesh, 28 × 3.5 cm) which was packed in 1:1 benzene–ethyl acetate. The column was washed with ethyl acetate (500 ml) and then with solvent *C*. Fractions (50 ml) were collected from the latter solvent mixture and fractions 12-18, which gave a homogeneous spot identical with that given by **4** obtained from Method A (R_F 0.20, solvent *C*), were combined and evaporated to dryness. A white crystalline mass formed which was triturated with ice-cold water and filtered off to give 350 mg. Recrystallization from methanol–water yielded 280 mg of rosettes in two crops, m.p. 226–228°. This product was identical in all respects with that obtained from Method A. Several minor products obtained from the silicic acid column were not investigated, but one of these may have been the β-L-anomer.

Method C. Syrupy 9-(5-*O*-acetyl-2,3-*O*-isopropylidene-L-rhamnofuranosyl)-6-benzamidopurine (7 g) was obtained by coupling **1** (5.4 g) with 6-benzamidochloromercuripurine (11.1 g) by the methods already described[7]. The acyl blocking groups were removed with hot sodium methoxide, and the solution was neutralized and evaporated to a syrup. This was dissolved in 0.05M sulfuric acid (200 ml) and kept for 5 days at room temperature. The mixture was treated with 1 equivalent of barium hydroxide in 50 ml of water and, after stirring for 1 h, the mixture was filtered and the filtrate was evaporated to a syrup. This was dissolved in 30% aqueous methanol and applied to a column of Bio-Rad AG 1-X2 (200–400 mesh. OH^-)[11]. Elution with 30% aqueous methanol gave a major peak which yielded 783 mg of crystals. Recrystallization gave **4**, identical with the previous samples.

9-α-L-Rhamnofuranosyladenine (**5**). — *Method A.* Elution of the resin column, described in the preceeding paragraph, with 60% aqueous methanol gave two minor peaks and a major peak. The latter crystallized from ethanol–2-butanone to give **5** (216 mg) which appeared to be identical with the product described by Baker and Hewson[8], m.p. 132–135°, $[\alpha]_D^{25}$ −10° (*c* 0.375, water); lit.[8] m.p. 132–135°, $[\alpha]_D^{27}$ −18° (*c* 0.3, water). The i.r. showed the C = O peak of the solvate at 1708 cm^{-1} as previously described[8].

Method B[9]. Compound **4** (248 mg) was dissolved in 90% trifluoroacetic acid (3 ml) over a period of 5 min. When the substance was completely dissolved, the solution was kept for 1 min, and then it was diluted with abs. ethanol (45 ml). The solvent was evaporated (bath temperature under 40°) and ethanol (2 ml portions) was added and evaporated 5 times. The residue was dissolved in water (20 ml), the

pH was adjusted to 6 with Dowex-1 (OH^-), and the resin was filtered off. Crystallization as described in Method A gave **5** (48 mg), identical in every test with the previous product. T.l.c. of the mother liquor showed that adenine was the main product.

9-(2,3-O-Isopropylidene-α-D-mannofuranosyl)adenine (**8**). — 1-*O*-Acetyl-2,3:5,6-di-*O*-isopropylidene-D-mannofuranose[15] (2.8 g) was coupled with 6-benzamidochloromercuripurine (5.6 g) as previously described[7]. A syrup was obtained which contained 6-benzamido-9-(2,3:5,6-di-*O*-isopropylidene-D-mannofuranosyl)purine. This was treated with sodium methoxide and neutralized, as previously described. The isopropylidene groups were hydrolyzed with 0.05M sulfuric acid (50 ml) for 5 days at room temperature. Barium hydroxide (1 equivalent in 40 ml of water) was added, and the mixture was stirred for 1 h and filtered. The water was evaporated, and the residual syrup was recrystallized from ethanol–water to give 50 mg of crystals. Recrystallization raised the m.p. to 248°. No depression of the m.p. was observed upon admixture with an authentic sample[1], and the i.r. spectra and t.l.c. were identical.

9-α-D-Mannofuranosyladenine (**9**). — The mother liquor from the preceding step was chromatographed on Bio-Rad AG 1-X2 (200–400 mesh, OH^-, 15 × 1.5 cm) using 60% aqueous methanol[11]. After two minor u.v.-absorbing peaks had been eluted (see the preparation of **10**, Method B), a third major peak was obtained, from which 90 mg was obtained in crystalline form. The m.p., mixed m.p., t.l.c., and i.r. spectrum established the identity of this compound[1].

9-β-D-Mannofuranosyladenine (**10**). — *Method A*. 6-Benzamidochloromercuripurine (15.6 g) and 2,3:5,6-di-*O*-isopropylidene-α-D-mannofuranosyl chloride (9.1 g) were coupled[1], and, after crystallization of **8**, the mother liquors were combined and the solvent was evaporated. The residue was treated with 25% acetic acid (100 ml) for 3 h at 100°, and the solvent was evaporated. From the residue a picrate was prepared, and the free nucleoside was regenerated in the usual manner[16]. The aqueous solution was concentrated to several ml and applied to a Bio-Rad AG 1-X2 column[11] (26 × 1 cm). The column was washed with 30% aqueous methanol (50 ml) and 60% aqueous methanol (100 ml), and the anomeric nucleosides were eluted with 90% aqueous methanol. The first peak contained **10** and the second peak contained **9** (25 mg). This order was unexpected since it has been reported that β anomers of nucleosides generally emerge after α anomers on these columns[17]. Compound **10** was purified further by a repeat of the chromatography with 60% aqueous methanol. Attempts to crystallize **10** from common solvents were unsuccessful. Evaporation of the solvent afforded a solid which was dried by several additions and evaporations of abs. ethanol. The product (23 mg) was dried for 48 h at 100° over phosphorus pentoxide under high vacuum; it became a viscous syrup at 130–140°; $[\alpha]_D^{25}$ −35 ±2° (*c* 0.69, M hydrochloric acid); u.v. data: $\lambda_{max}^{H_2O}$ 259 nm (ε 13,000); i.r. data: ν_{max}^{KBr} 3400–3100 (broad OH, NH), 1640, 1595, 1570 (purine ring), 1085, 1055 cm^{-1} (C–O). Compound **10** was homogeneous on F_{254} t.l.c. plates, R_{Ade} 0.64 (solvent *A*), R_{Ade} 1.20 (solvent *B*). Identical values were obtained for **9**.

Anal. Calc. for $C_{11}H_{15}N_5O_5$: C, 44.45; H, 5.09; N, 23.56. Found: C, 44.31; H, 4.97; N, 23.45.

Method B. The minor peak that was eluted from the Bio-Rad column just before the peak containing **9** in the titanium tetrachloride coupling experiment (see preparation of **9**), was rechromatographed using 60% aqueous methanol and 10 mg of **10** was obtained, identical with the compound described in Method A (i.r. and t.l.c).

ACKNOWLEDGMENT

This work was supported by Grant No. T-442 from the American Cancer Society.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

NOUVELLE VOIE D'ACCÈS AUX DÉRIVÉS MÉTHYL β-D-LYXOFURANOSIQUES À PARTIR DU MÉTHYL 2,3,5-TRI-*O*-*p*-TOLYLSULFONYL-β-D-RIBOFURANOSIDE*

J. HILDESHEIM**, J. CLÉOPHAX ET S. D. GÉRO
Institut de Chimie des Substances Naturelles, C.N.R.S., 91 Gif-sur-Yvette (France)
(Reçu le 12 janvier 1970)

ABSTRACT

The synthesis of derivatives of methyl β-D-lyxofuranoside has been accomplished starting from methyl tri-*O*-*p*-tolylsulfonyl-β-D-ribofuranoside. Treatment of this compound with sodium benzoate in *N*,*N*-dimethylformamide displaced the tosyloxy groups at C-3 and C-5. The resulting *trans*-benzoyloxysulfonate was further transformed stereospecifically into methyl 3,5-di-*O*-benzoyl-β-D-lyxofuranoside, after hydrolysis of the intermediate phenoxonium ion. A minor product, methyl tri-*O*-benzoyl-β-D-lyxofuranoside, was formed by benzoylation of the major product with *N*,*N*-dimethylformamide–sodium benzoate.

SOMMAIRE

La synthèse de dérivés du méthyl β-D-lyxofuranoside a été effectuée à partir du méthyl tri-*O*-*p*-tolylsulfonyl-β-D-ribofuranoside. Le traitement de ce dernier par le benzoate de sodium dans la *N*,*N*-diméthylformamide a causé le déplacement des groupements sulfonyloxy sur C-3 et C-5. Le dérivé *trans*-benzoyloxy-sulfonyle résultant a mené alors stéréospécifiquement au méthyl 3,5-di-*O*-benzoyl-β-D-lyxofuranoside après hydrolyse de l'ion intermédiaire phénoxonium. Un produit mineur, le méthyl tri-*O*-benzoyl-β-D-lyxofuranoside, a été formé par benzoylation du produit majeur par le réactif *N*,*N*-diméthylformamide–benzoate de sodium.

INTRODUCTION

Les méthyl β-pentofuranosides possédant les configurations *ribo*, *xylo* et *arabino* peuvent être préparés par glycosidification des pentoses correspondants avec l'acide chlorhydrique gazeux dans le méthanol[2,3]. Cette voie n'est cependant pas applicable à la préparation du méthyl β-D-lyxofuranoside. Il a en effet été montré que le méthyl α-D-lyxofuranoside est le produit majeur de la glycosidification du D-lyxose[3], l'anomère β n'étant isolé qu'avec un rendement négligeable (3–5%). Ce fait est

*Une communication préliminaire de ce travail a été publiée[1].
**Présente adresse : Department of Biophysics, The Weizmann Institute of Science, Rehovoth, Israel.

explicable si l'on considère les interactions qu'exercent entre eux les hydroxyles de C-1, C-2, C-3 et C-5, dues au fait que ces groupements se trouvent tous situés d'un même côté du plan du cycle lorsque la configuration sur le carbone anomérique est *β*.

Dans le cadre d'un programme ayant pour objet des modifications fonctionnelles dans des cycles pentofuranosique et tétrahydrofurannique, nous avions besoin de quantités importantes de méthyl *β*-D-lyxofuranoside. Les rendements obtenus par glycosidification du D-lyxose ne sont pas compatibles avec une utilisation préparative de cette dernière réaction. La voie que nous avons utilisée fait appel à une substitution du type S_N2 par l'ion benzoate sur les deux groupes tosyloxy d'un cycle ribofuranosique, suivie d'un déplacement intramoléculaire, avec assistance anchimérique du groupement benzoyloxy ainsi introduit.

RÉSULTATS ET DISCUSSION

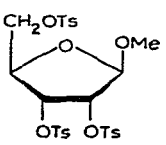
Récemment, nous avons montré[4] que l'ion azide déplaçait sélectivement, suivant un processus de type S_N2, les groupes sulfonyloxy de C-3 et C-5 du méthyl 2,3,5-tri-*O*-*p*-tolylsulfonyl-*β*-D-ribofuranoside (**1**). Bien que moins nucléophile que l'ion azide, il était raisonnable d'anticiper que le benzoate de sodium déplacerait les deux groupements tosyloxy des carbones 3 et 5 dans le système pertosylé **1**. Le dérivé dibenzoyltosylé de configuration *xylo* **3** ainsi obtenu posséderait la géométrie requise pour subir un déplacement intramoléculaire du groupe sulfonate de C-2 par assistance anchimérique du groupement benzoyloxy de C-3, menant finalement au dérivé *β*-D-lyxofuranosique **5**.

Lorsque le dérivé tri-*O*-*p*-tolylsulfonylé **1** est chauffé 20 h à 140° en présence de benzoate de sodium dans la *N*,*N*-diméthylformamide, on note sur couche mince la disparition totale de la substance de départ **1** et la formation de quatre nouveaux composés, *A*, *B*, *C* et *D*, ainsi nommés respectivement suivant l'ordre de R_F décroissant. Une séparation chromatographique sur colonne de gel de silice a permis de séparer ces produits à l'état pur.

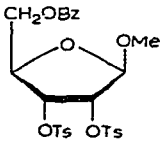
Le produit *A* est obtenu sous forme cristalline avec un rendement de 5%. Ce dernier montre l'absence de bande correspondant au groupe tosyloxy à 1590 cm^{-1} dans son spectre infrarouge. Le spectre de masse présente une fragmentation compatible avec un dérivé tri-*O*-benzoylé, ce que confirment son analyse élémentaire et son spectre de r.m.n. Par débenzoylation catalytique de *A* suivie d'une hydrolyse chlorhydrique, le D-lyxose a été obtenu et caractérisé. La substance *A* est par conséquent le méthyl 2,3,5-tri-*O*-benzoyl-*β*-D-lyxofuranoside (**7**).

Le produit *B* obtenu sous forme également cristalline avec un rendement de l'ordre de 17% révèle deux groupements benzoylés et un tosylé en r.m.n. Son spectre de masse montre un pic moléculaire à *m/e* 526, ainsi que les ions *a* et *b* correspondant à une coupure au niveau des chaînes latérales. Pour des raisons d'interactions dipolaires défavorables que nous avons explicitées dans une précédente publication[4], il est peu probable qu'un déplacement se soit produit sur C-2. Ceci se trouve confirmé par l'examen du spectre de r.m.n. Les protons de C-1 et C-2 se présentent sous forme

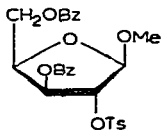
d'un doublet (τ 4,95, $J_{1,2}$ 1,5 Hz). La valeur de cette constante montre clairement que la stéréochimie sur les carbones 1 et 2 s'est maintenue au cours de la réaction. D'autre part, on observe un déplacement paramagnétique très net de H-3 qui résonne sous forme d'un doublet de triplet à τ 4,37 alors qu'il apparaissait dans la substance de départ **1** comme un multiplet non résolu centré à τ 5,25. Ces données suggèrent pour le produit *B* la structure du méthyl 3,5-di-*O*-benzoyl-2-*O*-*p*-tolylsulfonyl-β-D-xylofuranoside (**3**). Le produit **3**, par un temps de chauffage prolongé, peut être



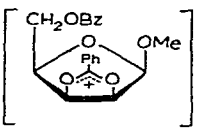
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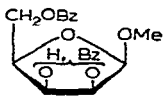
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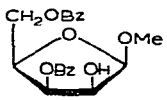
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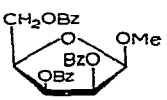
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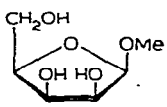
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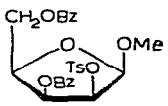
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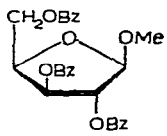
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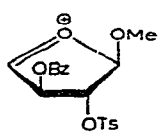
10

progressivement transformé dans le milieu réactionnel en la substance *D*. D'autre part si **3** est traité par le benzoate de sodium ou le fluorure de sodium dans la *N*,*N*-diméthylformamide dans les mêmes conditions que dans la réaction originale, on observe sa disparition quasi totale (sur couche mince) au profit du produit *D*. Ce dernier est donc issu de **3** dans le milieu réactionnel.

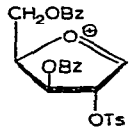
Le produit *C* cristallin, est isolé avec un rendement de 15%. Il a été identifié au méthyl 5-*O*-benzoyl-2,3-di-*O*-*p*-tolylsulfonyl-β-D-ribofuranoside (**2**) que nous avons déjà décrit dans une précédente publication[5]. Celui-ci peut être transformé totalement, par un temps de réaction prolongé, en dérivés *B* et *D*.

Le produit *D* n'a pu être cristallisé. Il est obtenu sous forme d'une huile homogène en chromatographie sur couche mince, avec un rendement de 30%. Son spectre i.r. montre clairement la présence d'un groupe hydroxyle vers 3500 cm^{-1} et l'absence de fonction tosyle à 1590 cm^{-1}. Le spectre de r.m.n. de *D* montre le proton glycosidique à τ 5 sous forme d'un doublet. La valeur de la constante de couplage $J_{1,2}$ 6 Hz est en accord avec une disposition « *cis* » des protons sur C-1 et C-2. A ce stade, la structure de *D* a été attribuée à un méthyl 2(ou 3), 5-di-*O*-benzoyl-β-D-lyxofuranoside (**5**). Le spectre de masse montre le pic moléculaire attendu à *m/e* 372 ainsi que les ions *c* et *d*, provenant de la coupure au niveau des chaînes latérales. Le produit *D* peut être benzoylé et le composé perbenzoylé ainsi obtenu est identique au

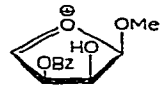
méthyl 2,3,5-tri-*O*-benzoyl-*β*-D-lyxofuranoside (**7**). D'autre part, une débenzoylation catalytique de **5** par le sodium méthanolique fournit quantitativement le méthyl *β*-D-lyxofuranoside (**8**) sous forme sirupeuse. Après purification chromatographique et



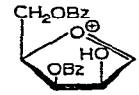
a *m/e* 391



b *m/e* 495



c *m/e* 237



d *m/e* 341

séchage prolongé, ce dernier possède un $[\alpha]_D$ −112°. Le pouvoir rotatoire de l'anomère α est : $[\alpha]_D$ +128° (Réf. 2). Ces données sont en accord avec les valeurs théoriques que l'on peut attendre en appliquant la règle d'Hudson[6,7] à l'un ou l'autre de ces anomères.

Les rendements en méthyl *β*-D-lyxofuranoside (**8**) à partir du méthyl 2,3,5-tri-*O*-*p*-tolylsulfonyl-*β*-D-ribofuranoside (**1**) dans les conditions décrites ci-dessus sont de l'ordre de 35%. En augmentant le temps de chauffage jusqu'à 35 h ce rendement atteint 48–50%.

La formation du méthyl 3,5-di-*O*-benzoyl-2-*O*-*p*-tolylsulfonyl-*β*-D-xylofuranoside (**3**) ne peut être expliquée que par une substitution de type S_N2 du groupe tosyloxy en C-3 du dérivé **2**. En effet, on pourrait envisager théoriquement un déplacement du groupe tosyloxy en C-3 de **2** avec participation du groupe benzoate en C-5, et dans la même perspective, on pouvait se demander si la formation du méthyl 2,3,5-tri-*O*-benzoyl-*β*-D-lyxofuranoside (**7**) ne serait pas également due à un intermédiaire orthobenzoate C-2–C-5.

La possibilité de réaction *via* un ion orthoester à 6 chaînons C-3–C-5 a déjà été discutée au sujet du traitement du même composé méthyl 5-*O*-benzoyl-2,3-di-*O*-*p*-tolylsulfonyl-*β*-D-ribofuranoside (**2**) par l'azothydrate de sodium[15], et nous avions exclu sans équivoque une assistance anchimérique du benzoate en C-5 vers le groupe tosyloxy de C-3.

En ce qui concerne plus particulièrement l'obtention de **3**, la présence d'une trace d'eau dans le milieu réactionnel* devrait fournir, pour une substitution avec assistance du groupe benzoate en C-5, en plus de **3**, les dérivés D-xylo et D-ribo dont l'hydroxyle en C-3 serait libre. De même, si la formation de **7** se faisait par un intermédiaire orthoester C-2–C-5 à 7 chaînons, elle conduirait à côté de **6** et de **7**, aux composés appropriés D-xylo et D-ribo hydroxylés en C-2. Or un ion orthoester à 7 chaînons nous semble très improbable et n'a jamais été signalé dans la littérature. Le fait qu'aucune substance autre que **2**, **3**, **6** et **7** n'ait été décelée lors de la réaction permet d'éliminer l'hypothèse d'une participation acyloxy du C-5 benzoate dans le déplacement du groupe *p*-tolylsulfonyle en C-3 ou en C-2.

*L'obtention du dérivé **6** indique la présence de traces d'eau dans la *N*,*N*-diméthylformamide utilisée. Ce solvant avait été distillé une fois sous pression réduite et séché sur tamis moléculaire en perles 4Å.

A l'appui de notre conclusion, on peut noter que l'existence d'un ion orthoester à 6 chaînons dans des cas semblables au nôtre, a été écartée par d'autres auteurs[16,17].

Deux points néanmoins restaient à éclaircir : (1) la voie d'obtention du méthyl 2,3,5-tri-*O*-benzoyl-β-D-lyxofuranoside (7); (2) la formation exclusive du méthyl 3,5-di-*O*-benzoyl-β-D-lyxofuranoside (6). Concernant le premier point, deux hypothèses peuvent être avancées :

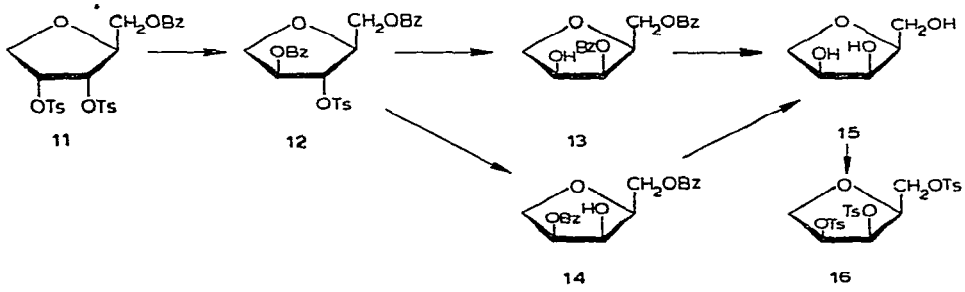
(a) Un double déplacement de type S_N2 sur C-2 et C-3 du dérivé 5-*O*-benzoyl-2,3-ditosyloxy **2**. Cette première hypothèse, si elle était concevable, ne résistait que difficilement à un examen attentif de la littérature. Le site C-2 est en effet inerte vis-à-vis de déplacements nucléophiles quelle que soit la configuration sur le carbone anomérique. A notre connaissance, un seul cas de déplacement par l'ion azide a été décrit lors d'une synthèse de la kasuganobiosamine[8]. Encore faut-il noter dans ce cas la présence dans le cycle hexopyranosique d'un méthylène sur C-3 et, d'autre part, le faible rendement global de cette dernière réaction.

(b) Une benzoylation dans le milieu réactionnel du di-*O*-benzoyllyxofuranoside formé **6**. En effet lorsque **6** est traité à 140° dans la *N*,*N*-diméthylformamide en présence d'anhydride benzoïque, le tribenzoate **7** est formé avec un rendement voisin de 60%. Parallèlement, cette même expérience effectuée en présence de benzoate de sodium permet d'obtenir **7** avec un rendement voisin de 40%. La formation du tribenzoate **7** peut être attribuée à l'acylation de l'hydroxyle libre de **6**, probablement par l'anhydride benzoïque formé in situ dans le milieu réactionnel à partir du benzoate de sodium.

Concernant le second point, il semble que l'étude de la composition des produits du type **5** provenant de l'ouverture d'un ion acyloxonium n'a pas encore été abordée. L'examen attentif du produit **5** sur couche mince dans différents systèmes de solvants n'a montré la présence que d'un produit unique, ce que confirme l'étude du spectre de r.m.n. de **3**, qui montre clairement que seul le proton de C-3 a subi un déplacement paramagnétique dû au groupement benzoyloxy géminal. Il apparaît sous forme de triplet à τ 4,13, J 6 Hz. Par *p*-toluènesulfonylation de **5**, un seul dérivé tosylé **9** est obtenu sous forme cristalline. Il était raisonnable d'anticiper que, si le groupe tosyloxy était situé sur C-3, un déplacement par l'ion benzoate dans la *N*,*N*-diméthylformamide pouvait être envisagé. Par contre, si le groupe tosyloxy était lié à C-2, aucun déplacement n'était attendu. De fait, l'action du benzoate de sodium dans la *N*,*N*-diméthylformamide sur **9** a fourni après 30 h à 140° un produit unique tribenzoylé **10** avec un rendement inférieur à 0,5%. Celui-ci, après séparation de la substance de départ **9** par chromatographie sur couche mince préparative, a été débenzoylé, puis hydrolysé par l'acide chlorydrique aqueux. Le D-xylose ainsi obtenu a été identifié par chromatographie sur papier. Cette suite de réactions conduisant au D-xylose permet d'attribuer à **10**, **9** et **6** respectivement les structures suivantes : méthyl 2,3,5-tri-*O*-benzoyl-β-D-xylofuranoside, méthyl 3,5-di-*O*-benzoyl-2-*O*-*p*-tolylsulfonyl-β-D-lyxofuranoside et finalement méthyl 3,5-di-*O*-benzoyl-β-D-lyxofuranoside. On peut noter le rendement négligeable du déplacement par l'ion benzoate sur **9**. Ceci est bien en accord avec la

présence du groupe tosyloxy sur C-2. Signalons que récemment la synthèse de dérivés du D-lyxofuranose[12] et du 5-amino-D-lyxofuranose[13,14] a été publiée.

La remarquable spécificité d'obention du méthyl 3,5-di-*O*-benzoyl-β-D-lyxofuranoside (**6**), lors du processus d'ouverture de l'ion benzoxonium **4**, nous a incités à examiner ce même type de réaction en série tétrahydrofurannique. Si le 2,5-anhydro-1-*O*-benzoyl-3,4-di-*O*-*p*-tolylsulfonyl-D-ribitol[5] (**11**) est traité par le benzoate de sodium dans la *N*,*N*-diméthylformamide, on obtient sélectivement le 2,5-anhydro-1,4-di-*O*-benzoyl-2-*O*-*p*-tolylsulfonyl-L-lyxitol (**12**). Un tel déplacement sélectif en position **4** est attendu pour des raisons purement stériques et a déjà signalé dans l'une de nos communications précédentes[9]. Les données de la r.m.n. confirment cette hypothèse. En effet le signal complexe, centré à τ 5,2, correspondant aux deux protons de C-3 et C-4 dans le produit de départ **11** est remplacé dans le « *trans* »-benzoyloxytosyle **12** par un quadruplet distinct à τ 4,88 dû au proton de C-3 et, d'autre part, par un multiplet attribuable au proton de C-4 qui a subi, après inversion de configuration, un déplacement paramagnétique prévisible. Ce dernier résonne à τ 4,58. L'arrangement « *trans* » des fonctions sur C-3 et C-4 est confirmé par la valeur du couplage de H-3 : $J_{3,4}$ 3 Hz. Or, le traitement de **12** par le fluorure de sodium dans la *N*,*N*-



diméthylformamide a mené avec un rendement de l'ordre de 60% à un mélange de deux « *cis* »-hydroxydibenzoates **13** et **14** dans le rapport approximatif 1 : 1. Aucun des deux isomères n'a pu être séparé à l'état pur, la chromatographie sur couche mince montrant un R_F très voisin pour ces deux dérivés. Une débenzoylation catalytique du mélange **13**, **14** a fourni un seul produit : le 2,5-anhydro-L-arabitol (**15**) sous forme de sirop. Ce dernier a été caractérisé par son dérivé tritosylé **16** cristallin qui a déjà été décrit par d'autres auteurs[10].

L'obtention spécifique de **6** est sans aucun doute imputable à la nature hémiacétalique de la molécule. Cette situation étant absente dans le cycle tétrahydrofurannique, les deux isomères **13** et **14** sont obtenus en proportions sensiblement égales.

PARTIE EXPÉRIMENTALE

Conditions générales. — Les chromatographies sur colonne ont été effectuées sur gel de silice Davison (100–200 Mesh). Les séparations en couche mince ont été

faites à l'aide de Kieselgel G (E. Merck, Darmstadt). Les chromatographies sur papier ont été effectuées sur papier Whatman No. 1 et ont été révélées soit par le nitrate d'argent, soit par le phtalate acide d'aniline. Les spectres de r.m.n. ont été mesurés dans le chloroforme, à la fréquence de 60 MHz avec un spectrophotomètre Varian A-60. Les déplacements chimiques sont mesurés à partir de la raie du tétraméthylsilane, prise comme zéro de référence, et sont exprimés en τ. Les spectres de masse ont été effectués dans le service de l'Institut sur un appareil MS 9 de la compagnie A.E.I. Les points de fusion ont été déterminés sur un appareil Koffler et sont corrigés; les pouvoirs rotatoires ont été mesurés sur un appareil « Quick » de Roussel et Jouan. Toutes les analyses ont été fournies par le service de microanalyse du C.N.R.S.

Action du benzoate de sodium sur le méthyl 2,3,5-tri-O-p-*tolylsulfonyl-β-*D-*ribofuranoside* (**1**). — Le composé[5] **1** (11 g) dans la *N,N*-diméthylformamide (100 ml) est additionné de benzoate de sodium (12,7 g, 5 éq.). Le tout est porté à 140°. Après 40 h le produit réactionnel est extrait au chloroforme et la phase organique est abondamment rincée à l'eau, séchée sur sulfate de sodium, filtrée, concentrée sous vide partiel, puis sous vide poussé. La chromatographie sur couche mince du sirop brun résultant montre alors la présence de 4 taches : (*A*) R_F 0,78; (B) R_F 0,68; (C) R_F 0,62; (D) R_F 0,38; solvant acétate d'éthyle–éther de pétrole (3:7). Le résidu brut ainsi obtenu est chromatographié sur une colonne de gel de silice (400 g) préalablement équilibrée à l'aide du système 15:85 acétate d'éthyle–éther de pétrole. Ce même solvant permet d'isoler les substances A, B, et C. La substance D est obtenue en augmentant la proportion d'acétate d'éthyle jusqu'à 40%.

*Méthyl 2,3,5-tri-*O-*benzoyl-β-*D-*lyxofuranoside* (**7**). — La substance A a été cristallisée à partir d'éther de pétrole chaud, 584 mg (7%), p.f. 83,5–85°, $[\alpha]_D^{23}$ −70° (*c* 0,45, chloroforme).

Anal. Calc. pour $C_{27}H_{24}O_8$: C, 68,10; H, 5,04. Trouvé : C, 68,50; H, 5,04.

Le tribenzoate 7 (10 mg) est traité par du sodium méthanolique (10 mg dans 2 ml) pendant une nuit à température ordinaire. La solution est neutralisée par de l'Amberlite IR-120 (H^+), filtrée et évaporée. Le résidu est chauffé pendant 30 min dans 0,5 ml d'acide chlorhydrique aqueux 5% à 100°. Après neutralisation par de l'Amberlite IR-45 (OH^-), le produit est identique au lyxose en chromatographie sur papier (R_F 0,356), dans le solvant 4:1:5 1-butanol–acide acétique–eau.

*Méthyl 3,5-di-*O-*benzoyl-2-*O-p-*tolylsulfonyl-β-*D-*xylofuranoside* (**3**). — La substance B cristallise à partir d'acétate d'éthyle–éther de pétrole, 816 mg (8%), p.f. 112–114°, $[\alpha]_D^{22}$ −4,2° (*c* 0,85, chloroforme).

Anal. Calc. pour $C_{27}H_{26}O_9S$: C, 61,59; H, 4,98; S, 6,08. Trouvé : C, 61,63; H, 5,06; S, 6,02.

Le composé 3 (20 mg) dans la *N,N*-diméthylformamide (0,5 ml) est additionné indifféremment de benzoate de sodium ou de fluorure de sodium (25 mg). Après une nuit à 140°, la chromatographie sur couche mince montre la conversion du produit de départ en dérivé **6** (rdt. estimé sur couche mince : 45–55%).

*Méthyl 5-*O-*benzoyl-2,3-di-*O-p-*tolylsulfonyl-β-*D-*ribofuranoside* (**2**). — La

substance C a été obtenue avec un rdt. négligeable dans cet essai (1 %), les temps de réaction étant très longs.

*Méthyl 3,5-di-*O*-benzoyl-β-*D*-lyxofuranoside* (**6**). — La substance D a été obtenue sous forme sirupeuse avec un rdt. de 2,58 g (39,6 %).

Le composé **6** (135 mg) dans la pyridine anhydre (1 ml) est additionné de chlorure de benzoyle (0,054 ml, 1,3 éq.). Après 19 h à température ordinaire, le produit réactionnel est extrait par le chloroforme comme pour **8**. Le produit ainsi obtenu est identique au méthyl 2,3,5-tri-*O*-benzoyl-β-D-lyxofuranoside (**7**).

Le méthyl 3,5-di-*O*-benzoyl-β-D-lyxofuranoside (**6**) dans le méthanol (10 ml) contenant du sodium (30 mg) est laissé 3 h à 50°. La solution est neutralisée par de l'Amberlite IR-120 (H^+), filtrée, concentrée à sec et le résidu est chromatographié sur une colonne de gel de silice (8 g) dans le solvant 85:15 acétate d'éthyle–méthanol. On recueille 178 mg (68 %) d'une substance vitreuse incolore, homogène en chromatographie sur couche mince; R_F 0,3, solvant 1:1 acétate d'éthyle–méthanol. Le méthyl β-D-lyxofuranoside (**8**) ainsi obtenu possède, après séchage prolongé, un pouvoir rotatoire $[\alpha]_D^{23}$ −112° (*c* 0,6, eau), (lit.[3] : $[\alpha]_D$ −84°).

L'hydrolyse du méthyl β-D-lyxofuranoside **8** ainsi obtenu, est effectuée dans les mêmes conditions que pour **7**. Le sirop résultant, après séchage prolongé sous bon vide, a un pouvoir rotatoire $[\alpha]_D^{23}$ −12,7° (*c* 2,2, eau), correspondant à celui du D-lyxose (lit.[12] : $[\alpha]_D$ −13,8°. Le produit d'hydrolyse est en outre identique au D-lyxose en chromatographie sur papier (R_F 0,368), solvant 4:1:5 butanol–acide acétique–eau.

Le composé **6** (60 mg) dans la *N,N*-diméthylformamide (1 ml) est divisé en deux parties égales. La première partie est additionnée de benzoate de sodium (30 mg) et la seconde d'anhydride benzoïque (30 mg). Après 22 h de chauffage à 140° les deux produits réactionnels sont extraits de façon habituelle par le chloroforme et les résidus respectifs déposés sur couche mince en présence d'un échantillon authentique de **7**. La plaque révèle une conversion de l'ordre de 60 % de **6** en méthyl 2,3,5-tri-*O*-benzoyl-β-D-lyxofuranoside (**7**) avec l'anhydride benzoïque. Avec le benzoate de sodium, la conversion est de l'ordre de 40 %.

*Méthyl 3,5-di-*O*-benzoyl-2-*O*-p-tolylsulfonyl-β-*D*-lyxofuranoside* (**9**). — Le composé **6** (600 mg) dans la pyridine (4 ml) est additionné de chlorure de *p*-toluènesulfonyle (612 mg) et le tout est gardé à température ordinaire (40 h). Après extraction par le chloroforme de la manière usuelle, on recueille 645 mg d'un sirop présentant une tache majeure de R_F 0,45 et une tache mineure de R_F 0,50 en couche mince, dans le rapport 20:1, en solvant 7:3 acétate d'éthyle–éther de pétrole. Le produit brut de la réaction est chromatographié sur une colonne de gel de silice (50 g) en solvant 4:1 acétate d'éthyle–éther de pétrole. On recueille un produit (580 mg) cristallisant dans un mélange de benzène et d'éther de pétrole, p.f. 95–97°, $[\alpha]_D^{23}$ −41,6° (*c* 0,48, chloroforme).

Anal. Calc. pour $C_{27}H_{26}O_9S_1$: C, 61,60; H, 4,94; S, 6,08. Trouvé : C, 61,78; H, 4,73; S, 6,14.

*Conversion du méthyl 3,5-di-*O*-benzoyl-2-*O*-p-tolylsulfonyl-β-*D*-lyxofuranoside* **9** *en* D-*xylose*. — Le composé **9** (195 mg) dans la *N,N*-diméthylformamide (2 ml) est

additionné de benzoate de sodium (160 mg) et le tout est chauffé à 130° pendant 30 h. Après ce laps de temps et après extraction du produit réactionnel, le résidu montre sur couche mince deux taches en solvant 3:1 acétate d'éthyle–éther de pétrole : l'une (R_F 0,3) est la substance de départ **9**, l'autre (R_F 0,65) est le méthyl 2,3,5-tri-*O*-benzoyl-β-D-xylofuranoside (**10**). Le rapport de ces deux substances est respectivement de 99:1. Le mélange obtenu est déposé sur une plaque de gel de silice préparative de 20×20 (40 g) et la plaque est éluée à l'aide du solvant décrit ci-dessus. On isole environ 1 mg du dérivé présumé **10**. Ce dernier est successivement débenzoylé et hydrolysé comme pour la substance **7**. Le produit résultant est alors chromatographié sur papier Whatman No. 1 en présence d'échantillons authentiques des quatre pentoses. Il possède une migration identique au D-xylose en solvant 8:2:1 acétate d'éthyle–pyridine–eau.

2,5-Anhydro-1,4-di-O-benzoyl-3-O-p-tolylsulfonyl-L-lyxitol (**12**). — Le 2,5-anhydro-1-*O*-benzoyl-3,4-di-*O*-*p*-tolylsulfonyl-D-ribitol[9] (**11**, 1 g) dans la *N*,*N*-diméthylformamide (15 ml) est additionné de benzoate de sodium (1,15 g, 4 équiv.). Le tout est porté à 120° pour 1 h 30 min. Le produit réactionnel est alors refroidi et extrait par le chloroforme de la manière habituelle. Le sirop résultant montre deux taches en chromatographie sur couche mince en solvant 3:7 acétate d'éthyle–éther de pétrole : R_F 0,5 (produit de départ **11**) et R_F 0,65 (produit formé **12**). Une chromatographie sur colonne de gel de silice avec l'éluant 3:17 acétate d'éthyle–éther de pétrole permet d'obtenir **12** sous forme d'un sirop. Celui-ci cristallise dans un mélange de chloroforme–éther de pétrole, p.f. 65–67°, $[\alpha]_D^{28}$ +60° (*c* 1, chloroforme).

Anal. Calc. pour $C_{20}H_{24}O_8S_1$: C, 62,90; H, 4,87; S, 6,45. Trouvé : C, 62,61; H, 4,98; S, 6,73.

2,5-Anhydro-1,3- et -1,4-di-O-benzoyl-L-arabinitol (**13** *et* **14**). — Le composé **12** (0,5 g) dans la *N*,*N*-diméthylformamide (7 ml) est additionné de 5 équivalents de fluorure de sodium et le tout est porté à 150° pendant 18 h. Après extraction par le chloroforme, le sirop résultant montre deux taches sur couche mince dans le rapport approximatif 1:1 (R_F 0,45 et 0,50) en solvant 3:7 acétate d'éthyle–éther de pétrole. Après filtration sur colonne de gel de silice dans le solvant 3:7 acétate d'éthyle–éther de pétrole, on recueille 207 mg (60 %) du mélange **13** et **14**.

2,5-Anhydro-L-arabinitol (**15**). — Le mélange de **13** et **14** obtenu ci-dessus est dissous dans le méthanol (20 ml) contenant du sodium (60 mg) et le tout est laissé 3 h à température ordinaire. La solution est alors neutralisée par de l'Amberlite MB-3 et concentrée sous vide réduit. Le sirop ainsi obtenu est très hygroscopique : $p.e._{0,01}$ 130–135°, n_D^{26} 1,494. Chromatographie sur papier : R_F 0,43 en solvant 40:11:19 1-butanol–éthanol–eau.

2,5-Anhydro-1,3,5-tri-O-p-tolylsulfonyl-L-arabinitol (**16**). — Le triol **15** (100 mg), préalablement séché au dessiccateur, dans la pyridine anhydre (5 ml) est additionné de chlorure de *p*-toluènesulfonyle (1,2 g, 3 équiv.). Après extraction de la manière habituelle par le chloroforme, le solide obtenu est recristallisé dans l'éthanol, p.f. 139°, $[\alpha]_D^{24}$ −24,5° (*c* 0,57, chloroforme); lit.[10] : p.f. 128°, $[\alpha]_D^{23}$ −27,4°.

Anal. Calc. pour $C_{26}H_{28}O_{10}S_3$: C, 52,35; H, 4,73; S, 16,08. Trouvé : C, 52,41; H, 4,74; S, 16,03.

REMERCIEMENTS

Ce travail a bénéficié d'une aide matérielle de la Ligue Nationale Française contre le Cancer. Nous remercions M. le Professeur E. Lederer pour l'intérêt qu'il a porté à ce travail.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

RÉACTIONS D'ÉLIMINATION ET DE SUBSTITUTION EN SÉRIE MÉTHYL β-D-LYXOFURANOSIQUE*

J. HILDESHEIM**, A. GAUDEMER ET S. D. GÉRO
Institut de Chimie des Substances Naturelles, C.N.R.S., 91 Gif-sur-Yvette (France)
(Reçu le 12 janvier 1970)

ABSTRACT

Reaction of methyl 2,3,5-tri-*O*-*p*-tolylsulfonyl-β-D-lyxofuranoside with sodium benzoate–*N*,*N*-dimethylformamide gave an "enol-tosylate" exclusively, whereas with sodium azide–*N*,*N*-dimethylformamide it gave a mixture of methyl 5-azido-5-deoxy-2,3-di-*O*-*p*-tolylsulfonyl-β-D-lyxofuranoside and methyl 3,5-diazido-3,5-dideoxy-2-*O*-*p*-tolylsulfonyl-β-D-arabinofuranoside. The general outcome of bimolecular nucleophilic displacement reactions on methyl 2,3,5-tri-*O*-*p*-tolylsulfonyl-β-D-lyxofuranoside is discussed and interpreted in terms of "steric compression", and comparative reactivity of the tolylsulfonyloxy groups at C-2, C-3, and C-5.

SOMMAIRE

La réaction du méthyl 2,3,5-tri-*O*-*p*-tolylsulfonyl-β-D-lyxofuranoside avec le benzoate de sodium dans la *N*,*N*-diméthylformamide donne exclusivement un énol-tosylate, alors qu'avec l'azothydrate de sodium dans ce même solvant, un mélange de méthyl 5-azido-5-désoxy-2,3-di-*O*-*p*-tolylsulfonyl-β-D-lyxofuranoside et de méthyl 3,5-diazido-3,5-didésoxy-2-*O*-*p*-tolylsulfonyl-β-D-arabinofuranoside est obtenu. Le comportement général du méthyl 2,3,5-tri-*O*-*p*-tolylsulfonyl-β-D-lyxofuranoside vis-à-vis de réactifs nucléophiles est discuté et interprété en termes de « compression stérique » et de réactivité respective des groupements sulfonyloxy sur C-2, C-3 et C-5.

INTRODUCTION

Les déplacements bimoléculaires sur des sulfonates d'hexopyranosides et de pentofuranosides[2–7] sont devenus des réactions courantes depuis ces cinq dernières années. Certaines difficultés rencontrées dans le déroulement normal de telles réactions ont été attribuées à des effets d'origine stérique ou polaire[3] qui s'opposeraient à la formation d'un état de transition préalable lors du processus de substitution.

*Une communication préliminaire de ce travail a été publiée[1].
**Présente adresse : Department of Biophysics, The Weizmann Institute of Science, Rehovoth, Israel.

Le méthyl β-D-lyxofuranoside[8] **1** représente un système particulièrement encombré du point de vue stérique. L'arrangement spatial des groupes hydroxyle, tous situés de façon *cis* entre eux, est probablement responsable de la tension que l'on pouvait anticiper dans ce type de molécule. Il semblait intéressant d'examiner le comportement du tri-*p*-toluènesulfonate de configuration *lyxo* **2** vis-à-vis de réactifs tels que le benzoate de sodium et l'azothydrate de sodium dans la *N,N*-diméthylformamide.

Le travail que nous décrivons suggère qu'une compression stérique peut jouer un rôle important dans le déroulement d'un processus de solvolyse, ou de substitution.

RÉSULTATS ET DISCUSSION

Le méthyl 2,3,5-tri-*O*-*p*-tolylsulfonyl-β-D-lyxofuranoside (**2**) a été préparé par per-*p*-toluènesulfonylation dans la pyridine du méthyl β-D-lyxofuranoside[8] (**1**). Lorsque **2** est chauffé à 120° dans la *N,N*-diméthylformamide en présence de benzoate de sodium, on observe sur couche mince, dès les premières minutes de la réaction, la formation de deux nouvelles taches. Après deux heures, le produit de départ **2** a disparu au profit d'un seul composé. Par chromatographie sur colonne, une substance sirupeuse et incolore est isolée avec un rendement de 80%. Le spectre infrarouge de celle-ci révèle la présence d'un groupement benzoyloxy à 1720 cm^{-1}, d'une oléfine à 1670 cm^{-1} et d'un groupement tosyloxy à 1600 cm^{-1}. Le spectre ultraviolet effectué dans le méthanol présente deux maxima à 205 mμ (log ε 4,069) et à 229 mμ (log ε 4,35). Ces dernières données sont en accord avec une structure du type « énol-tosyloxy »[9]. Le spectre de masse montre un pic moléculaire à *m/e* 404 ainsi que les ions *a* et *b* issus de fragmentations au niveau des chaînes latérales. Sur la base des données fournies par le spectre de r.m.n.[1] nous avons attribué au dérivé « énol-tosyloxy » formé la structure d'un méthyl 5-*O*-benzoyl-3-désoxy-2-*O*-*p*-tolylsulfonyl-β-D-*glycéro*-pent-2-énofuranoside (**3**). Dans le spectre qui présente les signaux usuels des groupements tosyloxy, méthoxy et benzoyloxy, les protons H-1, H-3 et H-5 apparaissent respectivement à 5,38, 5,87, et 4,98 p.p.m. avec des constantes de couplage $J_{1,3}$ 0,85 Hz, $J_{3,4}$ 1,80 Hz et $J_{1,4}$ 1,15 Hz. L'ensemble de ces valeurs est compatible avec une structure telle que **3** dans laquelle le groupement OCH_3 en C-1 a conservé sa configuration β. La formation stéréosélective de **3** peut s'expliquer par un déplacement initial, par l'ion benzoate, du groupement sulfonate de C-5, suivie d'une élimination d'acide *p*-toluènesulfonique de C-2 et C-3. Le fait que **3** soit le produit unique de la

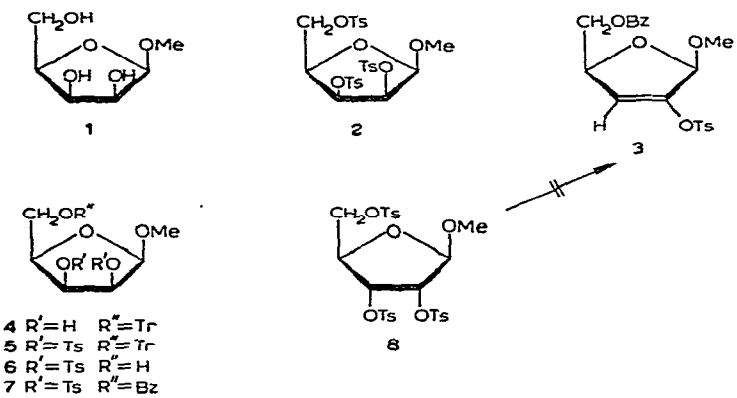


2 **3**

réaction suggère que ce processus qui soulagerait la tension stérique présente dans **2** est une voie qui serait préférée à celle faisant intervenir un déplacement de type S_N2

sur C-3. L'abstraction initiale du proton de C-2 peut s'expliquer par la présence dans la proximité immédiate de ce proton du méthoxyle anomérique, de l'oxygène de l'hétérocycle et du groupement sulfonyloxy géminal. Des réactions similaires d'élimination à partir de sulfonates adjacents ont déjà été décrites en séries cyclique et acyclique[7,10].

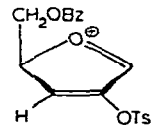
Afin de confirmer la mobilité exceptionnelle du proton de C-2 dans le système **2**, nous avons cherché à obtenir le méthyl 5-*O*-benzoyl-2,3-di-*O*-*p*-tolylsulfonyl-β-D-lyxofuranoside (**7**) qui doit probablement se former en premier dans la réaction conduisant de **2** à **3**. Le dérivé 5-*O*-benzoyl **7** n'a pu être obtenu par le déplacement usuel du sulfonate de C-5 par l'ion benzoate sur **2**. De même, une tentative de benzoylation sélective de l'hydroxyle primaire de **1**, qui aurait par la suite été suivie d'une sulfonylation pour fournir **7**, est demeurée infructueuse. La chlorotriphénylméthylation de **1** n'a fourni qu'en rendement moyen (50%) le méthyl 5-*O*-trityl-β-D-lyxofuranoside (**4**) qui a été per-*p*-toluènesulfonylé dans la pyridine pour mener au méthyl 2,3-di-*O*-*p*-tolylsulfonyl-5-*O*-trityl-β-D-lyxofuranoside (**5**) sirupeux. On peut noter la faible accessibilité de l'hydroxyle primaire, lors de la chlorotriphénylméthylation, réaction considérée normalement comme étant quasi quantitative. L'hydrolyse ménagée de **5** mène au méthyl 2,3-di-*O*-*p*-tolylsulfonyl-β-D-lyxofuranoside (**6**), qui



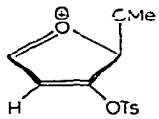
par benzoylation dans la pyridine, fournit le produit **7** désiré. Si **7** est chauffé pendant deux heures dans la *N*,*N*-diméthylformamide seule à 120°, le dérivé énol-tosyloxy[11] **3** est obtenu avec un rendement de l'ordre de 10%. Ainsi ce simple traitement de **7**, lié à l'instabilité d'un système tendu, est suffisant pour provoquer une élimination. En présence de benzoate de sodium, cette même réaction fournit l'oléfine **3** avec un rendement de l'ordre de 70%. A la lumière de ces résultats en quelque sorte inattendus, nous avons décidé de réexaminer la réaction du méthyl 2,3,5-tri-*O*-*p*-tolylsulfonyl-β-D-ribofuranoside (**8**) avec le réactif benzoate de sodium–*N*,*N*-diméthylformamide. L'examen attentif sur couche mince des produits de la réaction n'a pas révélé la formation d'autres produits que ceux déjà décrits antérieurement[8]. Récemment, des dérivés « énol-sulfonyloxy » de type méthyl 4,6-*O*-benzylidène-α-D-*erythro*-hex-2-énopyranoside ont été suggérés comme intermédiaires possibles lors du traitement de

méthyl 4,6-*O*-benzylidène-2,3-di-*O*-méthylsulfonyl-α-D-hexopyranosides par l'hydrazine anhydre[11].

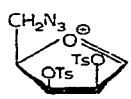
Etant donné la réactivité particulière du méthyl 2,3,5-tri-*O*-*p*-tolylsulfonyl-β-D-lyxofuranoside (**2**) vis-à-vis de l'ion benzoate dans la *N*,*N*-diméthylformamide, il paraissait intéressant d'examiner le comportement de **2** en présence d'un nucléophile puissant comme l'ion azide. Lorsque le dérivé **2** est traité à 100° par l'azothydrate de sodium dans la *N*,*N*-diméthylformamide, on observe sur couche mince la formation simultanée de deux taches prédominantes dès les premières minutes de la réaction. Après deux heures, le milieu réactionnel devient complexe et les deux composés ont été séparés avant ce stade par chromatographie. L'un d'eux a été isolé sous forme cristalline avec un rendement de 12%. Son analyse élémentaire, son spectre infrarouge qui présente une bande azide à 2100 cm^{-1}, ainsi que son spectre de masse, suggèrent pour ce dérivé la structure d'un méthyl 5-azido-5-désoxy-2,3-di-*O*-*p*-tolylsulfonyl-β-D-lyxofuranoside (**9**). Les fragmentations observées pour le spectre de masse de **9**,



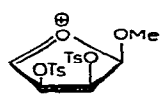
a *m/e* 373



b *m e* 269

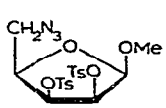


c *m/e* 466

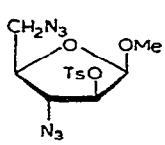


d *m/e* 441

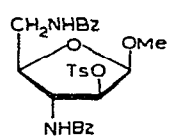
notamment les ions *c* et *d*, respectivement $M-OCH_3$ et $M-CH_2N_3$ sont compatibles avec la structure proposée. Le second produit principal de la réaction est isolé sous forme sirupeuse avec un rendement de 32%. Il présente une forte bande azide dans l'infrarouge à 2100 cm^{-1} et un groupement tosyloxy à 1590 cm^{-1}. Par hydrogénation catalytique suivie d'une benzoylation, il a été converti en méthyl 3,5-dibenzamido-3,5-didésoxy-2-*O*-*p*-tolylsulfonyl-β-D-arabinofuranoside (**11**) cristallin. La configuration *arabino* de celui-ci s'est trouvée être confirmée par l'examen de son spectre de r.m.n. En particulier, la valeur de la constante de couplage du proton anomérique est restée identique à celle observée pour le méthyl 2,3,5-tri-*O*-*p*-tolylsulfonyl-β-D-lyxofuranoside (**2**) de départ ($J_{1,2}$ 6 Hz). Cette valeur est en accord avec une disposition *cis* des protons de C-1 et C-2. En conséquence, le produit majeur de la réaction, formé avec un rendement de 32%, est le méthyl 3,5-diazido-3,5-didésoxy-2-*O*-*p*-tolylsulfonyl-β-D-arabinofuranoside (**10**).



9



10



11

Les quelques expériences décrites suggèrent que la disposition stérique des substituants du méthyl 2,3,5-tri-*O*-*p*-tolylsulfonyl-β-D-lyxofuranoside (**2**) confère à cette molécule un comportement particulier des groupes sulfonate de C-2, C-3, et C-5. On peut noter également la faible différence de réactivité des groupes sulfonate de

C-3 et C-5 vis-à-vis de nucléophiles comme l'ion azide et l'ion benzoate. Un tel comportement peut être une conséquence directe de l'arrangement spatial des substituants dans **2**. Ce phénomène a déjà été observé[2,12] lors de déplacements sélectifs de sulfonates primaires dans des molécules possédant une stéréochimie semblable à **2**.

PARTIE EXPÉRIMENTALE*

Méthyl 2,3,5-tri-O-p-*tolylsulfonyl-β*-D-*lyxofuranoside* (**2**). — Méthyl β-D-lyxofuranoside[8] (**1**, 330 mg) en solution dans la pyridine anhydre (5 ml) est additionné de chlorure de *p*-toluènesulfonyle (2,3 g) et le tout est laissé à température ordinaire pendant 48 h sous agitation. Le mélange réactionnel est extrait par le chloroforme et la phase organique est lavée à l'eau, séchée sur sulfate de sodium, filtrée et concentrée sous vide partiel. Le résidu solide est alors recristallisé dans un mélange de chloroforme–éther de pétrole (1050 mg, 84%), p.f. 148–149°; $[\alpha]_D^{23}$ −26° (*c* 0,69, chloroforme).

Anal. Calc. pour $C_{27}H_{30}O_{11}S_3$: C, 50, 97; H, 4,79; S, 15,30. Trouvé : C, 51,17; H, 4,80; S, 15,42.

Méthyl 5-O-benzoyl-3-désoxy-2-O-p-*tolylsulfonyl-β*-D-glycéro-*pent-2-énofuranoside* (**3**). *A partir de* **2**. — Le composé **2** (800 mg) dans la *N,N*-diméthylformamide (10 ml) est additionné de benzoate de sodium (970 mg). Le tout est chauffé à 120° sous agitation. Après 1 h 40 min on observe sur couche mince la disparition totale du produit de départ **2** au profit d'une nouvelle tache. Après 2 h, le mélange réactionnel est refroidi et extrait par le chloroforme comme pour **2**. Le résidu brun est chromatographié sur une colonne de gel de silice (100 g) avec le solvant acétate d'éthyle–éther de pétrole (3:17). Le sirop ainsi obtenu (414 mg, 78%) est homogène sur couche mince (R_F 0,55) en acétate d'éthyle–éther de pétrole (3:17). Le produit **3** est instable à la température ordinaire, $[\alpha]_D^{22}$ −55° (*c* 0,46, chloroforme), spectre de r.m.n. (chloroforme-*d*) : τ 7,60 (singulet 3H, $CH_3C_6H_4SO_3$), 6,72 (singulet 3H, OCH_3), 5,65 (doublet, H-5–H-5'), 5,02 (multiplet, H-4), 4,62 (triplet, H-1), 5,87 (quadruplet, H-3), 2,80–1,85 (multiplets, 9 protons aromatiques).

A partir de **7**. — (*a*). Une solution de **7** (20 mg) dans la *N,N*-diméthylformamide (0,2 ml) est chauffée à 120° pendant 2 h. Le mélange réactionnel est extrait de façon usuelle. Un examen visuel de la plaque de chromatographie en couche mince montre que 90% du produit de départ n'ont pas réagi. L'unique produit de la réaction (10%) possède un R_F identique à l'oléfine **3**.

(*b*) Une solution de **7** (20 mg) dans la *N.N*-diméthylformamide (0,3 ml) est additionnée de benzoate de sodium (20 mg) et le tout est chauffé 1 h à 120°. Le mélange réactionnel est extrait de manière habituelle pour fournir un produit montrant sur couche mince environ 30% de produit de départ inchangé et 70% de l'oléfine **3**.

Méthyl 2,3-di-O-p-*tolylsulfonyl-β*-D-*lyxofuranoside* (**6**). — Le methyl β-D-lyxofuranoside (**1**, 327 mg) dans la pyridine anhydre (5 ml) est additionné de chloro-

*Pour les conditions générales, voir Réf. 8.

triphénylméthane (612 mg). Après 72 h à température ordinaire, du chlorure de *p*-toluènesulfonyle (1,530 g) est ajouté et le mélange réactionnel est laissé 48 h à la température du laboratoire. Après extraction par le chloroforme suivant le processus habituel, le résidu cristallise dans un mélange de chloroforme et d'éther de pétrole pour donner 400 mg du dérivé tritosylé **2**. Les eaux mères (1,5 g) sont alors chromatographiées sur une colonne de gel de silice (100 g). Le méthyl 2,3-*O*-*p*-tolylsulfonyl-5-*O*-trityl-β-D-lyxofuranoside (**5**) ainsi isolé n'a pu être cristallisé et a été traité directement par une solution à 0,2% d'acide chlorhydrique dans 100 ml de méthanol. Après une nuit à température ordinaire, la détritylation est complète. La solution est neutralisée par de l'Amberlite IR-45 (OH^-). Après filtration et évaporation du solvant, le résidu est chromatographié sur une colonne de gel de silice (80 g). Un lavage au benzène permet d'éluer les produits tritylés et le dérivé tritosylé **2** restant. L'alcool **6** (480 mg, 48%) est obtenu sous forme solide avec le système benzène–méthanol (99:1). Une recristallisation dans le mélange acétate d'éthyle–éther de pétrole fournit l'échantillon analytique, p.f. 142,5–144°, $[\alpha]_D^{24}$ −62° (*c* 0,6, chloroforme).

Anal. Calc. pour $C_{20}H_{24}O_9S_2$: C, 50,84; H, 5,08; S, 13,55. Trouvé : C, 51,17; H, 5,19; S, 13,34.

*Méthyl 5-*O*-benzoyl-2,3-di-*O*-*p*-tolylsulfonyl-β-*D*-lyxofuranoside* (**7**). — L'alcool ditosylé **6** (160 mg) dans la pyridine anhydre (0,8 ml) est additionné de chlorure de benzoyle (0,065 ml). Après une nuit à température ordinaire, on ajoute une solution d'hydrogènocarbonate de sodium et laisse le tout 1 h sous agitation. Le mélange réactionnel est traité de manière habituelle. Le résidu cristallise dans un mélange de benzène–éther de pétrole (161 mg 80%), p.f. 152–153°; $[\alpha]_D^{25}$ −47° (*c* 0,66, chloroforme).

Anal. Calc. pour $C_{27}H_{28}O_{14}S_2$: C, 56,28; H, 4,86; S, 11,11. Trouvé : C, 56,28; H, 5,07; S, 11,11.

*Méthyl 5-azido-5-désoxy-2,3-di-*O*-*p*-tolylsulfonyl-β-*D*-lyxofuranoside* (**9**) *et méthyl 3,5-diazido-3,5-didésoxy-2-*O*-*p*-tolylsulfonyl-β-*D*-arabinofuranoside* (**10**). — Le dérivé tritosylé (**2**, 900 mg) dans la *N*,*N*-diméthylformamide (10 ml) est additionné d'azothydrate de sodium (1,3 g) et le tout est porté à 120° sous agitation. Après 10 min de réaction la chromatographie sur couche mince montre la présence de deux taches dans le rapport approximatif 1:1. Ce rapport se conserve durant 1 h, après quoi la tache possédant le R_F le plus élevé augmente d'intensité vis-à-vis de celle ayant le R_F le plus bas. Après 1 h 45 min de réaction, on extrait par le chloroforme de la façon habituelle. Le résidu est chromatographié sur une colonne de gel de silice (50 g) pour fournir deux fractions principales en solvant acétate d'éthyle–éther de pétrole (1:9). Le diazide **10** est élué en premier (75 mg, 32%). Ce dernier n'a pu être cristallisé, R_F 0,9 en solvant acétate d'éthyle–éther de pétrole (1:9). Le monoazide **9** est obtenu sous forme de solide (85 mg, 12%), R_F 0,5 en solvant acétate d'éthyle–éther de pétrole (1:9). Ce dernier après une recristallisation dans un mélange d'acétate d'éthyle–éther de pétrole donne un p.f. 128–129°, $[\alpha]_D^{21}$ −4,3° (*c* 0,8, chloroforme).

Anal. Calc. pour $C_{20}H_{23}N_3O_8S_2$: C, 48,29; H, 4,62; N, 8,45; S, 12,87. Trouvé : C, 48,14; H, 4,72; N, 8,63; S, 12,81.

*Méthyl 3,5-dibenzamido-3,5-didésoxy-2-*O*-p-tolylsulfonyl-β-*D*-arabinofuranoside* (**11**). — Le diazide **10** obtenu est hydrogèné pendant 1 h dans le méthanol (10 ml) en présence de platine d'Adams (50 mg). La diamine obtenue est ensuite benzoylée par l'anhydride benzoïque (80 mg) dans le méthanol (10 ml). Après filtration sur Célite et évaporation du solvant, le solide résultant est recristallisé dans un mélange de chloroforme–éther de pétrole pour donner un produit ayant un p.f. 164–166°, $[\alpha]_D^{24}$ −54,8° (*c* 0,8, chloroforme).

Anal. Calc. pour $C_{27}H_{28}N_2O_7S_1$: C, 61,82; H, 5,38; N, 5,34; S, 6,10. Trouvé : C, 62,04; H, 5,24; N, 5,59; S, 6,13.

REMERCIEMENTS

Ce travail a bénéficié d'une aide matérielle de la Ligue Nationale Française contre le Cancer. Nous remercions M. le Professeur E. Lederer pour l'intérêt qu'il a porté à ce travail.

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RECOVERY AND RE-USE OF WATER-INSOLUBLE AMYLASE DERIVATIVES

S. A. BARKER, P. J. SOMERS,
Department of Chemistry, The University, Birmingham 15 (Great Britain)

AND R. EPTON
Department of Chemistry and Biology, The Polytechnic, Wolverhampton (Great Britain)

(Received January 23rd, 1970)

ABSTRACT

The activity retained by water-insoluble derivatives of alpha- and beta-amylase on repeated re-use was investigated. Derivatives of both enzymes were prepared by diazo and isothiocyanato coupling with appropriate cellulose or copolyacrylamide (Enzacryl AA) derivatives. In addition, a copolyacrylamide (Enzacryl AH)-based derivative of alpha-amylase, in which the enzyme was attached by acid azide-coupling, was investigated. Although considerable desorption of physically bound enzyme took place on initial re-use, the cellulose-bound derivatives were subsequently re-used repeatedly, with comparatively little loss of activity. The Enzacryl-based derivatives were superior, in that recovery of activity was good on initial as well as repeated re-use.

INTRODUCTION

We have previously reported the preparation of water-insoluble derivatives of alpha- and beta-amylase by coupling with chemically active derivatives of cellulose[1, 2] and cross-linked polyacrylamide (Enzacryl)[3]. In general, attachment of an enzyme to a water-insoluble support may confer upon the enzyme such properties as enhanced heat stability, shifts in pH optimum, and changes in specificity. However, the most obvious advantage is that the insolubilised enzyme may be simply recovered from digests by filtration and subsequently re-used. Surprisingly, although numerous water-insoluble derivatives of enzymes have been described[4, 5], very little information concerning their recovery and re-use has been published. It seemed of interest, therefore, to investigate the recovery and re-use of alpha- and beta-amylase derivatives based on cellulose and Enzacryl.

EXPERIMENTAL

Materials. — Crystalline alpha-amylase (*B. subtilis*) and crystalline beta-amylase (sweet potato) were obtained from the Sigma Chemical Company. The 3-(*p*-aminophenoxy)-2-hydroxypropyl ether hydrochloride of cellulose (20.7 μequiv.

of ether linkage/g) was that described in a previous paper[1]. Cross-linked copolymers of acrylamide (Enzacryl AA and Enzacryl AH) incorporating, respectively, arylamino groups (0.27 mequiv./g) and acid hydrazide groups (0.33 mequiv./g) were obtained from Koch–Light Laboratories.

Preparation of water-insoluble enzyme derivatives. — (*a*) *By coupling alpha- and beta-amylase with activated 3-(*p*-aminophenoxy)-2-hydroxypropyl ethers of cellulose.* Samples of the cellulose ether were activated either with thiophosgene to give reactive 2-hydroxy-3-(*p*-isothiocyanatophenoxy)propyl ethers of cellulose or with nitrous acid to give reactive, diazotised 3-(*p*-aminophenoxy)-2-hydroxypropyl ethers of cellulose. Samples of alpha- and beta-amylase were separately coupled with both reactive cellulose derivatives. Subsequently, the derivatives were washed alternately with buffer (phosphate, 0.02M, pH 6.9, for alpha-amylase; acetate, 0.02M, pH 4.8, for beta-amylase) and buffered sodium chloride solution (0.5M for alpha-amylase, M for beta-amylase). The precise experimental procedures were similar to those detailed previously[1,2].

(*b*) *By coupling alpha- and beta-amylase with activated copolyacrylamide derivatives (Enzacryl AA and Enzacryl AH).* Samples of Enzacryl AA were activated either with thiophosgene or by diazotisation. Thus, the aryl amino groups of the copolymer were converted into aryl isothiocyanato groups and aryl diazo groups, respectively. Samples of Enzacryl AH were activated with nitrous acid which converted the acid hydrazide groups of the copolymer into acid azide groups. Derivatives of alpha-amylase were prepared by coupling with all three reactive Enzacryl derivatives. Beta-amylase was coupled with the activated derivatives of Enzacryl AA only, since the derivative based on Enzacryl AH had previously been shown to be inactive[3]. The coupling and subsequent washing procedures were as detailed previously.

Determination of amylolytic activity. — Amylolytic activity was determined against soluble starch in an appropriate buffer (phosphate, 0.02M, pH 6.9, for alpha-amylase; acetate, 0.02M, pH 4.8, for beta-amylase) at 20° with magnetic stirring at a constant rate.

The degree of hydrolysis in the various digests was determined by centrifuging down the water-insoluble enzyme at intervals and assaying samples of the supernatants for reducing sugar by means of the Bernfeld dinitrosalicylate assay[6].

Determination of amylolytic activity of cellulose-bound alpha- and beta-amylase derivatives on successive occasions of re-use. — Samples (2–10 mg) of cellulose-bound (diazo and isothiocyanato coupled) alpha-amylase and cellulose-bound (diazo and isothiocyanato coupled) beta-amylase were centrifuged down from their various buffer suspensions. To each solid derivative, a solution (5 ml, 1%) of starch in the appropriate buffer was added, and the digest was stirred magnetically for a few seconds, after which it was centrifuged. A sample (1 ml) of the supernatant was pipetted into an equal volume of dinitrosalicylate reagent. The digest was then stirred magnetically for 15 min at 20°, after which it was again centrifuged, and a sample (1 ml) of the supernatant was taken for dinitrosalicylate assay. The remaining supernatant was further incubated and tested for amylolytic activity at 15-min intervals.

The remaining solid was then stirred briefly with buffer solution (15 ml), the suspension was again centrifuged down, and, after the washings had been discarded, a solution (5 ml, 1%) of starch in the appropriate buffer was added. Thus, the digestion procedure was repeated.

In all, the digestion procedure was carried out eight times for each cellulose-bound derivative. Dinitrosalicylate assay of the samples taken from the digests before and after incubation enabled the total maltose (mg/ml) produced in each digest to be determined. Thus, the activity of the bound enzyme in successive digests was determined and expressed as a percentage of the activity in the initial determination. The results are presented in Fig. 1 (for alpha-amylase derivatives) and Fig. 2 (for beta-amylase derivatives).

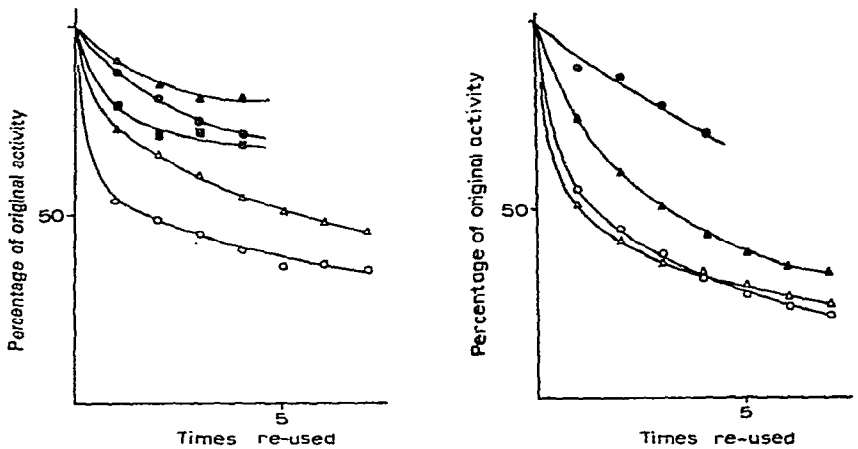


Fig. 1. Retention of activity of water-insoluble alpha-amylase derivatives on re-use; ○——○——○, cellulose-bound (diazo-coupled) alpha-amylase; △——△——△, cellulose-bound (isothiocyanato-coupled) alpha-amylase; ●——●——●, Enzacryl-bound (diazo-coupled) alpha-amylase; ▲——▲——▲, Enzacryl-bound (isothiocyanato-coupled) alpha-amylase; ■——■——■, Enzacryl-bound (acid azide-coupled) alpha-amylase.

Fig. 2. Retention of activity of water-insoluble beta-amylase derivatives on re-use; ○——○——○, cellulose-bound (diazo-coupled) beta-amylase; △——△——△, cellulose-bound (isothiocyanato-coupled) beta-amylase; ●——●——●, Enzacryl-bound (diazo-coupled) beta-amylase; ▲—▲—▲, Enzacryl-bound (isothiocyanato-coupled) beta-amylase.

The supernatants from the final digests involving each cellulose-bound enzyme were further incubated and tested for amylolytic activity at 15-min intervals.

Determination of amylolytic activity of Enzacryl-bound alpha- and beta-amylase derivatives on successive occasions of re-use. — The experimental procedures were similar to those outlined for the cellulose-bound enzymes. However, in the case of the more finely dispersed derivatives (those based on Enzacryl AA), the need for rapid (high-speed) centrifugation necessitated carrying out the digestion procedure in polypropylene centrifuge tubes. The results are presented in Fig. 1 (alpha-amylase derivatives) and Fig. 2 (beta-amylase derivatives).

DISCUSSION

In contrast to the Enzacryl-based alpha- and beta-amylase derivatives, the activity of the cellulose-based enzymes fell sharply on initial re-use (see Figs. 1 and 2). Thereafter, the drop in activity recovered was comparatively slight. Since, in the case of the cellulose-bound enzymes, a considerable proportion of the amylolytic activity lost on initial re-use was apparent in the supernatants from the initial digests, the drop in activity was due to physically adsorbed enzyme. However, no more than marginal activity could be detected in supernatants from subsequent digests and no activity at all in supernatants from the final digests. Thus, almost all of the physically bound enzyme was detached on the first treatment with starch.

We have previously reported[3] that extended washing with buffer and sodium chloride solution leads to decrease of activity of cellulose-bound alpha-amylase. However, even on prolonged incubation of the washings with starch solution, no re-solubilised activity was evident. It would appear, therefore, that the popular practice of washing water-insoluble derivatives of enzymes until no activity is apparent in the washings is no guarantee that all of the insolubilised enzyme is covalently bound. Washing with a solution of a macromolecular substrate would seem to be much more effective.

In the case of derivatives of alpha- and beta-amylase based on diazotised Enzacryl AA, and the derivative of alpha-amylase based on Enzacryl AH, no activity could be detected in supernatants from the initial or subsequent digests. However, marginal activity could be detected in supernatants from digests involving isothiocyanato-coupled enzyme derivatives based on Enzacryl AA. Since the isothiocyanato-coupled derivatives were particularly finely dispersed, it is likely that the activity arose from the experimental difficulties in quickly and completely recovering the water-insoluble enzymes from their digests. It appears that Enzacryl possesses many of the advantages of cellulose as an enzyme carrier, without having the disadvantage of physically binding enzymes. The present results are all the more significant, since the preparation of the Enzacryl-based enzymes involved considerably less washing than the corresponding cellulose derivatives.

ACKNOWLEDGMENTS

The authors thank Dr. P. B. Koch and Koch–Light Laboratories Ltd. for their interest in this work.

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STRUCTURAL AND IMMUNOLOGICAL STUDIES OF CHICK ALLANTOIC ANTIGEN

PART II. ACID HYDROLYSIS

M. J. How* AND J. D. Higginbotham†
Department of Chemistry, The University, P. O. Box 363, Birmingham 15 (Great Britain)

(Received December 23rd, 1969; in revised form, February 2nd, 1970)

ABSTRACT

Mild hydrolysis of chick allantoic antigen with acid liberated, among other products, terminal non-reducing residues of fucose and D-galactofuranose. D-Galactofuranose residues were also present in the interior of carbohydrate chains. Cleavage of such residues by acid liberated not less than 10% of the molecule as oligosaccharides. Partial structures of some of the oligosaccharides have been determined.

INTRODUCTION

Previous workers[1,2] have shown that chick allantoic antigen, which combines with antibody to influenza-virus host material and inhibits the agglutination of red blood-cells by influenza virus[3], is a sulphated glycopeptide that contains D-galactose, 6-deoxygalactose (fucose), 2-amino-2-deoxyglucose, 2-amino-2-deoxygalactose, and acetyl groups. Sugars, tentatively identified[2] (paper chromatography) as galactose and fucose, were liberated on treatment of the antigen with 0.04M hydrochloric acid for 10 min at 100°; more-drastic hydrolysis (0.1M hydrochloric acid, 100°, 6 h) liberated 2-amino-2-deoxyglucose 6-sulphate. This sulphate and galactose 6-sulphate were detected (paper chromatography and paper electrophoresis) in hydrolysates (0.25M sulphuric acid) of a chemically and immunologically related glycopeptide isolated from papain-digested allantoic fluid[4]. The present paper describes more-detailed studies of the degradation of native, chick allantoic antigen by mild treatment with acid.

MATERIALS AND METHODS

An apparently homogeneous preparation of chick allantoic antigen was a generous gift from Dr. A. Harboe.

Identification of monosaccharides liberated on mild hydrolysis of the antigen with acid. — Antigen (2.28 mg) was hydrolysed (0.04M hydrochloric acid, 0.6 ml, 30 min,

*Present address: Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford, Beds., Great Britain.
†Present address: Department of Pathology, New York University Medical School, New York, U.S.A.

100°) in a sealed tube under nitrogen. The hydrolysate was eluted with water from a column of Sephadex G-25 (superfine grade, 100 × 0.6 cm) that had been calibrated previously by elution with water of a solution of D-galactose in 0.04 M hydrochloric acid. D-Galactose was recovered quantitatively (cysteine–sulphuric acid method[5] and D-galactose oxidase assay[6]) in the calibration experiment. The eluted hydrolysate was scanned for carbohydrate (cysteine–sulphuric acid) up to the point of elution of monosaccharides. The material eluted in the same region as D-galactose, as established by the calibration experiment, was concentrated by rotary evaporation at 30°, and the sugar components of one aliquot of the concentrated solution were converted into *O*-trimethylsilyl (TMS) derivatives[7] and analysed by using a Pye 104 gas chromatograph with a column of 10% SE-30 on siliconised Celite (100–200 mesh) at 180°. Other aliquots were analysed for galactose and fucose (cysteine–sulphuric acid) and for D-galactose (D-galactose oxidase).

Smith degradation of the antigen. — Antigen (2.17 mg) was treated with 0.01 M sodium metaperiodate (2 ml) for 5 min at room temperature. 0.4 M Ethylene glycol (0.5 ml) was added, and the solution was dialysed at 4° against 0.1 M sodium chloride followed by water. The non-diffusible product was concentrated, and excess of sodium borohydride was added during 3 h. The pH of the solution was maintained at 7–7.5 by using carbon dioxide. After a further 18 h at 4° and pH 7, the solution was acidified with Dowex-50 (H^+) resin, filtered, and dialysed as described. The non-diffusible product was recovered in essentially quantitative yield by freeze-drying. The product was hydrolysed (2 M hydrochloric acid, 0.5 ml, 5.5 h, 100°) in a sealed tube, the hydrolysate was diluted to 2 ml with water, and neutral and basic components were separated by elution from Dowex-50 (H^+) resin with water and 2 M hydrochloric acid, respectively. The components of separate aliquots of the aqueous eluate were converted into TMS derivatives[7] and alditol acetates[8]. TMS derivatives were analysed by g.l.c. as described above, and alditol acetates by g.l.c. on a column of 2% ECNSS-M on Gas Chrom Q (60–80 mesh, Applied Science Lab. Inc.) heated from 150–200° at 1.5°/min.

Isolation and partial characterisation of oligosaccharides produced by mild hydrolysis of the antigen by acid. — Antigen (11 mg) was hydrolysed (0.04 M hydrochloric acid, 30 min, 100°) in a sealed tube under nitrogen, and the hydrolysate was eluted with water from a column (123 × 1.3 cm) of Sephadex G-25 (fine grade). Fractions (0.95 ml) were collected automatically and were scanned by using cysteine–sulphuric acid. The contents of appropriate tubes were combined and concentrated, and certain of the products were hydrolysed (0.08 M hydrochloric acid, 1 ml, 30 min, 100°) and fractionated again as described. This procedure was repeated for certain fractions from the second hydrolysis.

Appropriate fractions from the three hydrolyses were analysed for sugars and amino acids liberated on hydrolysis in sealed tubes (2 M hydrochloric acid, 6 h, 100°, for sugars; 6 M hydrochloric acid 20 h, 110°, for amino acids). Prior to analyses for sugars, neutral and basic components of the hydrolysates were separated by elution from Dowex-50 (H^+) resin with water and 2 M hydrochloric acid, respectively, and

were separately converted into TMS derivatives[7] which were analysed by g.l.c. as described above. Amino acids liberated on hydrolysis were determined by using a Technicon autoanalyser.

Structural studies of oligosaccharides. — (*a*) *Formation of Morgan–Elson chromogen.* The reactions of certain of the oligosaccharides and of 2-amino-2-deoxy-D-glucose were investigated by using a modification[9] of the Morgan–Elson reaction[10].

(*b*) *Determination of reducing sugar end groups.* Oligosaccharides **1b** and **2c** (see Fig. 1) were combined for structural studies, and an aqueous solution of the two oligosaccharides (*ca.* 150 μg) was treated with excess of sodium borohydride which was added during 3 h at pH 7–7.5 (carbon dioxide). Oligosaccharide **2e** (see Fig. 1, 150 μg) was treated similarly. The solutions were separately acidified to pH 1 with hydrochloric acid and were eluted with water (5 ml) from columns (6 × 0.6 cm) of Dowex-50 (H^+) resin. The aqueous eluates were concentrated to dryness, and boric acid was removed by repeated distillation of methanol from the residues. The products were separately hydrolysed (2M hydrochloric acid, 0.25 ml, 5.5 h, 100°) in sealed tubes and were diluted to 1 ml with water. Neutral and basic components of the hydrolysates were separated by using Dowex-50 (H^+) resin and were converted into TMS derivatives and analysed by g.l.c.

RESULTS

Mild hydrolysis of the antigen with acid and identification of the liberated monosaccharides. — Hydrolysis of the antigen with 0.04M hydrochloric acid for 30 min at 100°, followed by fractionation of the hydrolysate on Sephadex G-25 and colorimetric and enzymic analyses of the monosaccharides thus obtained, showed that D-galactose (3.4% of the antigen) and fucose (3.4%) were liberated. These values represent 13.1 and 79% of the D-galactose and fucose contents, respectively. Essentially similar values were obtained by g.l.c. of the monosaccharides, which also showed that D-galactose and fucose were the *only* monosaccharides liberated. Elution of the hydrolysate from Sephadex G-25 showed that some glycosidic linkages located in the interior of the molecule were cleaved by this mild treatment with acid, with the concomitant production of oligosaccharides.

Smith degradation of the antigen. Confirmation of the presence of D-*galacto-furanose residues by conversion into arabinose.* — Treatment of the antigen with sodium metaperiodate for 5 min, followed by treatment of the product with sodium borohydride at neutral pH and acid hydrolysis, liberated arabinose, among other products.

Isolation and partial characterisation of oligosaccharides liberated on mild hydrolysis of the antigen with acid. — Mild hydrolysis of the antigen liberated, in addition to D-galactose (2.9% of the antigen) and fucose (2.7%), oligosaccharides (**1b**, **1c**) and material (**1a**) that was not fractionated on elution from Sephadex G-25 (Fig. 1). Further hydrolysis of Fraction **1a**, using 0.08M hydrochloric acid, liberated D-galactose (1.6% of the antigen), several oligosaccharides (**2b**–**2e**), one (**2c**) of which

had an elution position from Sephadex G-25 identical with that of oligosaccharide **1b**, and material (**2a**) which was not fractionated on elution from Sephadex G-25 (Fig. 1). Further mild hydrolysis of the latter material produced oligosaccharides (**3b–3e**, Fig. 1) and larger molecular weight "core" material (**3a**).

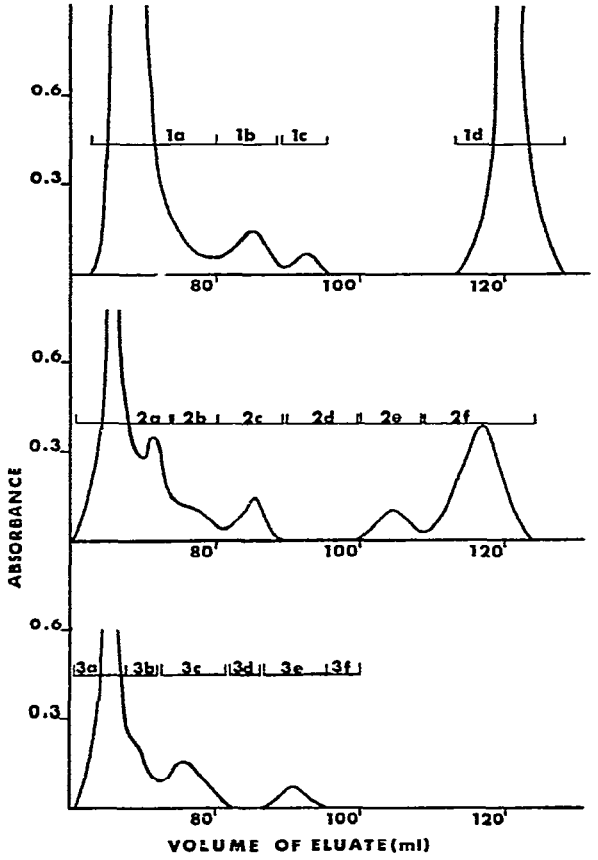


Fig. 1. Elution of acid hydrolysates of chick allantoic antigen from Sephadex G-25. Fractions assayed by reaction with cysteine–sulphuric acid.

The total amounts of D-galactose and fucose which were liberated by this sequence of mild hydrolyses with acid were 4.5 and 2.7%, respectively, of the antigen. The lower value that was obtained for the amount of fucose liberated by comparison with that obtained in the earlier experiment probably reflects the limitations of the analytical method. Analysis confirmed that D-galactose and fucose were the only monosaccharides liberated by mild hydrolysis with acid and showed that not less than 10% of the antigen was liberated as oligosaccharides (Table I). This figure does not take into account the acetyl and sulphate contents of the oligosaccharides. Fucose was not detected in hydrolysates of any of the oligosaccharides that were produced, thus indicating that oligosaccharides were not derived by acid hydrolysis

of fucosidic linkages located in the interior of carbohydrate chains. No monosaccharides were detected in hydrolysates of the combined, concentrated contents of those tubes that gave negative reactions with cysteine and sulphuric acid.

TABLE I

ANALYTICAL DATA FOR OLIGOSACCHARIDES LIBERATED ON MILD HYDROLYSIS OF CHICK ALLANTOIC ANTIGEN

Oligosaccharide[a]	*Yield*[b] (%)	*Molar proportions of sugars liberated on hydrolysis*		
		D-*Galactose*	*2-Amino-2-deoxyglucose*	*2-Amino-2-deoxygalactose*
1b plus **2c**	3	2	5	0
1c	0.3	1	1	0
2b	1.5	3	13	1
2e	0.9	3	6	1
3b	2	3	7	2
3c	2	3	11	1
3e	0.6	1	1	1

[a]No sugars were detected in hydrolysates of **2d**, **3d**, and **3f** (Fig. 1). Residual "core" material (**3a**) after three successive hydrolyses of the antigen contained all of the peptide, together with D-galactose, 2-amino-2-deoxyglucose, and 2-amino-2-deoxygalactose, in a molar ratio of 2:1:2. [b]No account is made for acetyl and sulphate contents.

Criteria of the heterogeneity of the oligosaccharides, other than their elution profiles from Sephadex G-25, were not obtained, since the yields of oligosaccharides were small and the amounts of antigen were limited. This fact necessarily precludes unequivocal interpretation of the analytical data (Table I) for the oligosaccharides. The results indicate, however, that the initial, mild hydrolysis of the antigen liberated D-galactose and fucose (**1d**, Fig. 1), and oligosaccharides (**1b** and **1c**) that were composed solely of D-galactose and 2-amino-2-deoxyglucose. The position of elution from Sephadex G-25 of oligosaccharide **1b**, which contained those sugars in the molar ratio of 2:5, suggests that the DP of the oligosaccharide is of the order of 12, whereas that of oligosaccharide **1c**, which contained equimolar amounts of the two sugars, is of the order of 6. Subsequent hydrolysis of the antigen liberated D-galactose (**2f**, Fig. 1) together with an oligosaccharide (**2c**) that was assumed, from its point of elution from Sephadex G-25, to be similar to, or identical with, oligosaccharide **1b**, and two oligosaccharides (**2b** and **2e**) that contained a small proportion of 2-amino-2-deoxygalactose in addition to D-galactose and 2-amino-2-deoxyglucose. Oligosaccharides **3b**, **3c**, and **3e** (Fig. 1) that contained different proportions of these three sugars were liberated on further hydrolysis of the antigen. Oligosaccharide **3e** contained equimolar amounts of the three sugars. The "core" material (**3a**) that was obtained after three mild treatments of the antigen with acid contained all of the peptide, in addition to D-galactose, 2-amino-2-deoxyglucose, and 2-amino-2-deoxygalactose, in a molar ratio of 2:1:2.

Structural studies of oligosaccharides. — Oligosaccharides **1b** and **2c** were

combined prior to structural studies. Treatment of oligosaccharides **2e** and (**1b** plus **2c**) with sodium borohydride at neutral pH, followed by acid hydrolysis, liberated galactitol as the sole alditol, thus confirming the presence of terminal, reducing D-galactose residues in these oligosaccharides. Oligosaccharides **2e** and (**1b** plus **2c**) gave positive reactions for 2-acetamido-2-deoxyhexose; the chromophores given by the oligosaccharides and by 2-acetamido-2-deoxy-D-glucose had identical absorption spectra in the region 450–650 nm. The colour yield obtained for oligosaccharide (**1b** plus **2c**) and for oligosaccharide **2e** corresponded to 26 and 23%, respectively, of their content of 2-amino-2-deoxyglucose.

DISCUSSION

These studies confirm previous conclusions that chick allantoic antigen contains some D-galactose and fucose residues that are extremely labile to acid. The present work shows also that these are the only monosaccharides that are liberated on hydrolysis of the antigen with 0.04M hydrochloric acid at 100°. The extreme acid lability of most of the fucose and some of the D-galactose residues in the antigen may be attributed to the presence of terminal, non-reducing fucose and D-galactofuranose residues, respectively. The presence of D-galactofuranose residues is confirmed by the production of arabinose from the antigen by a degradation sequence that involved brief treatment with sodium metaperiodate, treatment of the product with sodium borohydride, and finally acid hydrolysis. Arabinose may be produced, however, from such a reaction sequence with D-galactofuranosides in which HO-2 and/or HO-3 are substituted, as well as from terminal, non-reducing residues of D-galactofuranose. Evidence for the presence of both terminal, non-reducing, and internal residues of D-galactofuranose in the antigen is provided, since mild hydrolysis of the antigen with acid is accompanied by the liberation of not less than 10% of the antigen as oligosaccharides.

Detailed investigations of the heterogeneity of the oligosaccharides thus produced were precluded by the relatively low yields of oligosaccharides and by the small quantities of antigen that were available. Categorical evidence that oligosaccharides **1b** and **2c**, which were combined for structural studies, were identical was not obtained. Interpretation of the analytical and structural information obtained for these products of hydrolysis of the antigen is thus restricted. The following general conclusions may by made, however, about the sequential hydrolysis with acid of the antigen. Oligosaccharides that contain exclusively D-galactose and 2-amino-2-deoxyglucose are the initial products of mild hydrolysis, and the identification of D-galactose as the sole reducing end-group in these oligosaccharides confirms that they are produced by acid cleavage of galactofuranoside bonds. Carbohydrate chains that contain 2-amino-2-deoxygalactose residues in the antigen are present mainly in the "core" of relatively large molecular weight which contains all of the peptide. These monosaccharide residues may be involved in alkali-labile glycosidic linkages to serine and/or threonine (which form 50% of the peptide), thus comprising the carbohydrate–peptide linkage regions.

Certain, additional, structural features may be deduced for oligosaccharides **2e** and for (**1b** plus **2c**) which comprise approximately 4% of the antigen. They contain terminal, reducing residues of D-galactose, and their positive Morgan–Elson reaction implies that part of their content of 2-acetamido-2-deoxyglucose is exposed as a reducing sugar by an alkali-induced, peeling reaction and that those sugars thus exposed do not have an alkali-stable substituent at O-4. Reactions of the antigen with alkali[11] and periodate[12] provide more-detailed structural features of the antigen.

ACKNOWLEDGMENTS

We thank Dr. A. Harboe and his colleagues of the National Institute of Public Health, Oslo, Norway, for valuable discussions and a generous gift of chick allantoic antigen; Professors M. Stacey, C.B.E., F.R.S., and S. A. Barker for their interest in this work; and the Science Research Council for financial support.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

STRUCTURAL AND IMMUNOLOGICAL STUDIES OF CHICK ALLANTOIC ANTIGEN

PART III. THE CARBOHYDRATE–PEPTIDE LINKAGE REGIONS

M. J. How* AND J. D. Higginbotham†
Department of Chemistry, The University, P.O. Box 363, Birmingham 15 (Great Britain)

(Received December 23rd, 1969; in revised form, February 2nd, 1970)

ABSTRACT

Alkaline-degradation studies have shown that an antigenic, sulphated glycopeptide from chick allantoic fluid contains glycosidic linkages between 2-acetamido-2-deoxygalactose and serine and threonine. The 2-acetamido-2-deoxygalactose residues thus linked were also substituted at O-3 and O-6 by oligosaccharides. Structural features of the antigen are compared with those of an immunologically related glycopeptide from allantoic fluid.

INTRODUCTION

Mild hydrolysis with acid of a sulphated glycopeptide antigen from chick allantoic fluid liberated[1] at least 10% of the carbohydrate content as oligosaccharides, by cleavage of D-galactofuranosidic linkages located in the interior of carbohydrate chains. In addition to acetyl groups (6.5%), sulphate (12.0%), and peptide (13.0%), the antigen contains[2] D-galactose (26.0%), 6-deoxygalactose (4.3%), 2-amino-2-deoxyglucose (17.9%), and 2-amino-2-deoxygalactose (7.7%). The last sugar and the hydroxyamino acids serine plus threonine, which constitute *ca.* 50% of the peptide, are present in approximately equimolar amounts. Studies of the graded hydrolysis of the antigen showed[1] that the relatively acid-stable core of the antigen contained all of the peptide and was rich in 2-amino-2-deoxygalactose. More-definitive evidence for the nature of the carbohydrate–peptide linkage regions in the antigen is given in the present paper.

MATERIALS AND METHODS

The apparently homogeneous preparation of chick allantoic antigen used in the present work was a generous gift from Dr. A. Harboe. Since the antigen was obtained from a different batch of material than that described in earlier papers, it

*Present address: Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford, Beds., Great Britain.
†Present address: Department of Pathology, New York University Medical School, New York, U.S.A.

was analysed for components by using methods that are described in Ref. 2. In addition, the reaction of the antigen with alkaline ferricyanide[3] was investigated. Products that were obtained on treatment of the antigen with alkali were analysed by using cysteine–sulphuric acid[4] (Method *A*) and for Morgan–Elson chromogen [by using a modification of the method of Reissig *et al.*[5], in which the heating step after the addition of potassium tetraborate was omitted (Method *B*)]. The absorption at 241 nm was also measured (Method *C*).

*Treatment of antigen with 0.5*M *sodium hydroxide.* — M Sodium hydroxide (1.25 ml) was added to an aliquot (1.25 ml) of an aqueous solution of the antigen (2 mg/ml), and aliquots (0.5 ml and 0.1 ml) were withdrawn immediately and neutralised with 0.5M hydrochloric acid, prior to hydrolysis and analysis[2] for amino acids and 2-amino-2-deoxyhexoses. The remaining solution was saturated with nitrogen and heated at 25°. Aliquots were removed at intervals during 48 h and analysed by methods *B* and *C*. After 48 h, aliquots of the alkaline solution of the antigen were neutralised and analysed for amino acids and 2-amino-2-deoxyhexoses.

*Treatment of the antigen with 0.5*M *sodium hydroxide and sodium borohydride.* — 2M Sodium borohydride (0.25 ml) was added to an aliquot (0.25 ml) of an aqueous solution of the antigen (2 mg/ml). After 20 min at 25°, M sodium hydroxide (0.5 ml) was added to the solution which was then saturated with nitrogen and heated at 25°. After 48 h, the solution was acidified (pH 4) with Dowex-50 (H^+) resin, filtered, and concentrated by rotary evaporation at 25°. Boric acid was removed by repeated distillation of methanol from the residue. Aliquots of a solution of the residue were analysed for amino acids and 2-amino-2-deoxyhexoses.

*Fractionation of the products obtained on treatment of the antigen with 0.5*M *sodium hydroxide.* — A solution of the antigen (11.9 mg) in 0.5M sodium hydroxide (10 ml) was saturated with nitrogen and heated for 30 h at 25°, prior to neutralisation with 10M hydrochloric acid. The solution was concentrated to 2 ml by rotary evaporation at 25° and eluted with water from a column (80 × 1.3 cm) of Sephadex G-25 (superfine grade) that had been calibrated by elution of the antigen and of the Morgan–Elson chromogen derived from 2-acetamido-2-deoxy-D-glucose. Fractions were collected automatically and were analysed by Methods *A*, *B*, and *C*. Appropriate fractions were combined, concentrated to 1 ml by rotary evaporation at 25°, and eluted with water from a column (105 × 0.8 cm) of Sephadex G-10. Appropriate fractions from the column eluate were concentrated to 0.5 ml by freeze-drying and were eluted with water from a column (150 × 0.6 cm) of Sephadex G-50.

RESULTS

Quantitative analysis of the antigen for sugars, amino acids, and other components showed that it was essentially similar to that used in previous analytical and structural studies[1,2]. The antigen gave a high value for reducing sugar, as estimated from its reaction with alkaline ferricyanide. This suggested that alkali-labile bonds in the antigen were cleaved during the reaction, with concomitant exposure of reducing sugar end-groups.

Treatment of the antigen with 0.5M sodium hydroxide at 25° resulted in a marked increase in the absorbance of the solution at 241 nm and in the formation of the Morgan–Elson chromogen. Quantitative analyses (Table I) showed that 80% (0.24 μmole) of the serine content of the antigen, 71% (0.27 μmole) of the threonine content, and 89% (0.47 μmole) of the 2-amino-2-deoxygalactose content were destroyed on treatment of the antigen with 0.5M sodium hydroxide for 48 h. All other sugar and amino acid components were apparently unaffected by such treatment.

TABLE I

AMINO ACID AND 2-AMINO-2-DEOXYHEXOSE ANALYSES OF CHICK ALLANTOIC ANTIGEN

Component	*Content*[a] *(μmole/mg)*		
	A	B	C
Serine	0.30	0.06	0.06
Threonine	0.38	0.11	0.11
Alanine	0.12	0.13	0.25
2-Amino-2-deoxyglucose	0.99	1.0	not determined
2-Amino-2-deoxygalactose	0.53	0.06	not determined

[a] *A*, initially; *B*, after treatment with 0.5M sodium hydroxide for 48 h at 25°; *C*, after treatment with 0.5M sodium hydroxide and sodium borohydride for 48 h at 25°.

Similar analyses of the antigen following treatment with 0.5M sodium hydroxide and sodium borohydride showed decreased serine and threonine contents and increased alanine content (Table I). Two new compounds that were present in the hydrolysates were tentatively identified, from their elution positions on autoanalysis, as 2-aminobutyric acid and the reduced form of the Morgan–Elson chromogen. The amount of 2-aminobutyric acid and the increase in the amount of alanine produced on hydrolysis of the antigen, following treatment with alkali and borohydride, corresponded to *ca.* 19% of the threonine and 48% of the serine residues, respectively, that were destroyed by such treatment.

Reaction of the antigen with 0.5M sodium hydroxide at 25° was essentially complete after 30 h, as measured by the increase in absorbance at 241 nm and by production of the Morgan–Elson chromogen. Fractionation on Sephadex G-25 of the antigen before and after treatment with 0.5M sodium hydroxide for 30 h at 25° showed that considerable degradation had occurred (Fig. 1). The product mixture thus obtained was separated into three main fractions. Fraction *A* (Fig. 1), which was totally excluded from Sephadex G-25, gave positive reactions (cysteine and sulphuric acid) for D-galactose and fucose, and absorbed strongly at 241 nm. Morgan–Elson chromogen was present exclusively in Fraction *B* which contained material of apparently smaller molecular weight than the material in Fraction *A*. Fraction *C*, which was eluted in the same region as monosaccharides and oligosaccharides of small molecular weight, gave a positive reaction with cysteine and sulphuric acid. Subsequent fractionation using Sephadex G-10 and G-50 showed that Fraction *B* contained a complex mixture of products having a wide range of molecular weights. In a calibra-

tion experiment, the undegraded antigen was eluted as a single, sharp peak in the void volume of the column.

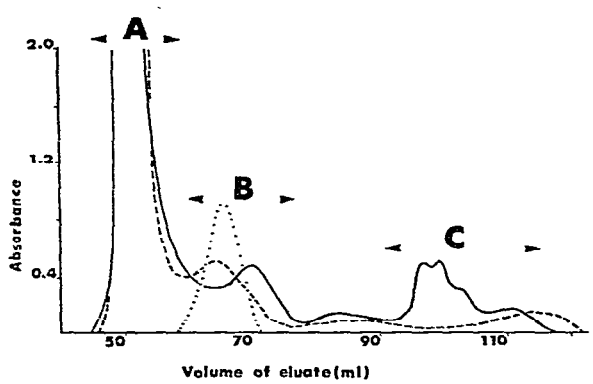


Fig. 1. Elution of alkali-treated chick allantoic antigen from Sephadex G-25. Fractions assayed by method *A* (———), method *B* (. . . . . .), and method *C* (– – – – –).

DISCUSSION

Preliminary indications that the carbohydrate–peptide linkage regions in chick allantoic glycopeptide consisted of alkali-labile, glycosidic linkages between 2-acetamido-2-deoxygalactose and serine and/or threonine were given by quantitative analyses of the antigen for component sugars and amino acids, by analysis of the products of graded hydrolysis of the antigen, and by the high value for reducing sugar given by the antigen on reaction with alkaline ferricyanide. Similarly, high values for reducing sugar have been reported[6] for other glycopeptides that contain alkali-labile linkages between 2-acetamido-2-deoxy-D-galactose and threonine. It is well established[7] that treatment of such *O*-glycosylated hydroxyamino acids with dilute, aqueous alkali results in β-elimination of the carbohydrate, which may subsequently undergo further alkali-induced reactions, and the formation of an α,β-unsaturated amino acid.

The present results confirm that the β-hydroxy groups of most of the serine and threonine residues in the chick allantoic antigen are glycosidically linked exclusively to 2-acetamido-2-deoxygalactose. Further evidence for these structures is provided by the presence of 2-aminobutyric acid and of increased amounts of alanine in hydrolysates of the antigen, following treatment with alkali and borohydride. These amino acids are formed by reduction of 2-aminocrotonic acid and 2-aminoacrylic acid that are produced from threonine and serine, respectively, by alkali-mediated β-elimination of the carbohydrate moiety in the proposed structures. The amounts of 2-aminobutyric acid and alanine that were produced in this reaction sequence were considerably lower than those expected from the decrease in the amounts of threonine and serine. Low yields of 2-aminobutyric acid, which have been reported[8,9] in similar studies of this type of carbohydrate–peptide linkage in other glycopeptides, have been attributed[10] to an inefficient hydrogenation reaction. This is the most

probable explanation of the low yields obtained in the present work. Wieland[11] has shown, however, that alkali treatment of *O*-glycosidically linked threonine residues in glycoproteins can produce glycine, but analytical data show that this reaction does not occur to any great extent on treatment of the chick allantoic antigen with alkali. 2-Acetamido-2-deoxygalactitol, which might be produced by the reduction with borohydride of some of the 2-acetamido-2-deoxygalactose residues that were liberated by alkali-mediated cleavage of the carbohydrate–peptide linkage region, was not resolved from 2-amino-2-deoxygalactose by using the autoanalyser.

Further evidence for *O*-(2-acetamido-2-deoxygalactosyl)threonine and *O*-(2-acetamido-2-deoxygalactosyl)serine in the carbohydrate–peptide linkage regions of the antigen is provided by the identification of the Morgan–Elson chromogen and its reduced form, respectively, in hydrolysates of the antigen, following treatment with alkali and with alkali and borohydride. The reduced form of the Morgan–Elson chromogen has been reported in acid hydrolysates of keratan sulphate and *N*-acetylchondrosine[12], and of sulphated glycopeptides from fish[9] after treatment with alkali and borohydride.

Fractionation, using Sephadex G-25, of the products of alkaline degradation of the antigen shows that the Morgan–Elson chromogen is attached to relatively large oligosaccharide(s) (Fraction *B*) which, in the intact antigen, must be linked to O-6 of 2-acetamido-2-deoxygalactose residues that are involved in the alkali-labile, carbohydrate–peptide linkage regions. Substitution of these 2-acetamido-2-deoxygalactose residues at O-4 would preclude chromogen formation. An O-3 substituent, which is imperative for chromogen formation under the conditions used[12], would be eliminated during alkaline degradation. Further fractionation studies of Fraction *B* showed that oligosaccharides that were linked to the Morgan–Elson chromogen had a wide range of molecular weights. The absence of free Morgan–Elson chromogen in the alkaline digests of the antigen indicates that none of the 2-acetamido-2-deoxygalactose residues in the alkali-labile, carbohydrate–peptide linkage region is substituted only at O-3.

The products of high molecular weight (Fraction *A*) that were produced by alkaline degradation of the antigen might contain undegraded antigen, oligosaccharide substituents of relatively high molecular weight which were eliminated from O-3 of 2-acetamido-2-deoxygalactose residues in the alkali-labile, carbohydrate–peptide linkage regions, and peptide involved in alkali-stable linkages to carbohydrate. The structural significance of the carbohydrates of smaller molecular weight (Fraction *C*) that were produced on alkaline degradation of the antigen is more difficult to interpret. They might be either substituents that are eliminated from O-3 of 2-acetamido-2-deoxygalactose residues in the carbohydrate–peptide linkage region or artefacts produced by alkali-mediated "peeling" reactions of larger oligosaccharides that were eliminated from that position.

This work reveals certain important structural differences between the chick allantoic antigen investigated by the present authors and a so-called "chick allantoic fluid saccharide" (CAFS) that was isolated by Meyer *et al.*[13] from papain and

pronase digests of allantoic fluid. Both materials contained the same sugar components, but CAFS was significantly richer in sialic acid(s) (*ca.* 13%) and contained less sulphate (*ca.* 7%). The peptide contents of the two materials were similar. Alkaline degradation of CAFS showed that it consisted of a peptide backbone to which multiple, short carbohydrate chains were attached by *O*-glycosidic linkages to serine and threonine. In contrast to the present findings, however, both 2-acetamido-2-deoxygalactose and 2-acetamido-2-deoxyglucose were involved in the carbohydrate–peptide linkage regions in CAFS. The antigenicity and higher immunological activity of the antigen used in the present work by comparison with CAFS may be ascribed, in part, to its higher molecular weight and to the presence of more complete peptide chains, but the present results show that there are, in addition, certain important structural differences between the two materials.

ACKNOWLEDGMENTS

We thank Dr. A. Harboe, National Institute of Public Health, Virus Department *B*, Oslo, Norway, for valuable discussion and for provision of chick allantoic antigen; Professors M. Stacey, C.B.E., F.R.S., and S. A. Barker for their interest in this work; and the Science Research Council for financial support.

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PROPERTIES OF A GLUCOAMYLASE FROM *Aspergillus phoenicis**

D. R. LINEBACK** AND W. E. BAUMANN
Department of Biochemistry and Nutrition, University of Nebraska, Lincoln, Nebraska 68503 (U. S. A)
(Received February 21st, 1970)

ABSTRACT

The extracellular glucoamylase [α-D-(1→4)-glucan glucohydrolase, E. C. 3.2.1.3] of *Aspergillus phoenicis* Staley 155, as purified by chromatography on ion-exchange columns of DEAE-cellulose consists of two forms. One of these was isolated in high purity, as indicated by sedimentation velocity and disc electrophoresis. The enzyme has a molecular weight of 63,600 by sedimentation approach-to-equilibrium studies. Optimal conditions for action on starch by the purified enzyme are 60° and pH 4.6. The glucoamylase loses 45% of its activity after 192 h at 25°. The enzyme is a glycoprotein containing 17% of total carbohydrate. The carbohydrate component was not dissociated by treatment with urea or during gel filtration in urea. D-Mannose is the preponderant sugar component and lesser proportions of D-glucose and D-galactose are present; sialic acid and hexosamines appear to be absent.

INTRODUCTION

Glucoamylases [α-D-(1→4)-glucan glucohydrolases, E. C. 3.2.1.3] convert starch almost quantitatively into D-glucose. As a result, these enzymes are important in the commercial production of crystalline D-glucose and in the manufacture of corn syrups of high D-glucose content. Extracellular glucoamylases have been isolated from *Aspergillus niger*[1–7], *A. oryzae*[8–10], *Rhizopus delemar*[11], *Coniophora cerebella*[12], and *Endomycopsis capsularis*[13], and their properties have been studied. Glucoamylases from the first three organisms above have been shown to be glycoproteins. Multiple forms of glucoamylases have been isolated from *A. niger*[1–3] and *A. oryzae*[8].

In our laboratory, the extracellular glucoamylase of *Aspergillus phoenicis* has been purified and two forms of the enzyme were obtained. One of these was isolated in high purity and was studied further. It is a glycoprotein and the carbohydrate components have been identified.

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*Published, with the approval of the Director, as Paper No. 2733 Journal Series, Nebraska Agricultural Experiment Station. Preliminary report: *Abstracts Papers Amer. Chem. Soc. Meeting*, 158 (1969) CARB 76.
**Present address: Department of Grain Science and Industry, Kansas State University, Manhattan, Kansas 66502, U. S. A.

RESULTS AND DISCUSSION

Purification of the enzyme. — Two forms of the glucoamylase of *Aspergillus phoenicis* were separable by chromatography of a crude enzyme preparation on ion-exchange columns of DEAE-cellulose. A typical pattern showing four protein components, obtained by chromatography of the crude enzyme, is given in Fig. 1. Only the third and fourth protein components possessed starch-hydrolyzing activity. Both of these components produced D-glucose as the sole product from substrates of starch and maltose, indicating that they were glucoamylases. Rechromatography of glucoamylase A (the starch-hydrolyzing component that was eluted first) and glucoamylase B produced patterns showing single major protein components.

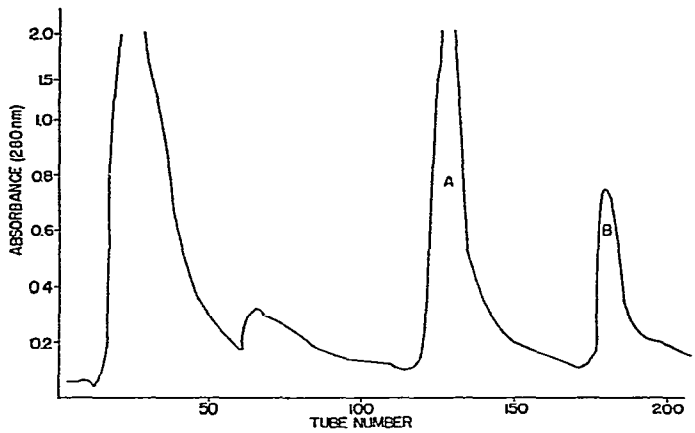


Fig. 1. Chromatography of the crude enzyme on DEAE-cellulose. The crude enzyme (100 ml) was chromatographed on a column of DEAE-cellulose (3 × 50 cm) at a flow rate of 1 ml/min by the elution procedure described in the experimental section. The fractions labeled A and B refer to glucoamylase A and B, respectively.

Test for heterogeneity. — Disc electrophoresis of the crude enzyme on 7% polyacrylamide gels showed several protein components. The electrophoretogram of purified glucoamylase A showed only a single component staining as protein, whereas purified glucoamylase B showed a major protein band and two clearly defined minor bands. Glucoamylase A migrated more slowly than the major protein component of glucoamylase B. Glucoamylase B was not studied further during the present investigation.

The efficiency of a typical purification procedure at each step is indicated in Table I. The activity of glucoamylase A was increased approximately 3.7-fold, as determined from the specific activity of the enzyme after the first fractionation. The specific activity of the crude enzyme cannot be used as the base figure since more than one starch-hydrolyzing enzyme was present. The low protein recovery for glucoamylase A during the chromatographic repurification was caused by precipitation of the protein. The pH of the enzyme solution was adjusted to 8.0 just prior to rechromato-

TABLE I

PURIFICATION OF GLUCOAMYLASE FROM *A. phoenicis*

Procedure	*Volume* (*ml*)	*Enzyme activity*		*Protein* (*mg/ml*)	*Sp. activity* (*units/mg*)	*Yield, (%)*	*Purification*[a]
		Concentration (*units/ml*)	*Total units* ($\times 10^{-3}$)				
Crude enzyme	100	60	60	20	3.0	100	—
Chromatography (DEAE-cellulose)							
Glucoamylase A	865	3.6	31	1.1	3.3	52	1.0
Glucoamylase B	400	0.74	3.0	0.58	1.3	5	1.0
Rechromatography of Glucoamylase A (DEAE-cellulose)							
Glucoamylase A	85	5.5	4.7	0.45	12.2	8	3.7
Rechromatography of Glucoamylase B (DEAE-celiulose)							
Glucoamylase B	65	2.4	1.6	0.36	6.7	3	5.1

[a]Reflects increase in specific activity over the enzyme obtained after the first chromatographic separation.

graphy, and precipitation occurred while the enzyme solution was being adsorbed onto the column.

On ultracentrifugal analysis (approximately 200,000 × g) glucoamylase A sedimented as a single, symmetrical boundary suggesting that it was not significantly contaminated by molecules having different sedimentation coefficients. Under the same conditions, glucoamylase B showed at least two peaks. A detailed boundary-analysis was performed on purified glucoamylase A by calculating the apparent diffusion coefficient from each picture and plotting these values as a function of time[14]. If a sample contains molecules of various sedimentation coefficients, the apparent diffusion coefficient increases linearly with time. Such an increase was not observed with glucoamylase A, indicating that the enzyme was highly purified.

Properties of glucoamylase A. — The molecular weight of glucoamylase A was determined by the Archibald approach-to-equilibrium procedure as described by Schachman[14]. Molecular weights calculated at the liquid meniscus or bottom of the cell were 63,600 ± 6,800. The partial specific volume of the protein, determined by the method of Schachman[14], was 0.73 ml/g. The molecular weight of purified glucoamylase A was also estimated by gel filtration on a calibrated column of Sephadex G-100 (Fig. 2); a value of 62,000 was obtained, in excellent agreement with that obtained by the approach-to-equilibrium procedure.

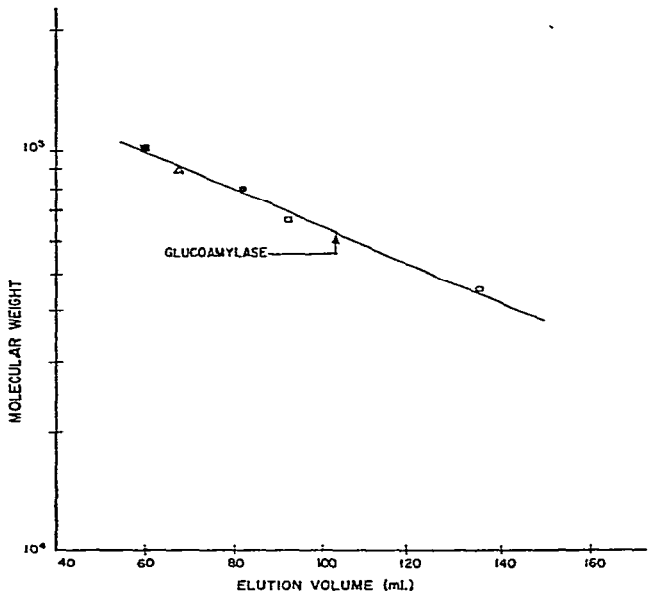


Fig. 2. Calibration of the column of Sephadex G-100 (2.5 × 65 cm); (■) lipoxidase, (△) D-amino acid oxidase, (●) alcohol dehydrogenase, (□) bovine serum albumin, and (○) ovalbumin. The column was eluted with 0.1M acetic acid at a flow rate of 6 ml/h.

The amino acid composition of glucoamylase A is shown in Table II based on a molecular weight of 63,600. All values were obtained by standard procedures on

TABLE II

AMINO ACID COMPOSITION OF GLUCOAMYLASE A FROM *A. phoenicis*

Amino acid	*Calc. residues 63,600 mol wt.*	*Nearest integer*
Arginine	15.1[a]	15
Histidine	4.3	4
Lysine	11.3	11
Phenylalanine	18.8	19
Tyrosine	18.6	19
Leucine	38.5	39
Isoleucine[b]	17.6	18
Methionine	1.4	1
Cystine/2	5.0	5
Valine[b]	34.6	35
Alanine	58.3	58
Glycine	43.9	44
Proline	20.2	20
Glutamic acid	38.4	38
Serine[c]	—	73
Threonine[c]	—	59
Aspartic acid	62.4	62
Ammonia[c]	—	105
Tryptophan[d]	0	0

[a]All values are the average of 24-, 48-, and 72-h hydrolyses in 6M HCl except where indicated. [b]Value from 72-h hydrolysis. [c]Extrapolated to zero time of hydrolysis. [d]Determined spectrophotometrically by the method of Goodwin and Morton[15].

acid hydrolyzates of the enzyme. The enzyme was not subjected to oxidation by peroxyformic acid prior to analysis, and the half-cystine value must be regarded as approximate only. Averaged values are given for each amino acid, except for serine and threonine, values for which were obtained by extrapolation to zero time, because of partial destruction of these amino acids during hydrolysis. The value for ammonia was also obtained by extrapolation to zero time, because its concentration increased during the course of the hydrolysis. Data from the 72-h hydrolysis only were used for isoleucine and valine, since these components were relatively resistant to hydrolytic release. The u.v. spectrum of purified glucoamylase A in 0.1M sodium hydroxide (pH 13) over the range 260–300 nm showed the peak expected for tyrosine at 290 nm[15] but showed no peak at 280 nm for tryptophan, suggesting that tryptophan is absent. A more-sensitive, micro method must be utilized before this absence can be confirmed.

The pH optimum for glucoamylase A acting on a starch substrate is shown in Fig. 3. Maximal activity was observed at pH 4.6, and there was approximately 25% of inactivation at pH values above 5.5 or below 3.5.

The optimum temperature for the action of glucoamylase A on a starch substrate was investigated over the range of 0–70°. Maximal activity was observed at about 60°.

The stability of glucoamylase A over prolonged periods of time at 25° was

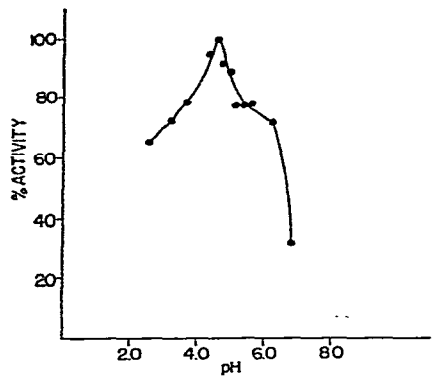


Fig. 3. pH Optimum for the action of glucoamylase A on starch. The enzyme (0.5 ml) was incubated with 4% starch (1.5 ml) at 30° for 1 h and the D-glucose produced was determined as described in the experimental section.

studied. The enzyme lost activity slowly; approximately 15% of the activity was lost after 72 h at 25° and 45% was lost after 192 h at 25°.

Glycoprotein nature of glucoamylase A. — Analyses[16,17], by the phenol–sulfuric acid method, of freeze-dried, dialyzed glucoamylase A revealed that the enzyme contained 17% of total carbohydrate (relative to a standard of D-mannose) indicating approximately 67 hexose residues per molecule of enzyme. A mannose and a glucose were readily identified by paper chromatography of acid hydrolyzates of the purified enzyme; the former was the preponderant hexose. Gas–liquid chromatographic analysis of the *O*-trimethylsilyl derivatives of an acid hydrolyzate of the purified enzyme gave products migrating as corresponding derivatives of mannose, glucose, and galactose. No amino sugars were detected by the paper or gas–liquid chromatograms of the acid hydrolyzates. Aliquots from a sample of glucoamylase A, hydrolyzed with 0.15M sulfuric acid, assayed exactly as the blanks in the thiobarbituric acid assay[18] indicating that sialic acid was absent from glucoamylase A. The *O*-trimethylsilyl derivatives of acid hydrolyzates and methanolysates of freeze-dried, dialyzed glucoamylase A were quantitatively analyzed by g.l.c. (Table III). The preponderant hexose was a mannose, and smaller proportions of a glucose and a galactose were also indicated, by both procedures. The values obtained for each hexose by methanolysis or hydrolysis were in reasonable agreement, except for

TABLE III

CARBOHYDRATE COMPOSITION OF GLUCOAMYLASE A FROM *A. phoenicis*

Procedure	*% Mannose*	*% Glucose*	*% Galactose*	*Total*
Methanolysis	7.3	3.9	.5	11.7
Hydrolysis	8.0	2.8	1.2	12.0
Phenol–sulfuric acid assay	—	—	—	16.9

galactose which is present in only small proportion. The discrepancy between the total carbohydrate as determined by the phenol–sulfuric acid procedure, and the total values from acid hydrolysis or methanolysis can most probably be attributed (*a*) to destruction of the sugars under the conditions of acid hydrolysis or methanolysis, (*b*) to incomplete release of the hexoses from the glycoprotein under these conditions, or (*c*) to contribution of the protein to color formation in the phenol–sulfuric acid assay.

That the carbohydrate is covalently bonded to the enzyme was indicated by the following. Dialysis of the purified enzyme in 4.0M urea for various times had no effect on the carbohydrate content of the enzyme. Purified glucoamylase A was chromatographed on a column of Sephadex G-100 that had been prepared in 4.0M urea and was eluted with 4.0M urea. The relative contents of protein and carbohydrate in the effluent fractions were measured and are shown in Fig. 4. The protein and

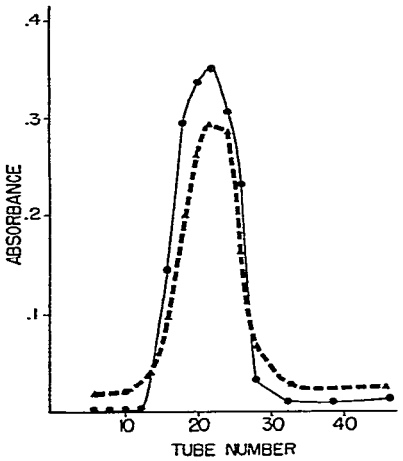


Fig. 4. Concentration of protein and carbohydrate in fractions obtained from gel filtration of glucoamylase A on a column (2.5 × 65 cm) of Sephadex G-100 equilibrated with 4M urea. The protein was eluted with 4M urea at a flow rate of 6 ml/h; (——) absorbance at 280 nm for protein, (– – – – –) absorbance at 489 nm for carbohydrate (phenol–sulfuric acid assay).

carbohydrate contents are eluted simultaneously, indicating that both are associated with the same component being eluted from the column. The same results were obtained when the purified enzyme was chromatographed on a column of Sephadex G-100 which had been prepared in 0.1M acetic acid and was eluted with the same solvent. The absence of separation of the components under these conditions indicates that the carbohydrate residues and the polypeptide chain are covalently linked.

The glucoamylase from *A. phoenicis* is similar, in many respects, to the glucoamylase from *A. niger*. The latter enzyme has been purified and found to consist of two forms[1–3] which are glycoproteins containing D-mannose, D-glucose and D-galactose[6]. Both enzymes possess pH optima[1,3] in the range 4.5–5.0 and temperature optima[19] of 60°. The carbohydrate moieties of the glucoamylase from *A.* niger have been reported to be *O*-glycosidically linked through mannose to the hydroxyl groups

of serine and threonine[7]. The amino acid analysis of glucoamylase A from *A. phoenicis* reveals the presence of relatively large proportions of serine and threonine, thus indicating the possibility that the same type of linkage may exist in this glycoprotein. The molecular weight of glucoamylase A from *A. phoenicis* is close to the value of 69,000 reported[9] for one of the four forms of the glucoamylase of *A. oryzae*, but is less than the value of 100,000 reported for the glucoamylases of *Rhizopus delemar*[11] and *A. niger*[5,11]. Interestingly, the glucoamylases of *A. phoenicis* and *A. niger*[6] do not contain amino sugars, whereas those from *A. oryzae*[10] and *R. delemar*[11] contain hexosamines. The carbohydrate–polypeptide linkage may be different in the latter enzymes. Multiple forms of fungal glucoamylases have now been isolated from culture filtrates of *A. phoenicis*, *A. niger*[1–3] and *A. oryzae*[8], whereas only single forms have been isolated from *R. delemar*[11], *Coniophora cerebella*[12], and *Endomycopsis capsularis*[13].

The specific functions of the carbohydrates in these glycoprotein glucoamylases are at present unknown. Further studies are in progress to elucidate the structure of the carbohydrate moieties of the glucoamylases to determine similarities between the enzymes from different sources and the relationship that this may have to the function of the carbohydrate residues.

EXPERIMENTAL

Materials. — A crude glucoamylase from *Aspergillus phoenicis* Staley 155 was provided by the A. E. Staley Manufacturing Company, Decatur, Illinois. Ovalbumin, bovine serum albumin, alcohol dehydrogenase, D-amino acid oxidase and soybean lipoxidase were obtained from Mann Research Laboratories, New York, New York. Pyridine (practical grade) was refluxed over barium oxide for 4–6 h and then distilled. The fraction boiling at 111–114° was stored over potassium hydroxide. Methanol (reagent grade) was dried by the magnesium procedure. All other chemicals used were reagent or analytical grade, unless otherwise specified.

Chromatographic procedures. — Ion-exchange columns of DEAE-cellulose (reagent grade, 0.9 meq/g, Brown Company, Berlin, New Hampshire) were prepared and operated as previously described[3].

Sephadex G-100 (40–120 μm, Pharmacia), was hydrated by stirring in 0.1M acetic acid. A thick slurry was poured into a column (2.5 × 80 cm) that had been pre-rinsed three times with 1% dichlorodimethylsilane in benzene at 60°, and dried. The gel was allowed to settle to 65–70 cm. Chromatography was performed at 4° with 0.1M acetic acid as eluant. Fractions (approximately 3 ml) were collected automatically at 30-min intervals. The flow rate was maintained with a Mariotte flask. A similar column of Sephadex G-100 was also prepared but with 4M urea in place of 0.1M acetic acid.

G.l.c. was performed with an F & M Model 5750 Gas Chromatograph equipped with flame-ionization detectors. A stainless-steel column (12 ft × 0.125 in) packed with a stationary phase of 3% SE-52 on Anakrom SD (90/100 mesh) was used to

separate the trimethylsilyl ethers of methyl glycosides. The column was operated at 145° with nitrogen as the carrier gas (22 ml/min). A stainless-steel column (9 ft × 0.125 in) packed with the same stationary phase was used to separate the trimethylsilyl ethers of free hexoses. Nitrogen was used as carrier gas at a flow rate of 10 ml/min. The chromatograph was programmed to maintain the temperature at 120° for 3 min after injection of the sample, and then to increase at 6°/min to 185°, at which point the temperature was maintained at 185° until the components had emerged from the column (33–39 min).

Enzyme assays. — Starch-hydrolyzing activity was qualitatively assayed by incubating a 0.5-ml aliquot of the enzyme solution with 1.5 ml of 4% starch (soluble, Lintner) at room temperature. The formation of D-glucose was detected with reagent strips of D-glucose oxidase (Clinistix, Miles Chemical Co., Elkhart, Indiana).

Enzymic activity was assayed quantitatively as previously described[3]. One unit of glucoamylase activity was defined as the amount of enzyme required to liberate 1 μmole of glucose per min under the defined reaction conditions[3]. Specific activity was expressed as units per mg of protein, as determined by the Lowry method[20].

Glucoamylase activity was identified by incubating aliquots (0.1 ml) of the enzyme solution with 0.1 ml of 10% maltose and with 0.1 ml of 4% starch in 0.1M acetate buffer, pH 4.8, at room temperature. Aliquots of the solutions were spotted on Eaton–Dikeman 613 chromatography paper after 0, 1, 2, 4, 8, and 24 h of incubation. The papers were developed by three ascents of a 6:4:3(v/v/v) butyl alcohol–pyridine–water solvent system[21] and the carbohydrate areas were located by silver nitrate[22]. The presence of a glucose as the only product from maltose or starch was indicative of glucoamylase activity.

Purification at the enzyme. — Crude glucoamylase from *Aspergillus phoenicis* Staley 155 was supplied as a dark brown solution. The crude enzyme (100 ml) was dialyzed against running tap water for 12 h. The dialysis casing was changed after 2 h to prevent its rupture. Sufficient 0.2M citrate–phosphate buffer (pH 8.0) was added to the dialyzed enzyme to yield a solution that was 0.025M. After adsorption onto the DEAE-cellulose, the constituents of the crude enzyme preparation were eluted by successive passage through the column of 500 ml of 0.025M citrate–phosphate buffer (pH 8.0), 500 ml of 0.05M citrate–phosphate buffer (pH 8.0), 750 ml of the same buffer (pH 6.0), and 1500 ml of the same buffer (pH 4.0). Each buffer was added when 75–100 ml of the previous buffer remained in the chromatographic column. Starch-hydrolyzing activity was observed in two protein peaks, one eluted at a pH of approximately 6.3 (glucoamylase A) and a second eluted at about pH 4.7 (glucoamylase B). The effluents containing glucoamylase A were combined and rechromatographed. Glucoamylase A was adsorbed onto the DEAE-cellulose at pH 8.0 and was recovered by successive elution with 750 ml of 0.025M citrate–phosphate buffer (pH 8.0), and 1000 ml of the same buffer (pH 6.0). The eluates containing glucoamylase B were combined and further purified by chromatography on a column of DEAE-cellulose. Adsorption of this enzyme was effected from 0.025M citrate–phosphate buffer (pH 6.0), and elution was performed in a manner analogous to that

described for glucoamylase A by using 0.025M citrate–phosphate buffers of pH 6.0 (750 ml) and 4.0 (1500 ml). Prior to rechromatography, both glucoamylases were dialyzed against running tap-water and adjusted to the desired ionic strength (0.025M) and pH by using a concentrated (0.2M) buffer.

Purified glucoamylase A was dialyzed against 0.025M citrate–phosphate buffer (pH 5.0), for 3–7 days to remove dialyzable, carbohydrate-positive material. The protein concentration of the purified solutions was determined by the Lowry method[20] by using bovine serum albumin as a standard.

Disc electrophoresis. — Disc electrophoresis was performed on 7% polyacrylamide gels by the method of Davis[23].

Sedimentation velocity and equilibrium measurements. — Purified enzyme solutions were concentrated to approximately 0.5 to 1% of protein by evaporation at 4°, and were dialyzed against 0.1M sodium chloride. Experiments on sedimentation velocity were conducted at a constant temperature of 20° with a rotor speed of 50,740 r.p.m. with a Spinco ultracentrifuge, Model E. Photographs were obtained at 16-min intervals after the centrifuge had reached maximum speed.

The homogeneity of the purified enzyme during sedimentation was evaluated by a detailed boundary analysis in the sedimentation velocity experiment. The apparent diffusion coefficient was calculated from each picture obtained during the experiment by the height–area method and was plotted *versus* time[14].

Experiments on sedimentation equilibrium were conducted at a constant temperature of 20° with rotor speeds of 5784 r.p.m. After the ultracentrifuge had reached full speed, ten photographs were taken at 16-min intervals. The initial concentration, c_0, was determined by an experiment in a synthetic-boundary cell with a rotor speed of 12,590 r.p.m. The weight-average molecular weights at the meniscus and bottom of the cell were calculated for each photograph as described by Schachman[14].

Determination of the partial specific volume. — Density measurements were made in a Sprengel–Ostwald type of pycnometer and weighings were performed with a Sartorius Selector balance sensitive to 0.025 mg. Distilled water was redistilled from glass and this redistilled water was used in all subsequent measurements. Density measurements were made at 25° on water, 0.100M NaCl, 0.5%, 0.4%, and 0.2% solutions of protein. The protein solutions were prepared by successive dialysis against distilled water and against 0.100M NaCl in redistilled water. The volume of the pycnometer was obtained by dividing the weight of water in the pycnometer at 25° by the density of water[24] at 25°. Protein concentrations were determined by weighing aliquots of the enzyme solutions before and after evaporation. Solutions in the weighing bottles were evaporated to dryness in a desiccator under diminished pressure over potassium hydroxide and phosphorus pentaoxide. Drying was continued to constant weight.

Gel-filtration chromatography. — A column (2.5 × 65 cm) of Sephadex G-100 was calibrated by determining the elution volumes for Blue Dextran (mol.wt. 2,000,000; void volume), soybean lipoxidase[25] (mol.wt. 102,000), D-amino acid oxidase[26]

(mol.wt. 90,000), alcohol dehydrogenase[27] (mol.wt. 80,000), bovine serum albumin[28] (mol.wt. 67,000), and ovalbumin[28] (mol.wt. 45,000). The proteins were located in the effluent fractions by measuring absorbance at 280 nm or by an appropriate enzymic assay. Blue Dextran was located by determining the absorbance of the fractions at 254 nm. The elution volume for purified glucoamylase A was determined on this column. All proteins were eluted with 0.1M acetic acid.

Purified glucoamylase A was also chromatographed on a similar column of Sephadex G-100, prepared in 4M urea. The protein was eluted with 4M urea. Protein was located in the effluent fractions by measuring the absorbance at 280 nm, and carbohydrate components were located by the phenol–sulfuric acid procedure[16,17].

Amino acid analysis. — Purified glucoamylase A was dialyzed against distilled water for at least 48 h at 4°, and then freeze-dried. Samples (*ca.* 35 mg) were hydrolyzed in sealed tubes in constant-boiling hydrochloric acid for periods of 24, 48, and 72 h. The acid was removed under diminished pressure, the residue was dissolved in water and the solution was again evaporated under diminished pressure. Amino acid analyses were performed according to the method of Spackman *et al.*[29] with a Spinco Model 120C amino acid analyzer. Tryptophan was assayed by the method of Goodwin and Morton[15].

Dialysis against urea. — Purified glucoamylase A was dialyzed for 12, 24, and 48 h at 4° against 4.0M urea. At each of these times, the carbohydrate content of the enzyme solution was determined by the phenol–sulfuric acid procedure[17].

pH optimum. — The activity of glucoamylase A was determined by using 4% starch at various pH values. The enzyme solutions were dialyzed against distilled water for 2 days at 4° and then diluted to 0.1 mg protein/ml. Aliquots (2.0 ml) were dialyzed for 3 days at 4° against 0.025M citrate–phosphate buffers of pH 2.6, 3.2, 3.8, 4.4, 4.6, 4.8, 5.0, 5.2, 5.4, 5.6, 6.2, and 6.8. Starch solutions (4%) were prepared in 0.025M citrate–phosphate buffers corresponding to each of the above pH values. Starch-hydrolyzing activity was assayed at each pH value in the manner previously described.

Temperature stability. — The stability of glucoamylase A was determined at 25°. The enzyme, in 0.05M citrate–phosphate buffer (pH 4.8) was kept at 25° and 0.5 ml aliquots were removed at 0, 24, 48, 72, and 192 h. The starch-hydrolyzing activity of these aliquots was determined in the manner previously described.

Temperature optimum. — Glucoamylase A (0.5 ml, approximately 0.1 mg protein/ml) in 0.025M citrate–phosphate buffer (pH 4.8) was incubated for 30 min with 1.5 ml of 4% starch in 0.025M citrate–phosphate buffer (pH 4.8) at temperatures of 0, 10, 20, 30, 40, 50, 60, and 70°. Reaction was then stopped by the addition of 5.0 ml of absolute ethanol to precipitate the enzyme and unreacted starch. The mixture was centrifuged and D-glucose was determined in the supernatant solution by using the glucose oxidase–chromophore (Glucostat, Worthington Biochemical Corp., Freehold, New Jersey) procedure previously described[3].

Carbohydrate content. — Total carbohydrate content of the enzyme was determined by the phenol–sulfuric acid procedure[17] by using 1.0 ml of 8.0% phenol

and 1.0 ml of sample solution. D-Mannose and D-glucose (0–150 μg) were used to prepare standard curves. Individual carbohydrate components were determined by g.l.c. following hydrolysis or methanolysis.

Glucoamylase A (30 mg of freeze-dried, dialyzed protein) was hydrolyzed with 1.0M HCl for 6 h at 100°. The hydrolyzate was diluted with water and brought to pH 6 by stirring with Bio-Rad AG 3-X4 (OH^-) resin (Bio-Rad Laboratories, Richmond, California). The resin was filtered off and washed with water. The combined washings and filtrate were freeze-dried. In initial experiments for investigating the presence of amino sugars, the freeze-dried residue was *N*-acetylated with acetic anhydride (0.1 ml) in methanol (2.5 ml) for 24 h at room temperature. The solvents were evaporated with a stream of air. The sugars were trimethylsilylated by treating the residue with a solution of hexamethyldisilazane and chlorotrimethylsilane in pyridine (TRI-SIL, Pierce Chemical Co., Rockford, Illinois). Aliquots of the solutions were then analyzed by g.l.c. For quantitative analysis, D-xylose was added as an internal standard prior to trimethylsilylation.

Aliquots of the acid hydrolyzates were also spotted on Eaton–Dikeman 613 chromatography paper, together with suitable reference monosaccharides, and subjected to four ascents of a butyl alcohol–ethanol–water (4:1:1 v/v/v) solvent[30]. Carbohydrates were located on the dried papers with the permanganate–periodate spray reagent[31].

Methanolysis of freeze-dried, dialyzed glucoamylase A was accomplished in a manner similar to that reported by Clamp *et al.*[32] Freeze-dried, purified enzyme (5 mg) was refluxed in dry, methanolic hydrogen chloride (1.87M) for 24 or 48 h. The solution was neutralized with silver carbonate and filtered. The precipitate was washed with dry methanol and the combined washings and filtrate were evaporated to dryness at 40–50° with a stream of filtered air. The residue was dried overnight at room temperature under diminished pressure over phosphorous pentaoxide and potassium hydroxide. Trimethylsilyl ethers of the methyl glycosides were prepared as described for the free sugars and analyzed by g.l.c. D-Xylose (1 mg) was added as an internal standard prior to methanolysis.

The thiobarbituric acid assay for sialic acid[18] was performed on a sample (81 mg) of purified glucoamylase A that had been hydrolyzed in 0.15M sulfuric acid for 60 min at 80°.

ACKNOWLEDGMENTS

This investigation was supported by a Grant from the Agricultural Research Service, U.S. Department of Agriculture, Grant No. 12-14-100-9143 (71), administered by the Northern Utilization Research and Development Division, Peoria, Illinois, U. S. A. We express our appreciation to the A. E. Staley Company for their generous gift of the glucoamylase from *A. phoenicis* Staley 155 and to the Department of Grain Science and Industry, Kansas State University for performing the amino acid analyses. The ultracentrifuge experiments were performed in the Department of Chemistry, University of Nebraska.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

EINE VERBESSERTE SYNTHESE VON 2-ACETAMIDO-1-*N*-(4-L-ASPARTYL)-2-DESOXY-β-D-GLUKOPYRANOSYLAMIN UND DIE STEREOCHEMIE SEINER DERIVATE

MORIO KIYOZUMI*, KEITARO KATO, TETSUYA KOMORI, AKIRA YAMAMOTO*, TOSHIO KAWASAKI, UND HISAO TSUKAMOTO

Fakultät für Pharmazeutische Wissenschaften der Universität Kyushu, Fukuoka (Japan)

(Eingegangen den 11. Juli, 1969; in modifizierter Form den 2. März, 1970)

ABSTRACT

Crystalline 2-acetamido-2-deoxy-β-D-glucosylamine was prepared by deacetylation of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl azide followed by catalytic hydrogenation. An improved synthesis of 2-acetamido-1-*N*-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine is described. The configuration and conformation of its derivatives are discussed on the basis of the n.m.r. and o.r.d. spectra.

ZUSAMMENFASSUNG

Durch Deacetylieren und anschliessende katalytische Hydrierung von 2-Acetamido-3,4,6-tri-*O*-acetyl-2-desoxy-β-D-glukopyranosyl-azid wurde kristallines 2-Acetamido-2-desoxy-β-D-glukopyranosylamin synthetisiert. Eine verbesserte Methode zur Synthese von 2-Acetamido-1-*N*-(4-L-aspartyl)-2-desoxy-β-D-glukopyranosylamin wird beschrieben. Die Konfiguration und die Konformation der Derivate werden auf Grund von N.m.r.- und O.r.d.-Daten diskutiert.

EINFÜHRUNG

In vielen Glykoproteinen ist 2-Acetamido-1-*N*-(4-L-aspartyl)-2-desoxy-β-D-glukopyranosylamin (8) als Baustein der Kohlenhydrat-Peptid-Bindung anzutreffen. Die Synthese dieser im Eieralbumin erstmalig nachgewiesenen Gruppierung wurde früher[1–6] durch Kondensation von α-Benzyl-*N*-benzyloxycarbonyl-L-aspartat mit 2-Acetamido-3,4,6-tri-*O*-acetyl-2-desoxy-β-D-glukopyranosylamin (4) durchgeführt. Das hierbei erhaltene Zwischenprodukt, 2-Acetamido-3,4,6-tri-*O*-acetyl-1-*N*-(1-benzyl-*N*-benzyloxycarbonyl-4-L-aspartyl)-2-desoxy-β-D-glukopyranosylamin[1–6] (7), ließ sich durch katalytische Hydrogenolyse und Deacetylierung in die Verbindung 8 überführen.

In der vorliegenden Arbeit beschreiben wir nun die Verbesserung der Dar-

*Gegenwärtige Adresse: Fakultät für Pharmazeutische Wissenschaften der Universität Kumamoto, Kumamoto, Japan.

stellung der Verbindung 8, ausgehend vom Zwischenprodukt 2-Acetamido-2-desoxy-β-D-glukopyranosylamin (3), anstatt von Verbindung 4.

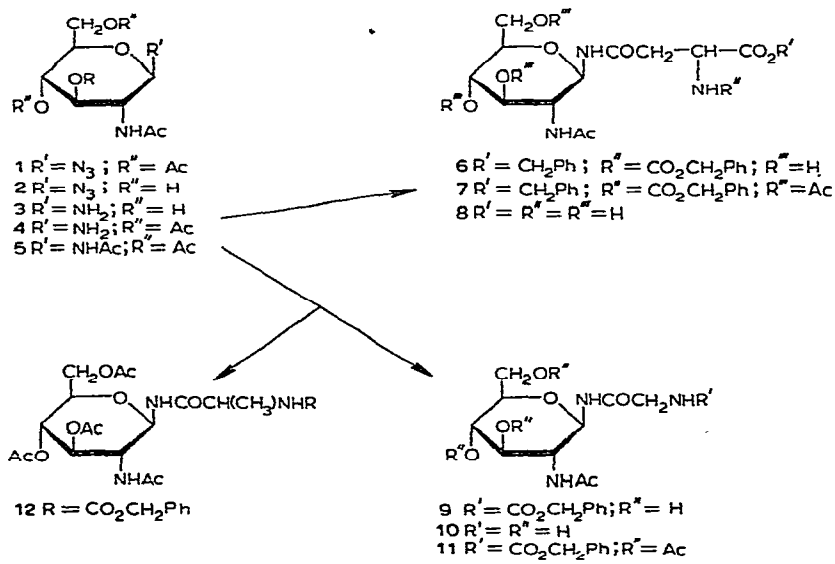
Wegen der biochemischen Bedeutung wird weiter die Stereochemie der Verbindung 8 und ihrer Derivate im Detail diskutiert.

DISKUSSION

Die erste unserer Synthesen von 3 geht auf die Arbeit von Makino und Mitarb.[7] zurück, die diese Substanz aber nicht kristallin isolieren konnten. Nach der katalytischen Hydrierung von 2-Acetamido-2-desoxy-β-D-glukopyranosyl-azid (2) wurde nun erstmals die Verbindung 3 kristallin erhalten. Das Peracetat von 3 schmolz im Gemisch mit dem früher synthetisierten Acetat von 4 ohne Depression.

Verbindung 3 und 1-Benzyl-*N*-benzyloxycarbonyl-L-aspartat ließen sich in Gegenwart von *N,N'*-Dicyclohexylcarbodiimid in das Produkt, 2-Acetamido-1-*N*-(1-benzyl-*N*-benzyloxycarbonyl-4-L-aspartyl)-2-desoxy-β-D-glukopyranosylamin (6), überführen, aus dem durch katalytische Hydrogenolyse der Schutzgruppe des Aminosäure-Teiles die Verbindung 8 gewonnen würde.

Auf diesem Weg konnten die Verbindung 7, 2-Acetamido-1-*N*-glycyl-2-desoxy-β-D-glukopyranosylamin (10), 2-Acetamido-3,4,6-tri-*O*-acetyl-1-*N*-(*N*-benzyloxycarbonylglycyl)-2-desoxy-β-D-glukopyranosylamin (11) und 2-Acetamido-3,4,6-tri-*O*-acetyl-1-*N*-(*N*-benzyloxycarbonyl-L-alanyl)-2-desoxy-β-D-glukopyranosylamin[8] (12)



aus Verbindung 3 leicht dargestellt werden. Die einzelnen Substanzen wurden durch Vergleich mit den Originalpräparaten[3,8,9] identifiziert.

Vergleicht man die Ergebnisse dieser modifizierten Reaktionsfolge mit denen der früher beschriebenen Methode, so zeigt sich, daß die Verbindung 8 in bester

Ausbeute (62%, früher 32%) erhalten und die Gesamtsynthese stark vereinfacht wurde.

Es wurde sodann die Konformation und Konfiguration der biochemisch wichtigen Substanz **8** geklärt. Die Verbindung **8** selbst ist in Deuterochloroform oder in anderen organischen Lösungsmitteln schwer löslich. Daher wurde die Konfiguration von **7**, **11** und **12** n.m.r.-spektrometrisch untersucht; denn die Konfiguration dieser Vergleichs-Verbindungen **7**, **11** und **12** ist aufgrund der Synthese mit der von **8**, identisch. Als grundlegendes Experiment wurde zuerst die Konfiguration von 2-Acetamido-3,4,6-tri-*O*-acetyl-2-desoxy-β-D-glukopyranosyl-azid (**1**) und 2-Acetamido-3,4,6-tri-*O*-acetyl-1-*N*-acetyl-2-desoxy-β-D-glukopyranosylamin (**5**) untersucht.

Das N.m.r.-Spektrum der Verbindung **1** in Deuterochloroform (60 MHz) (Abb. 1) zeigt bei δ 6,57 p.p.m. ein Dublett für eine NH-Gruppe, das bei Deuteriumoxyd-Behandlung verschwindet. Es scheint, daß das Multiplett bei δ 4,20 p.p.m. den zwei Protonen H-6 und H-6′ zuzuordnen ist.

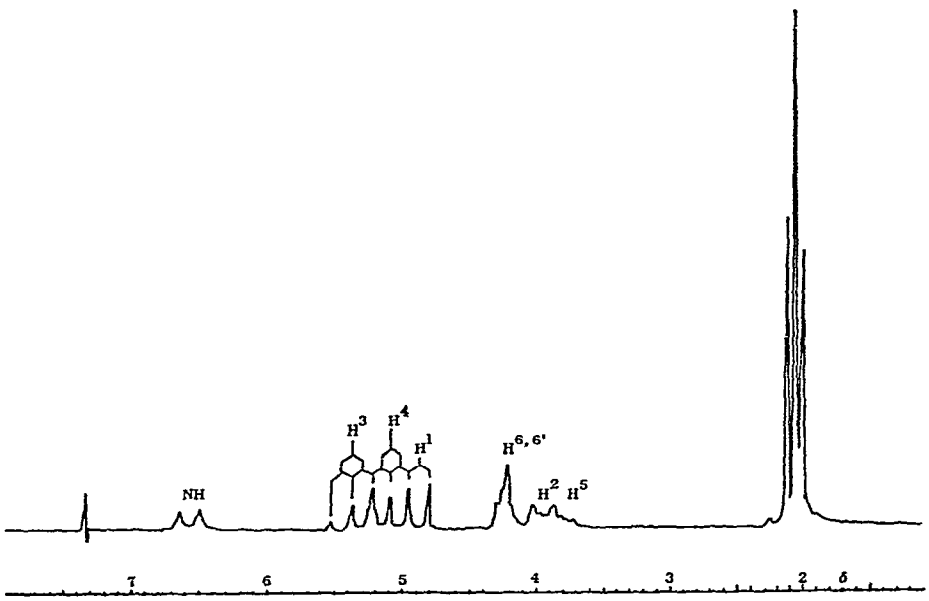


Abb. 1. N.m.r.-Spektrum von 2-Acetamido-3,4,6-tri-*O*-acetyl-2-desoxy-β-D-glucopyranosyl-azid (**1**) in Deuterochloroform (60 MHz).

Die Zuordnung der Resonanzen bei δ 3,7–4,1 p.p.m. konnte auf folgende Weise getroffen werden. Bei dem verwandten Methyl-2,3,4,6-tetra-*O*-acetyl-β-D-glukopyranosid tritt in Deuterochloroform (100 MHz) das Signal für H-5 bei δ 3,70 p.p.m. als Oktett auf. Außerdem fanden Liebert und Mitarb.[10] in *O*-Acetyl-β-D-galaktose-Derivaten, daß das Signal des Protons am C-5 des Pyranose-Rings am stärksten nach höherem Feld verschoben ist. Deshalb gehört ein Teil des Signals für zwei Protonen bei δ 3,70–4,10 p.p.m. zu H-5.

Sodann wurde das Triplett bei δ 5,38 p.p.m. ($J_{2,3} = J_{3,4} = 9$ Hz) für das

Proton am C-3, das Triplett bei δ 5,1 p.p.m. ($J_{4,3} = J_{4,5} = 9$ Hz) für das Proton des C-4 angenommen, weil die Protonen H-3 und H-4 der β-D-Glukopyranosyl-tetraacetat-Derivate im allgemeinen ungefähr bei δ 5,5 p.p.m. beziehungsweise bei δ 5,15 p.p.m. liegen[11]. Das Dublett bei δ 4,88 p.p.m. ($J_{1,2}$ 9 Hz) stammt von dem Proton in Stellung 1, da das Proton H-1 im Spektrum des Vergleichspräparats Methyl-2,3,4,6-tetra-*O*-acetyl-β-D-glukosid bei δ 4,43 p.p.m. vorkommt und das anomere Proton einer glykosylamin Bindung etwa die gleiche chemische Verschiebung aufweist wie bei einer glykosyl Bindung. Deswegen mußte der andere Teil des Signals δ 3,70–4,10 p.p.m. auf dem Proton H-2 beruhen. Eine transdiaxiale Stellung von H-1, H-2, H-3 und H-4 war daher bewiesen. Wir schlagen eine *C1* Konformation und β-D-Konfiguration für die Verbindung **1** vor. Bolton und Mitarb.[5] hatten schon früher mit Hilfe der Kernresonanz eine β-D-Konfiguration für **1** bewiesen.

Man sollte nun für die Verbindung **3** die gleiche Konfiguration wie für **1** finden, da keine Umlagerung des Ringprotons durch das Deacetylieren und die Reduktion zu erwarten ist. Statt der Verbindung **3** wurde weiter die Konfiguration ihres Acetats **5** n.m.r.-spektrometrisch untersucht. Aus den N.m.r.-Spektren von Derivaten des Methyl-2,3,4,6-tetra-*O*-acetyl-β-D-glukopyranosid und des α-D-Anomeren ist die chemische Verschiebung des β-H-1 (etwa δ 5,70–5,85 p.p.m.) und α-H-1 (etwa δ 6,40 p.p.m.) bekannt. Das N.m.r.-Spektrum des Pentaacetats **5** (Abb. 2) zeigt die zwe

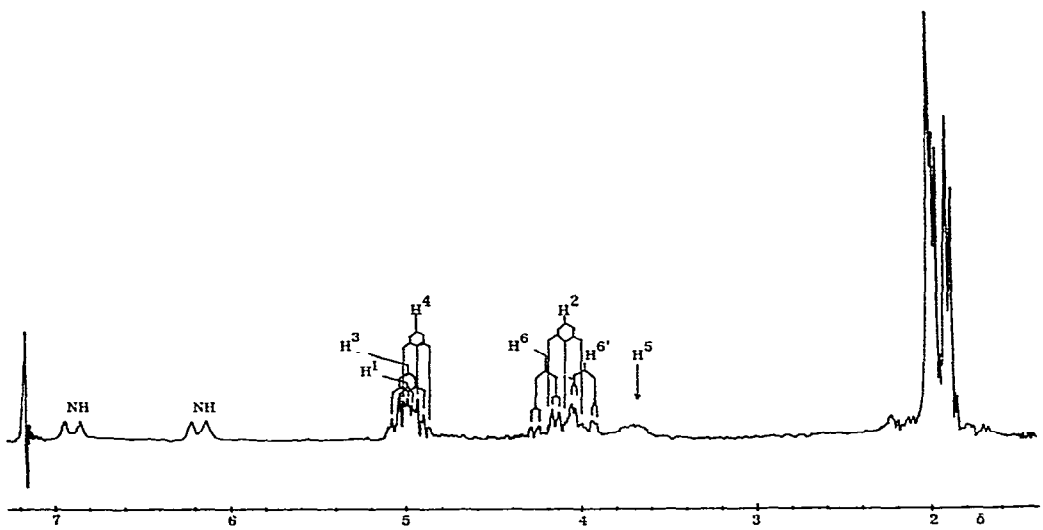


Abb. 2. N.m.r.-Spektrum von 2-Acetamido-3,4,6-tri-*O*-acetyl-1-*N*-acetyl-2-desoxy-β-D-glukopyranosylamin (**5**) in Deuterochloroform (100 MHz).

Dubletts bei δ 6,19 und 6,90 p.p.m. für die beiden NH-Protonen, die bei Deuteriumoxyd-Behandlung verschwinden. Da kein Ring-Proton bei niedrigeren Feld als δ 5,0 p.p.m. gefunden wurde, muß man nun annehmen, daß das Proton H-1 in β-D-Konfiguration steht und die Signale bei δ 5,0 p.p.m. drei Protonen für H-3, H-4 und H-1 anzeigen. Tatsächlich war das Signal von H-1 infolge Kopplung mit H-2 ein

Dublett bei δ 5,0 p.p.m. ($J_{1,2}$ 9 Hz); H-3 und H-4 zeigte je ein Triplett bei δ 4,99 p.p.m. ($J_{3,4}$ 8 Hz) und bei δ 4,94 p.p.m. ($J_{4,5}$ 8 Hz). Das Oktett bei δ 3,70 p.p.m. gehörte zu H-5 und die Protonen H-6 und H-6′ bildeten je ein Quadruplett im Bereich von δ 4,30–3,90 p.p.m. [H-6, δ 4,21 p.p.m. ($J_{5,6}$ 4 Hz); H-6′, δ 3,98 p.p.m. ($J_{5,6'}$ 2 Hz, $J_{6,6'}$ 12 Hz)]. Deshalb ergibt das Signal von H-2 infolge Kopplung mit H-1 und H-3 ein Quadruplett bei δ 4,10 p.p.m. ($J_{2,3}$ 8 Hz) und von dem Acetat **5** läßt sich ableiten, daß es die gleiche *C1* Konformation wie die Verbindung **1** besitzt.

Wir untersuchten nun weiter die Konfiguration der Verbindung **12**[8], die aus dem Ausgangsmaterial **3** über das Zwischenprodukt 2-Acetamido-1-*N*-(*N*-benzyloxycarbonyl-L-alanyl)-2-desoxy-β-D-glukopyranosylamin synthetisiert wurde.

Im Spektrum von **12** (Abb. 3) sind die drei Dubletts bei niederem Feld (δ 5,55, 6,52 und 7,40 p.p.m.) eindeutig den NH-Protonen zuzuordnen, weil sie durch Behandlung mit Deuteriumoxyd verschwinden. Das Signal der Methylenprotonen aus der Benzyloxycarbonyl-Gruppe zeigt ein scharfes Singulett bei δ 5,0 p.p.m. Die Signale der Ringprotonen sollten dieselben Systeme wie in Verbindung **5** aufweisen. Die Resonanzlagen von H-5–H-4, H-2–H-1 und H-2–H-3 wurden durch Entkopplungs-Experimente ermittelt. Bei Einstrahlung der Frequenz des Oktetts bei δ 3,72 p.p.m. für H-5 wird das Signal bei δ 4,94 p.p.m. (H-4) deformiert; in diesem Fall waren die Signale bei δ 5,10 p.p.m. (H-3) und bei δ 5,06 p.p.m. (H-1) unverändert. Bei Einstrahlung der Frequenz des Quadrupletts bei δ 4,07 p.p.m. waren das Signal bei δ 4,94 p.p.m. (H-4) unverändert, die Signale bei δ 5,10 p.p.m. (H-3) und δ 5,06 p.p.m. (H-1) jedoch deformiert. Deshalb spricht das Quadruplett bei δ 4,07 p.p.m. für ein axiales Proton H-2. Aus Abb. 3 ergeben sich die Kopplungs-

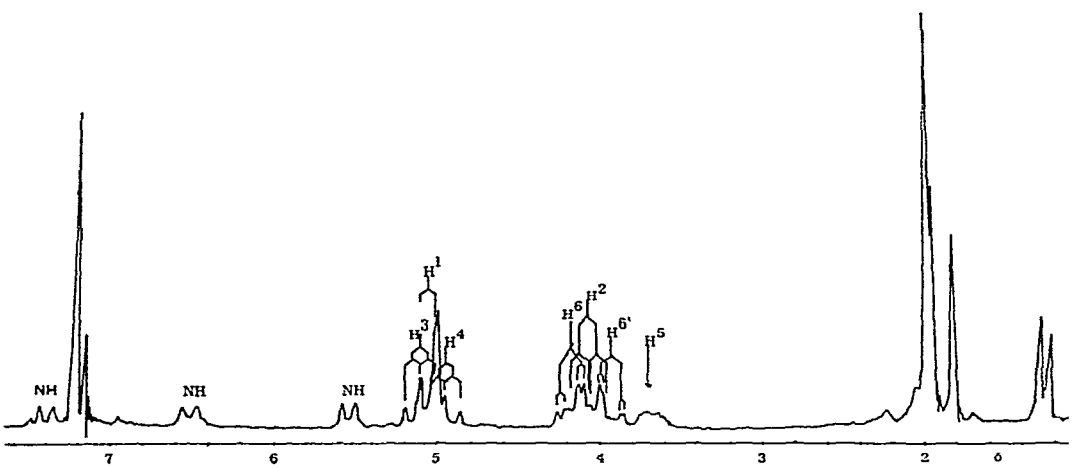


Abb. 3. N.m.r.-Spektrum von 2-Acetamido-3,4-6-tri-*O*-acetyl-1-*N*-(*N*-benzyloxycarbonyl-L-alanyl)-2-desoxy-β-D-glucopyranosylamin (**12**) in Deuterochloroform (100 MHz).

konstanten und δ-Werte der chemischen Verschiebung der Ring-Protonen von **12** wie folgt: H-1, δ 5,06 p.p.m. ($J_{1,2}$ 8 Hz); H-2, δ 4,07 p.p.m. ($J_{2,3}$ 9 Hz); H-3,

δ 5,10 p.p.m. ($J_{3,4}$ 9 Hz); H-4, δ 4,94 p.p.m. ($J_{4,5}$ 9 Hz); H-5, δ 3,72 p.p.m. (Oktett); H-6, δ 4,18 p.p.m. ($J_{5,6}$ 4 Hz); H-6', δ 3,93 p.p.m. ($J_{5,6'}$ 2 Hz) ($J_{6,6'}$ 12 Hz).

Da die Verbindungen **7** und **11** in Deuterochloroform schwer löslich waren, wurde die N.m.r.-Untersuchung in Dimethylsulfoxyd-d_6 durchgeführt. Die Signale der Ringprotonen im Spektrum von **11** (Abb. 4) zeigen große Ähnlichkeit mit dem von **12**; H-1, δ 5,04 p.p.m., Dublett, $J_{1,2}$ 8 Hz; H-2, δ 3,92 p.p.m., $J_{2,3}$ 8 Hz; H-3, δ 5,10 p.p.m., Triplett, $J_{3,4}$ 8 Hz; H-4, δ 4,78 p.p.m., Triplett, $J_{4,5}$ 9 Hz. Daraus kann man schließen, daß die Verbindungen **11** und **12** von gleicher β-D-Glukopyranosid-Struktur sind.

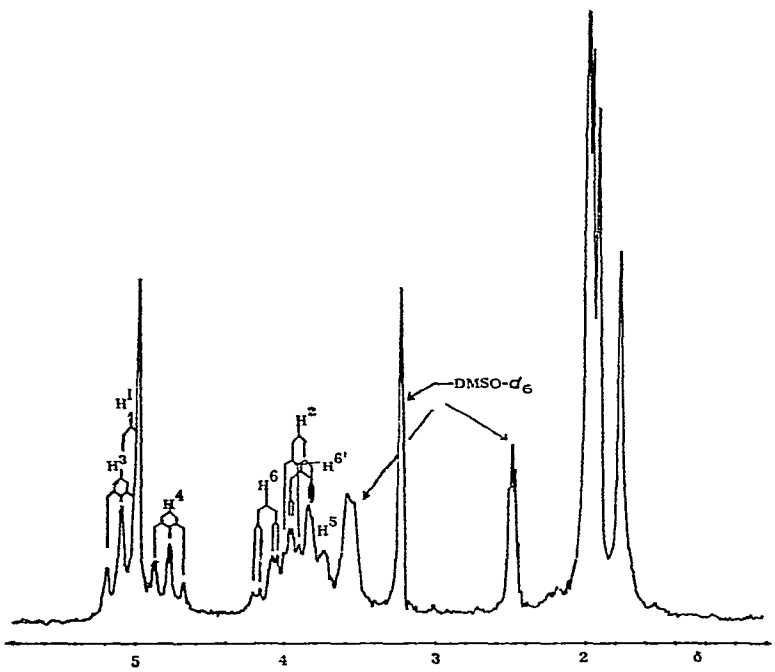


Abb. 4. N.m.r.-Spektrum von 2-Acetamido-3,4,6-tri-*O*-acetyl-1-*N*-(*N*-benzyloxycarbonylglycyl)-2-desoxy-β-D-glucopyranosylamin (**11**) in Dimethylsulfoxyd-d_6 (100 MHz).

Die Signale für die zwei Methylenprotonen der Benzylgruppe von **7** (Abb. 5) fallen mit den Signalen der Ringprotonen bei etwa δ 5,0 p.p.m. zusammen, während die Signale der Ringprotonen von **7** mit den von **12** fast übereinstimmen. Dies war auch infolge der alltrans-Konfiguration der Ringprotonen von **7** zu erwarten.

In Übereinstimmung mit der alltrans-Konfiguration der Ringprotonen der Verbindungen **1**, **5**, **7**, **11** und **12** stehen auch die chemischen Verschiebungen der Acetylgruppen. Zur Bestimmung der Konfiguration der axialen und äquatorialen *O*-Acetylgruppen aus n.m.r.-Daten wurden die Beobachtungen von Sowden *et al.*[12] herangezogen, die für die axiale Gruppierung δ 2,15 p.p.m. und für die äquatoriale Anordnung δ 2,03–2,10 p.p.m. finden. Da diese Autoren aber kein Lösungsmittel angegeben hatten, wurden die chemischen Verschiebungen der Acetylgruppe von

Methyl-2,3,4,6-tetra-*O*-acetylglykosiden in Deuterochloroform weiter untersucht und die gleichen Resultate wie oben erhalten.

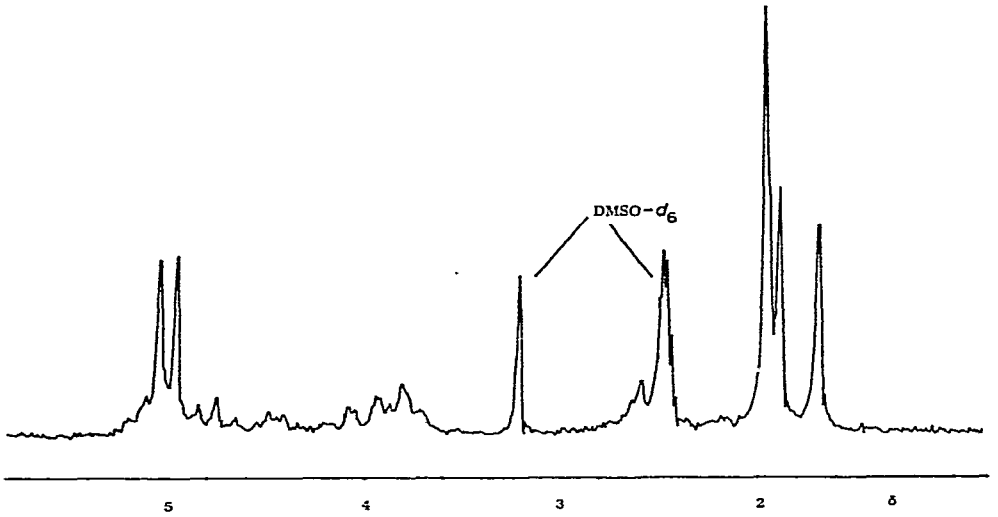


Abb. 5. N.m.r.-Spektrum von 2-Acetamido-3,4,6-tri-*O*-acetyl-1-*N*-(1-benzyl-*N*-benzyloxycarbonyl-4-L-aspartyl)-2-desoxy-β-D-glukopyranosylamin (7) in Dimethylsulfoxyd-d_6 (100 MHz).

TABELLE I

CHEMISCHE VERSCHIEBUNG (δ-WERTE) DER ACETYL-PROTONEN

Verbindung	O-*Acetyl*	NH-*Acetyl*	*Lösungsmittel*
1	2,10 (3 H, s[a]) 2,03 (6 H, s)	1,97 (3 H, s)	$CDCl_3$
5	2,01 (3 H, s) 1,99 (3 H, s) 1,97 (3 H, s)	1,90 (3 H, s) 1,88 (3 H, s)	$CDCl_3$
12	1,97 (6 H, s) 1,93 (3 H, s)	1,80 (3 H, s)	$CDCl_3$
7	1,94 (6 H, s) 1,88 (3 H, s)	1,68 (3 H, s)	DMSO-d_6
11	1,98 (3 H, s) 1,96 (3 H. s)	1,76 (3 H, s)	DMSO-d_6

[a]s, Singulett.

Wie aus Tab. I zu ersehen ist, stimmen die δ-Werte der *O*-Acetylgruppen alle mit einer äquatorialen Konfiguration überein. Dieser Befund erhärtet die trans-diaxiale Konfiguration der Ringprotonen der untersuchten Verbindungen.

Listowsky *et al.*[13] beobachteten kürzlich, daß die Circulardichroismus-Spektren von Methyl-2-acetamido-2-deoxy-β-D-glukopyranosid einen Minus-Cotton-Effekt bei 220 mμ und das α-D-Anomere einen Plus-Cotton-Effekt bei 225 mμ zeigten. Die O.r.d.-Spektren der Verbindung 3 weisen bei 220 mμ einen Minus-Cotton-Effekt auf (Abb. 6). Deshalb ist die β-D-Glukopyranose-Form für 3 zu fordern.

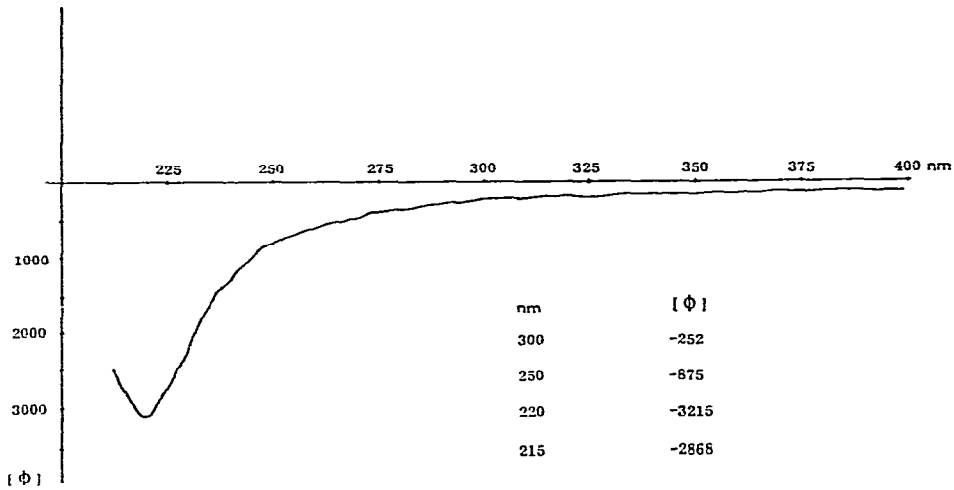


Abb. 6. O.r.d.-Spektrum von 2-Acetamido-2-desoxy-β-D-glukopyranosylamin (3) in Wasser. Nach der Auflösung der Substanz ist das Spektrum sofort aufgenommen worden.

Die erzielten experimentellen Ergebnisse bestätigen die alltrans Konfiguration der Ringprotonen und eine *C1* Konformation für **1**, **3**, **7**, **11** und **12**. Damit ist die gleiche Struktur auch für die Verbindung **8** sicher gestellt.

EXPERIMENTELLER TEIL

Die Schmelzpunkte sind nicht korrigiert. Die n.m.r.-Spektren wurden mit dem Varian-Instrument HA-100 oder mit dem JEOL-JNM-C-60 H, in Deuterochloroform oder in Dimethylsulfoxyd-d_6 (Tetramethylsilan als innerer Standard) gemessen. Die O.r.d.-Spektren sind mit dem Gerät JASCO, Modell ORD/CD-1 aufgenommen worden.

2-Acetamido-2-desoxy-β-D-glukopyranosyl-azid (**2**). — 2-Acetamido-3,4,6-tri-*O*-acetyl-2-desoxy-β-D-glukosyl-azid **1** (5 g) wurde über Phosphor-pentoxyd getrocknet und in getrocknetem Methanol (125 ml) gelöst. Zu dieser Lösung wurde die äquiv. Menge einer 2%-Lösung von Magnesiummethylat in absol. Methanol bei 0° zugesetzt. Nach 1 Std. Stehen bei 0° wurde die Lösung mit wenig Wasser und mit Dowex-50 (H^+, 140 ml) versetzt, und dann 10 Min. lang kräftig umgerührt. Das Harz wurde abfiltriert und mit Methanol gewaschen. Das mit dem Waschmethanol vereinigte Filtrat wurde i. Vak. abdestilliert. Der Rückstand wurde aus 2-Propanol umkristallisiert, wobei farblose Nadeln (3,2 g), Schmp. 145–146° (Zers.), $[\alpha]_D^{16}$ −27,5° (*c* 2,8, Wasser), erhalten wurden; Lit.[14]: Schmp. 142° (Zers.), $[\alpha]_D$ −30° (≤ 1,0, Wasser).

Anal. Ber. für $C_8H_{14}N_4O_5 \cdot 0{,}5\ H_2O$ (255,2): C, 37,64; H, 5,93; N, 21,95. Gef.: C, 37,93; H, 6,18; N, 21,92.

2-Acetamido-2-desoxy-β-D-glukopyranosylamin (**3**). — In Methanol (84 ml) wurde **2** (2 g) unter Zusatz von Platinoxid (200 mg) bei Raumtemp. 2 Stdn. lang katalytisch hydriert. Der Katalysator wurde abfiltriert. Das unter vermindertem

Druck eingeengte Filtrat lieferte die Substanz, die aus Methanol umkristallisiert wurde; dabei wurden farblose Nadeln (1,6 g) erhalten; Schmp. 140–143° (Zers.), $[\alpha]_D^{19}$ −9,9° (*c* 1,9, Wasser, bestimmt sofort nach der Auflösung), $[\alpha]_D^{19}$ −3,4° (*c* 1,9, Wasser, bestimmt nach 24 Stdn.). Unter diesen Bedingungen erhielt man kein Diglycosylamin.

Anal. Ber. für $C_8H_{16}N_2O_5 \cdot 0{,}5\ H_2O$ (229,2): C, 41,91; H, 7,48; N, 12,22. Gef.: C, 42,18; H, 7,36; N, 12,66.

*2-Acetamido-1-*N-(*1-benzyl-*N-*benzyloxycarbonyl-4-*L-*aspartyl*)-*2-desoxy-β-*D-*glukopyranosylamin* (**6**). — Zu einer Lösung von **3** (0.2 g) und 1-Benzyl-*N*-benzyloxycarbonyl-L-aspartat (0,3 g) in 4:1 Pyridin–Wasser (6 ml) wurden *N,N'*-Dicyclohexylcarbodiimid (0,22 g) gegeben. Es wurde bei Zimmertemp. 2 Stdn. lang gerührt und über Nacht stehengelassen. Dann wurde 1 Tropfen Essigsäure hingefügt und 10 Min lang gerührt. Das durch Abfiltrieren des Dicyclohexylharnstoffs erhaltene Filtrat wurde unter vermindertem Druck abdestilliert. Der hierbei hinterbliebene amorphe Rückstand wurde ohne Reinigung weiter verarbeitet.

*2-Acetamido-1-*N-(*4-*L-*aspartyl*)-*2-desoxy-β-*D-*glukopyranosylamin* (**8**). — Unter Zusatz von 0,11 g Paladium-Kohle (10%) wurde die Verbindung **6** (0,5 g) in Methanol (65 ml) 5 Stdn. lang katalytisch hydriert. Das Lösungsmittel wurde entfernt und der Rückstand aus heißem wässrigen Äthanol umkristallisiert; 0,18 g, Schmp. 250° (Zers.), $[\alpha]_D^{22}$ +24.9° (*c* 1,0, Wasser). Lit.[1]: Monohydrat, Schmp. 220,5° (Zers.). Lit.[5]: Monohydrat, Schmp. 219–221° (Zers.), $[\alpha]_D^{26}$ +24° (*c* 1,0, Wasser). Wenn **8** aus kaltem wässrigen Äthanol umkristallisiert wurde, erhielt man ein Additionsprodukt mit 3 Mol Wasser. Es schmilzt bei 220-222° (Zers.). Siehe Ref. 4.

Anal. Ber. für $C_{12}H_{21}N_3O_8$ (335,3): C, 42,98; H, 6,31; N, 12,53. Gef.: C, 42,86; H, 6,23; N, 12,45.

*2-Acetamido-3,4,6-tri-*O-*acetyl-1-*N-*acetyl-2-desoxy-β-*D-*glukopyranosylamin* (**5**). — Verbindung **3** (0,5 g) wurde in Pyridin (12 ml) mit Acetanhydrid (38 ml) bei 0° acetyliert. Nach drei Tagen wurde die Lösung in Eiswasser (100 ml) gegossen und mit Chloroform extrahiert. Man wusch den Extrakt mit M Salzsäure, Wasser, wässeriger Lösung von Natriumhydrogencarbonat und mit Wasser, trocknete mit Natriumsulfat and engte i. Vak. ein, wobei eine farblose Masse verblieb. Aus Äthanol wurden Nadeln (0,5 g) vom Schmp. 239–240° (Zers.), $[\alpha]_D^{22}$ +17,9° (*c* 1,0, Pyridin) erhalten. Lit.[5]: Schmp. 236–237°, $[\alpha]_D^{24}$ +12,5° (*c*, 2,0, chloroform).

Anal. Ber. für $C_{16}H_{24}N_2O_9$ (388,4): C, 49,48; H, 6,23; N, 7,21. Gef.: C, 49,08; H, 6,01; N,7,17.

*2-Acetamido-1-*N-(N-*benzyloxycarbonylglycyl*)-*2-desoxy-β-*D-*glukopyranosylamin* (**9**). — Analog Verbindung **6** wurde **9** aus **3** und *N*-Benzyloxycarbonylglycin dargestellt. Das nach dem Abfiltrieren des *N,N'*-Dicyclohexylureas erhaltene Filtrat wurde unter vermindertem Druck eingedampft. Aus dem Rückstand wurde **9** aus Methanol umkristallisiert. Farblose Nadeln, Schmp. 230° (Zers.), $[\alpha]_D^{13}$ +41,5° (*c* 1,0, Pyridin).

Anal. Ber. für $C_{18}H_{25}N_3O_8$ (411,4): C, 52,55; H, 6,13; N, 10,21. Gef.: C, 52,66; H, 6,10; N, 10,26.

*2-Acetamido-1-*N-*glycyl-2-desoxy-β-*D-*glukopyranosylamin* (**10**). — Wie die

Verbindung **8** wurde **10** aus der Verbindung **9** durch Hydrierung gewonnen. Die aus wässrigem Äthanol umkristallisierte Verbindung **10** zeigte den Schmp. von 236° (Zers.), $[\alpha]_D^{19}$ +25,4° (*c* 1,0, Wasser).

Anal. Ber. für $C_{10}H_{19}N_3O_6$ (277,3): C, 43,31; H, 6,91; N, 15,16. Gef.: C, 43,12; H, 6,93; N, 14,89.

*2-Acetamido-3,4,6-tri-*O*-acetyl-1-*N*-(1-benzyl-*N*-benzyloxycarbonyl-4-*L*-aspartyl)-2-desoxy-β-*D*-glukopyranosylamin* (**7**). — Die Acetylierung von **6** wurde entsprechend der für **3** beschriebenen durchgeführt. Der hierbei erhaltene Rückstand wurde aus Äthanol umkristallisiert, wobei farblose Nadeln, Schmp. 213–217° (Zers.), $[\alpha]_D^{20}$ +12,0° (*c* 0,5, Chloroform) erhalten wurden. Lit.: Schmp. 221–222° (Ref. 1); Schmp. 220–221°, $[\alpha]_D^{26.5}$ +17° (*c* 2,0, Chloroform) (Ref. 5).

Anal. Ber. für $C_{33}H_{39}N_3O_{13}$ (685,7): C, 57,80; H, 5,73; N, 6,13. Gef.: C, 57,74; H, 5,83; N, 5,98.

*2-Acetamido-3,4,6-*O*-tri-acetyl-1-*N*-(N-benzyloxycarbonylglycyl)-2-desoxy-β-*D*-glukopyranosylamin* (**11**). — Durch Acetylierung von **9** wurde **11** ähnlich der Überführung von **3** in **5** gewonnen. Kristallisation aus Äthanol nach üblichem Aufarbeiten, Schmp. 195–196° (Zers.), $[\alpha]_D^{20}$ +12,8° (*c* 1,1, Pyridin).

Anal. Ber. für $C_{24}H_{31}N_3O_{11}$ (537,5): C, 53,62; H, 5,81; N, 7,82. Gef.: C, 53,47; H, 5,83; N, 7,83.

DANK

Wir möchten Herrn Dr. H. Morimoto, Forschungslaboratorium für Chemie der Takeda Chemische Industrie A.G., Osaka, für die Aufnahme von 100 MHz N.m.r.-Spektren sowie dem Forschungsinstitut der Firma Shionogi, Osaka, für die Aufnahme der O.r.d-Spektren herzlich danken.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

EVALUATION OF THE PROPORTION OF THE *keto* FORM OF D-FRUCTOSE AND RELATED 2-KETOHEXOSES PRESENT IN AQUEOUS SOLUTION*

GAD AVIGAD, SASHA ENGLARD, AND IRVING LISTOWSKY**

Department of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, Bronx, New York 10461 (U. S. A.)

(Received March 9th, 1970)

ABSTRACT

The acyclic forms of D-fructose and some related compounds have been studied by absorption spectroscopy and circular dichroism. These compounds exhibit absorption and ellipticity bands near 275 nm attributed to the *keto* group of the acyclic form. At mutarotational equilibrium in aqueous solution, D-fructose exists to the extent of ~2% in the acyclic form, whereas solutions of D-fructose 1-phosphate, D-fructose 6-phosphate, and D-fructose 1,6-diphosphate contain up to 10 times this proportion of the acyclic form. On the basis of circular dichroism spectra, these phosphoric esters, as well as certain disaccharides of D-fructose, could be differentiated with respect to the position of substitution. The differences in position and magnitude of the dichroic bands were related to specific, conformational features of these molecules.

INTRODUCTION

It is generally considered that the acyclic (*aldehydo* or *keto*) forms of reducing sugars in neutral, aqueous solution are present only in minute proportions at equilibrium[1–5]. Attempts to quantitate the amount of free *aldehydo* form of several aldoses, by measuring the interaction with hydrocyanic acid[6] and by polarography[7–10], indicated that its concentration is extremely low, for example, 0.0026% for D-glucose[9]. As the acyclic form rapidly equilibrates with the cyclic forms, conditions employed for the chemical or electrochemical analyses may cause changes in the equilibrium concentration of the aldehyde[9,10]. Such physical methods as u.v. absorption[11–14] and n.m.r. spectroscopy, and chromatography[4,5], do not permit accurate determination of the extremely low concentrations of acyclic form present in a solution, and the presence of trace contaminants may complicate any reliable quantitative evaluation.

*This work was supported by Grants HE 11511 from the National Institutes of Health, Grant IN-281N-2 from the American Cancer Society, and Grant GB 12670 from the National Science Foundation.

**Senior Investigator, New York Heart Association.

Earlier studies of the u.v. absorption spectrum of solutions of D-fructose[15–17] suggested the presence of a small proportion of the *keto* form. The molar extinction at 278 nm varied with temperature and concentration, and values between 0.38 and 0.60 $cm^{-1} \cdot mol^{-1}$ were obtained[17–20], consistent with a concentration of ~1–2% of the *keto* form. By direct acetylation[21,22], D-fructose yields the acyclic pentaacetate, and the infrared absorption spectrum of D-fructose lyophilized from aqueous solution indicates the presence of some free carbonyl group[23]. G.l.c. analysis of permethylated D-fructose shows the presence of 1.6–3.4% of penta-*O*-methyl-*keto*-D-fructose[24], but, in view of the drastic procedures employed in this analysis, it is difficult to extrapolate this value to give the actual proportion of *keto*-D-fructose present in the solution. Indeed, the values obtained[24] for the proportions of *aldehydo*-D-glucose and -D-galactose were much higher than those estimated by polarography[7,9] or by g.l.c.; the latter method did not show any detectable amount of the acyclic form of these aldohexoses[25–28].

Per(trimethylsilyl)ation of crystalline D-fructose followed by g.l.c. analysis showed the presence of the β-D-pyranose component only[25,29,30]. A similar analysis of D-fructose equilibrated in aqueous solution indicated the presence of several components[31,32], one of which was unequivocally identified as the *keto* form[29,33]. An accurate estimate of the proportion of this form was not made, but, based on the relative intensity of its peak, it is probably $\leqslant$ ~2% of the total D-fructose in solution. By use of an electron-capture detector, it was estimated that the proportion of penta-*O*-(trimethylsilyl)-*keto*-D-fructose was ~0.5–0.6% of the total trimethylsilylated D-fructose[34]. A ketose recently found[35] to exist, in solution, to an appreciable extent in the *keto* form is D-*altro*-3-heptulose ("coriose").

In the course of our studies on the optical properties of carbohydrates, we noticed that D-fructose and certain of its phosphoric esters exhibit dichroic bands near 275 nm that are most probably associated with the carbonyl absorption of the acyclic form of the ketose. The present report describes some of these observations, and their significance in evaluating the conformation of reducing sugars in solution.

RESULTS AND DISCUSSION

The absorption and corresponding ellipticity bands near 280 nm for the sugars listed in Table I are, in all probability, associated with the $n \rightarrow \pi^*$ transition of an *aldehydo* or *keto* carbonyl group. A neutral, aqueous solution of D-fructose has been reported to contain ~25% of the pyranose and 75% of the furanose form at equilibrium[2,31,33], and the temperature markedly affects the relative distribution of these two forms[31,36,37]. On the basis of the molar extinction coefficient at 275 nm (see Table I), with 1,3-dihydroxy-2-propanone as a standard assumed to represent the approximate absorption intensity of the carbonyl group of D-fructose and related 2-ketohexoses, we calculated that ~2% of the D-fructose in solution was the *keto* form. This estimate agrees closely with the proportion of the acyclic form ascertained in previous studies (see Introduction).

TABLE I

ABSORBANCE AND ELLIPTICITY OF SEVERAL 2-KETOHEXOSES AND RELATED COMPOUNDS[a]

Compound	pH	*Absorbance*		*Ellipticity*		
		λ_{max} (*nm*)	ε_{max}	λ_{max} (*nm*)	$[\theta]_\lambda$	g × *10*$^{-3}$
D-Fructose	7.4	275	0.45	275	+33	22.2
1-phosphate	7.7	285	4.8	275–285	+26	1.6
6-phosphate	7.4	280	4.9	275	+280	17.3
1,6-diphosphate	7.4	280	5.0	275–280	+106	6.4
L-Sorbose	7.5	280	0.54	273	−12.3	−7.0
D-Tagatose	6.7	285	0.6[b]	282	+16.8	8.3[c]
D-*threo*-2,5-Hexodiulose	4.5	260	7.8	295	−47	−1.8
D-Glucose	7.1	280	<0.02	—	—	—
6-phosphate	6.8	300[d]	0.85	305	−0.11	−0.4
		275	1.2	275	+3.7	0.9
6-*O*-α-D-Glucopyranosyl-D-fructose	7.3	275	2.5	275	+95	11.5
4-*O*-α-D-Galactopyranosyl-D-fructose	7.1	275	1.5[b]	273	+10	2.0[c]
3-*O*-α-D-Glucopyranosyl-D-fructose	7.1	270	1.2	285	+56	14.2
1-*O*-β-D-Glucopyranosyl-D-fructose	7.3	270	2.8[b]	275	±5	<1.0
D-Glyceraldehyde	6.0	275	15	290	−112	−2.3
Acetone	7.0	265	16			
1,3-Dihydroxy-2-propanone phosphate	7.2	270	19.2			
	2.5	260	25[b]			

[a]Compounds were dissolved in water to give concentrations of 0.04 to 0.3M; D-glucose was measured at a concentration of 3.0M. The sodium salts of the phosphoric esters were used. [b]The presence of small proportions of optically inactive, u.v.-absorbing contaminants prevented an exact determination of the absorbance for these compounds. The numbers reported are approximate, and are maximal values obtained for the sugar after treatment with charcoal in 1:9 ethanol–water. [c]Minimal value. [d]A shallow shoulder.

The three phosphoric esters of D-fructose (see Table I), irrespective of the position(s) of esterification, show a marked increase (about 10-fold) in the content of acyclic form. These observations are in agreement with the conclusions of Model *et al.*[38] based on the enhanced, nonenzymic rate of oxygen exchange of D-fructose 1-phosphate and D-fructose 1,6-diphosphate with water. A similar increase in the content of acyclic form induced by the introduction of phosphate at C-6 of D-glucose can be noted by comparing the extinction coefficient of D-glucose with that of D-glucose 6-phosphate (see Table I). External phosphate ions have no appreciable effect on the equilibrium concentration of acyclic forms, as evidenced by the fact that the u.v. spectrum of D-fructose is not materially affected by the presence of 0.1M potassium phosphate buffer at pH 7.4.

To a much smaller extent than for the phosphoric esters, glycosyl substitutions at O-1, O-3, O-4, and O-6 of D-fructose all increase the molar extinction coefficient,

indicating the presence of greater proportions of the *keto* form (as compared to D-fructose). The *keto* form is also detectable in solutions of L-sorbose[33] and D-tagatose, and, in particular, large proportions of the *keto* form are present in solutions of D-*threo*-2,5-hexodiulose ("5-keto-D-fructose"), an observation compatible with the previously reported properties of this sugar[39].

In view of the different proportions of acyclic form present in solution at equilibrium, the molar ellipticities (see Fig. 1 and Table I) do not accurately reflect

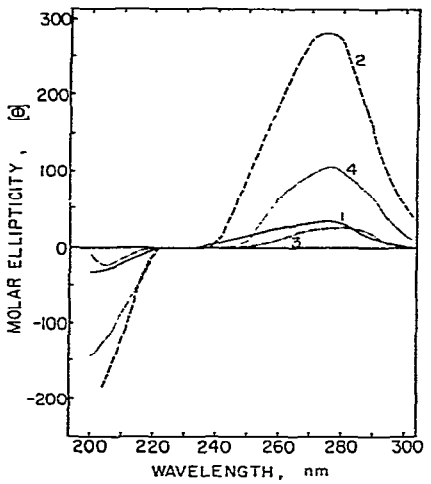


Fig. 1. C.d. spectra of D-fructose and its phosphoric esters; measurements were conducted on mutarotated, aqueous 0.05–0.10M solutions at pH 7.4; 1, ———, D-fructose; 2, ———, D-fructose 6-phosphate; 3, —·—·—, D-fructose 1-phosphate; 4, ..., D-fructose 1,6-diphosphate.

the rotational power associated with the asymmetric perturbation of the $n \rightarrow \pi^*$ transition of the carbonyl group of the acyclic form of the sugars listed. The data are, therefore, also reported in terms of dissymmetry factors or anisotropy ratios (g), which permit a more precise comparison of the rotational power of the various sugars under consideration (see Experimental section). From the data presented in Table I, it is evident that D-fructose, in comparison to its phosphoric esters, has the highest rotational power, followed by D-fructose 6-phosphate, D-fructose 1,6-diphosphate, and D-fructose 1-phosphate, in that order. Compared to the presence of a glycosyl group on O-1 and O-4 of D-fructose, equivalent substitutions at O-3 and O-6 give rise to significantly higher rotational powers associated with the carbonyl chromophore. In this connection, it should be noted that phosphate or glycosyloxy groups at C-1 or at C-6 have similar effects on the rotational power of D-fructose. Thus, the g value of 22.0 for D-fructose is decreased to 17.3 and 11.5 for D-fructose 6-phosphate and isomaltulose, respectively, and to 1.6 and <1.0 for D-fructose 1-phosphate and 1-*O*-β-D-glucopyranosyl-D-fructose, respectively.

The substitutions at C-1 may directly affect the transition moments of the carbonyl group or, more probably, modify the asymmetric perturbants thereof. The asymmetric environment about the carbonyl group (in particular, the 3-dimensional

structure at C-3) should be the primary factor that determines the shape of the 275-nm ellipticity band. The favored situation at C-3 will, in turn, be influenced by the relative disposition of its neighboring groups. The lower rotational power of the 1-*C*-substituted D-fructoses may, therefore, be explained in terms of interactions between the C-1 and C-3 substituents that give rise to changes in the relative populations of rotational isomers at C-3. A net cancelling effect will result if two rotational isomers contribute ellipticity bands of opposite sign. It is, therefore, suggested that a large substituent, such as a phosphate or a glycosyloxy group at C-1 of D-fructose, by non-bonding or polar interactions with its substituents, lessens the population of the rotational isomer at C-3 ordinarily favored.

The sign of the ellipticity band associated with the $n \rightarrow \pi^*$ transition of the carbonyl group cannot be predicted solely on the basis of the absolute configuration at C-3, because of the influence of the environment on the dispositional possibilities at this carbon atom. Thus, L-sorbose and "5-keto-D-fructose" have negative ellipticity bands, whereas D-fructose and D-tagatose, having identical configurations at C-3, show positive ellipticity bands. D-Glyceraldehyde, in which the configuration of the carbon atom adjacent to the carbonyl chromophore is the opposite of that of these 2-ketohexoses, has a negative ellipticity band in this spectral region[40].

Temperature has been shown to affect the pyranose–furanose equilibrium of sugars, such as D-fructose, that have the *arabino* group-configuration[31,37]. Solvents, also, have been reported to cause changes in the ring size, owing to specific solvation effects[41,42]. In particular, methyl sulfoxide, a solvent in which mutarotation is extremely slow[43], induces an increase in the proportion of the furanose form in solutions of sugars having the *arabino* configuration[2,44]. In addition, both the solvent *and* the temperature appear to have a considerable effect on the conformation of simple ketones and aldehydes[45–49]. In view of this evidence, similar effects of solvent and temperature on the abundance of the *keto* form in solutions of D-fructose and on the relative distribution of the different conformers of the *keto* form may be anticipated. Table II presents data on the wavelengths of maximum absorption and the corresponding extinction coefficients of D-fructose in different solvents. Significant

TABLE II

U.V. ABSORPTION OF D-FRUCTOSE IN DIFFERENT SOLVENTS

Solvent[a]	λ_{max} (*nm*)	ε at λ_{max}
Water[b]	278	0.46
Methanol[b]	280	0.48
p-Dioxane, 90%[c]	278	0.56
Acetonitrile–95% methanol (5:2)[c]	280	0.59
Methyl sulfoxide, 98%[b]	288	1.31

[a]Mutarotation in methyl sulfoxide was complete only after 24 h, whereas, in the other solvents, mutarotational equilibrium was attained within 2 h at room temperature. [b]277 mM solution. [c]177 mM solution.

changes are not observed when D-fructose is dissolved in methanol, *p*-dioxane, or acetonitrile–methanol. In methyl sulfoxide, however, a significant shift of the absorption maximum to higher wavelengths, and an increased extinction coefficient, indicate the presence of larger proportions of the *keto* form.

In contrast to the absorption spectra, the circular dichroism (c.d.) spectra of D-fructose in the different solvents (see Fig. 2) show much greater variability and increased complexity. Most notable is the appearance, in methanol and methyl sulfoxide, of a negative ellipticity band centered near 300 nm and a significant decrease in the positive ellipticity band at ~270 nm. In *p*-dioxane–water and in

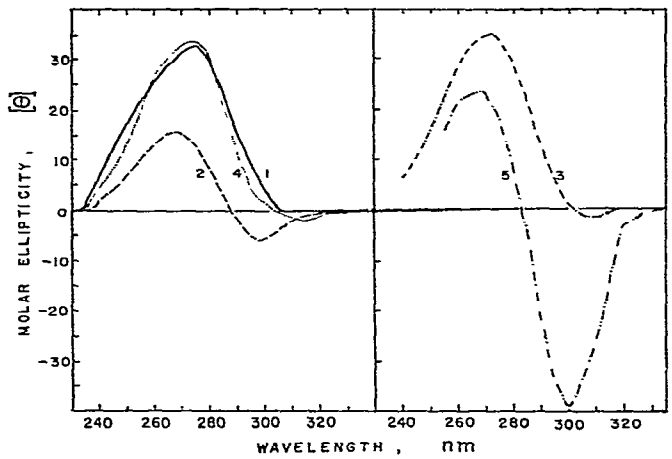


Fig. 2. C.d. spectra of D-fructose in different solvents; measurements were made on the solutions, described in Table II: 1, ————, water; 2, – – – –, methanol; 3, —·—·—, *p*-dioxane; 4, ... acetonitrile–methanol; 5, —··—··—, methyl sulfoxide.

acetonitrile–methanol, the positive ellipticity band at shorter wavelength is essentially unaffected, but a small negative ellipticity band also appears at ~310 nm. Overlapping ellipticity bands of opposite sign that are centered about a single electronic transition can be attributed to dipole-coupled transition-moments[50] or vibrational structure[51], or both. For carboxylic acids[52], this phenomenon has been interpreted in terms of conformational equilibria. The possible conformations of the *keto* form differ in polarity depending on the spatial disposition of the hydroxyl groups with respect to (*a*) each other, and (*b*) the carbonyl group (that is, conformations having *trans*-adjacent hydroxyl groups are more polar than their rotational isomers having *gauche* hydroxyl groups). It is, therefore, conceivable that changes in the polarity of the solvent may induce changes in the relative populations of certain rotational isomers, and the overlapping ellipticity bands (see Fig. 2) may be attributable to conformational equilibria that cannot at present be defined.

The results of the present study, based on an analysis of both the u.v. absorption and the c.d. spectra of the phosphoric esters of D-fructose, suggest that substantial proportions of the *keto* form are present in solution at equilibrium. G.l.c. analysis of

esterified and trimethylsilylated D-fructose phosphates (after dissolution) has revealed the presence of several components[53,54] that have not as yet been rigorously characterized; it would be interesting to determine whether any of them is the *keto* form of the hexose phosphate.

Acyclic forms of D-fructose phosphates may play a significant role in several enzymically catalyzed reactions. For example, the *keto* form has been considered to be the molecular species involved in the mechanism of action of D-fructose 1,6-diphosphate aldolase[55]; this suggestion has received indirect qualitative support from the observation that a Schiff base involving the *keto* carbonyl group and an L-lysine residue of the enzyme is involved in the aldolase reaction[56,57]. More-direct experimental evidence for the participation of *keto*-D-fructose 1,6-diphosphate in the aldolase reaction was obtained from a study of the 2-C–O exchange patterns of D-fructose during the course of this reaction[38].

EXPERIMENTAL

Materials. — Sugar phosphates as the sodium salts were purchased from the Sigma Chemical Co., St. Louis, Missouri, and from Boehringer und Sohne, GmbH, Mannheim, Germany. Three different batches of each material were examined. Other sugars studied were obtained from Fluka AG, Buchs, Switzerland, and from Sigma Chemical Co. D-*threo*-2,5-Hexodiulose was prepared as previously described[39]. 6-*O*-α-D-Glucopyranosyl-D-fructose ("isomaltulose" or "palatinose") was obtained from Dr. R. Weidenhagen, and 1-*O*-β-D-glucopyranosyl-D-fructose was a sample obtained from the late Dr. E. Pacsu. The organic solvents employed were reagents of spectrophotometric grade and were of high purity.

Methods. — U.v. absorption spectra were recorded with a Cary 14 spectrophotometer calibrated with a standard potassium chromate solution. The solutions that had been employed for determination of the absorption spectra were generally diluted for subsequent measurements of ellipticity. All measurements were made at 25–27°. The c.d. spectra were obtained with a Cary 60 spectropolarimeter having a 6001 CD attachment, calibrated with 5α-cholestan-3-one solution[46,58]; this instrument records the angle of ellipticity (θ) in degrees, and molar ellipticities were calculated from the relationship $[\theta] = \theta \cdot \text{mol.wt.}/10 \cdot l \cdot c$. A cuvet of 1.0-cm pathlength ($l = 1$) was employed for all of these studies, and the concentration (c) in g/ml of solution was varied, depending on the signal-to-noise ratios of the measurements and the availability of the compound studied. The anisotropy ratios (g) were calculated from $\Delta\varepsilon/\varepsilon$ (see Refs. 58 and 59), the $\Delta\varepsilon$ values being calculated from the molar ellipticities ($[\theta] \approx 3300 \cdot \Delta\varepsilon$). All values given are for the completely mutarotated compound, as checked by the $[\alpha]_D$ value and the appearance of maximal absorption at 280 nm.

ACKNOWLEDGMENTS

We thank Miss J. Bohm and Mr. H. Horwitz for their assistance in these studies.

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NOTE ADDED IN PROOF.

While this paper was in press, Gray and Barker[60] published evidence, based on i.r., u.v., and n.m.r. spectroscopy, that phosphoric esters of several ketoses exist to varying degrees in *keto* and hydrated *keto* forms in solution. This publication drew our attention to an important paper by McGilvery[61] that had escaped our notice. In the latter study, it was suggested, on the basis of u.v. and particularly o.r.d. spectroscopy, that D-fructose 1,6-diphosphate exists in solution mostly in an acyclic form.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

A CRITICAL RE-EXAMINATION OF PROBLEMS INHERENT IN COMPOSITIONAL ANALYSIS OF HEMICELLULOSES BY GAS–LIQUID CHROMATOGRAPHY

J. D. BLAKE AND G. N. RICHARDS*
Chemistry Department, James Cook University, Townsville, Queensland (Australia)
(Received February 16th, 1970)

ABSTRACT

Because of conflicting observations in published methods of compositional analysis of polysaccharides by gas–liquid chromatography of alditol acetates and our own interest in the hemicellulose constituents of grasses, an examination of problems inherent in the analysis of acidic polysaccharides was undertaken.

Following acid hydrolysis of the polysaccharide, the hydrolysis of lactones and the ion-exchange separation of acidic components have been incorporated into a scheme of analysis which works adequately for neutral components. Reduction schemes for uronic acids have been examined but have not been successfully applied to hemicelluloses. The uronic acids were best determined by titration.

INTRODUCTION

In recent years, there have been marked advances in the development of rapid and accurate methods of analysis by gas–liquid chromatography (g.l.c.) of the hydrolytic products obtained from polysaccharides. Trimethylsilyl (TMS) ethers of reducing sugars or the acetates of the derived alditols have proved to be the most successful derivatives for routine work[1].

Sweeley and co-workers[2] demonstrated the scope of TMS derivatives, and their application to the quantitative analysis of monosaccharides[3,4] and to polysaccharide hydrolysates[5,6] has since been reported. Shortcomings in the use of such derivatives include difficulty in the preparation of suitable, crystalline, standard compounds and some lack of reproducibility in quantitative conversion. A more important problem in this type of analysis of hydrolysates containing a number of different reducing sugars is the multiplicity of peaks produced from each aldose by anomeric and different ring-forms of the TMS derivatives. This problem can be overcome by reduction of aldoses to alditols. Gunner *et al.*[7,8] demonstrated the feasibility of using these derivatives, and, with the improved separations made by Sawardeker and co-workers[9], reports of quantitative measurement of the neutral-sugar components of wood pulps have been published[10,11].

*To whom communications should be addressed.

Most published work utilising g.l.c. has referred to neutral-sugar components of polysaccharides. A new avenue was opened when Perry and Hulyalkar[12] attempted to analyse hexuronic acids by g.l.c. They separated the 2,3,5,6-tetrakis-*O*-(trimethylsilyl)aldono-1,4-lactones derived from the uronic acids, but experienced difficulties in making the chemical conversion procedures quantitative. However, Sjostrom *et al.*[13] have reported a conversion of uronic acids into acetylated alditols and subsequent g.l.c., and our interests in the hemicelluloses of plants and the conflict in published methods prompted an examination of some alternative approaches to this problem.

EXPERIMENTAL AND RESULTS

Apparatus. — The gas chromatograph used was an Aerograph Autoprep Model 705 equipped with flame-ionization detector. The collector exit was sealed to ensure that all column effluent passed through the detector. A glass-liner insert was placed in the injection port to minimise contact between vapour and metal[8]. The column was operated isothermally at 185°, with injection port at 255° and detector at 240°.

Nitrogen carrier gas was used at 45 ml/min. Two different stationary phases were used: (*1*) 3% ECNSS-M on Gas Chrom Q, 100–120 mesh (supplied precoated by Applied Science Laboratories); (*2*) a mixture of 1.5% ethylene glycol succinate (LAC-4R-886) and 1.5% silicone oil XF-1150 on acid-washed Chromosorb W, 100–120 mesh. Column packing (*1*) was used in a coiled 210 × 0.7-cm column, and packing (*2*) in a 130 × 0.7-cm column. Both columns were glass.

Materials. — Commercial monosaccharides (British Drug Houses Ltd.) were dried and used without purification They were demonstrated to be chromatographically homogeneous by paper chromatography.

Alditol acetates were prepared by treatment of alditols with acetic anhydride in dry pyridine and purified by recrystallisation. All of the samples used were obtained in crystalline condition, except for rhamnitol pentaacetate which failed to crystallise (*cf.* Ref. 9).

Reagent-grade Amberlite ion-exchange resins were used and were prepared as recommended[14]. Acetic anhydride was redistilled before use, and pyridine was dried over potassium hydroxide pellets and then distilled from phosphoric oxide.

Hemicellulose samples were obtained by chlorite delignification of grasses and subsequent extraction with sodium hydroxide. They are described in detail elsewhere[15].

Procedure. — A generalised analytical procedure is shown in Fig. 1. This represents the preferred, total procedures, based on the detailed investigations of variables described below.

Hydrolysis, and separation of neutral sugars from uronic acids. — Primary hydrolysis was performed in 72% sulphuric acid (300 mg of polysacchaccharide/ml) for 1 h in a Pyrex tube at 30°. Agitation with a glass rod ensured complete dissolution. After dilution with water (28 ml/ml of acid), the tube was sealed, and secondary hydrolysis was carried out by heating in a boiling-water bath for 3 h.

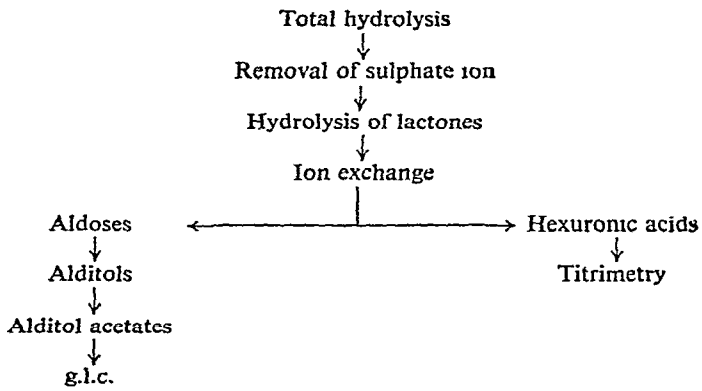


Fig. 1. The generalised analytical procedure adopted for the analysis of hemicelluloses.

The sample was washed into a tared vessel, and the volume determined by reweighing. Titration on a hot-plate magnetic stirrer at 60–70° with 0.25M barium hydroxide to a Congo Red end-point (pH 3–4) removed sulphate ions. The indicator was adsorbed on the precipitate. Filtration of the hot, uniformly mixed hydrolysate into a tared vessel allowed an aliquot portion to be used for the remainder of the analysis without washing of the precipitate. It was shown that losses by sorption on the product were not significant.

Further titration, using a pH meter to monitor the addition of 0.1M potassium hydroxide to maintain the solution in the pH range 8.5–9.0, converted the lactones into the corresponding hexuronic acid salts[16].

Separation of the samples into aldose and uronic acid fractions was performed by passage through columns of Amberlite IR-120(H^+) and IRA-400(Ac^-) resins mounted in tandem, so that the effluent from the cation exchanger passed directly into the anion-exchange unit. After collection of 10–12 bed-volumes containing the aldose fraction, the upper column was removed, and hexuronic acids were eluted from the anion-exchange column with 16 bed-volumes of 10% acetic acid (v/v). Uronic acids were recovered by evaporation of the resultant acetic acid effluent at 40° and final drying over potassium hydroxide pellets. They were measured initially by colorimetry (phenol–sulphuric acid procedure[17]) and, in later instances, by titration with alkali to equilibrium value on a pH stat (Metrohm Combititrator 3D) at pH 8.0.

Reduction of aldoses. — The solution of aldoses from the preceding experiment was concentrated at 40°, and freshly prepared 0.3M sodium borohydride was added (100 mg of borohydride/150 mg of original polysaccharide). After 3 h, excess reagent was destroyed by acetic acid, sodium ions were removed by passage through a column of Amberlite IR-120(H^+) resin (1 ml of resin/mequiv. of Na^+), and 5 bed-volumes of effluent and water washings were collected. After evaporation to dryness under diminished pressure, boric acid was removed by several distillations with dry methanol (5 × 20 ml). The alditols were next treated with a mixture of acetic anhydride and pyridine (1 ml each) for >1 h at room temperature, and the resulting solution was

used for chromatographic analysis, after addition of a weighed amount of an appropriate alditol acetate as internal standard.

Reduction of uronic acids. — Hexuronic acids were reduced to aldonic acids by sodium borohydride, using the procedure outlined for aldoses. Lactonisation was effected by heating under reduced pressure[12].

The use of boric acid buffer in the reduction of lactones was investigated as follows: D-glucono-1,5-lactone (360 mg) and D-mannitol (360 mg) were dissolved in 0.4M boric acid (20 ml), and 0.3M sodium borohydride (40 ml) was added slowly. Samples (3 ml), which were removed at intervals for analysis, were adjusted to pH 9 with aqueous sodium hydroxide, and more sodium borohydride was added to each of the initial samples to ensure completion of the reduction of the aldose to the alditol. Sodium ions and boric acid were removed, and the mixture was acetylated. Arabinitol pentaacetate was then added as internal standard, after preliminary analysis by g.l.c. had demonstrated the absence of any component having a similar retention time. Peak areas in the initial chromatograms were difficult to measure because of overlapping components. Fig. 2 shows the reduction efficiency achieved in conversion of aldonolactone into alditol. D-Mannitol was added to the initial reaction mixture as an additional, internal standard to permit calculation of the degree of conversion in samples which were not accurately known aliquot-portions of the reaction mixture.

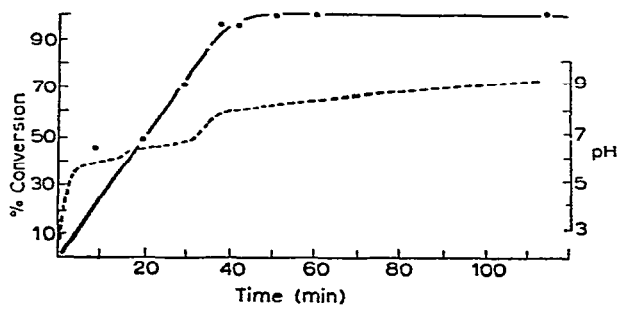


Fig. 2. The reduction efficiency of D-glucono-1,5-lactone by aqueous sodium borohydride in 0.4M boric acid, as determined by g.l.c. of the acetylated alditol product (– – –, pH change).

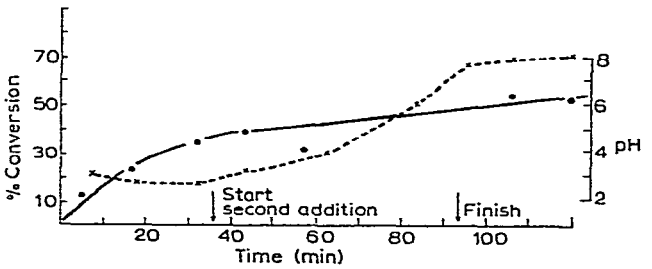


Fig. 3. The reduction efficiency of D-glucono-1,5-lactone by aqueous sodium borohydride in the presence of Amberlite IR-120(H^+) resin (× ——× ——×, change in solution pH).

Frush and Isbell[19] have previously utilised Amberlite IR-120(H^+) resin as buffer in such reductions, and we have now followed the course of this reaction as follows: a solution of 0.05M boric acid (20 ml), D-glucono-1,5-lactone (182 mg), and D-mannitol (236 mg) in water was stirred magnetically with 4 ml of Amberlite IR-120(H^+) resin in a flask packed in an ice bath. 0.3M Sodium borohydride (20 ml) was added slowly, and the pH was monitored. Frush and Isbell suggested the addition of borohydride solution over a 5–6-min period, but it was found that attempts to maintain this rate of addition resulted in sharp rises in pH. It was therefore decided to control the rate of addition in order to maintain a pH of *ca.* 3. Consequently, 28 min were required for the first addition, after which the solution was stirred for 7 min, and further borohydride (20 ml) was added. Samples were withdrawn during the course of the reaction and analysed as above. The results are shown in Fig. 3.

The addition of a freshly prepared solution of sodium borohydride requires careful rate-control, and it was found necessary to avoid clamps and orifices in order to check surges of reagent due to accumulation of gas bubbles at these points. A closed system proved to be best, whereby the pressure of the evolved hydrogen forced the reagent to the reaction vessel by upward displacement.

Removal of sulphate by ion exchange. — In an evaluation of this approach, D-glucuronic acid (5 mg) was dissolved in 0.5 ml of 0.5M sulphuric acid and applied to various volumes of Amberlite IRA-400(Ac^-) resin in columns of 1-cm diameter. After washing of the columns with 5 bed-volumes of water, 16 bed-volumes of 10% acetic acid eluted the D-glucuronic acid which was measured colorimetrically by using the phenol–sulphuric acid procedure. No sulphate was detected in the effluent at any stage. The results were as follows:

Resin volume (ml/mequiv. of sulphate)	1.5	1.9	3.0	4.0
Recovered uronic acid (%)	48	87	102	102

In a second study, using different loadings of D-glucuronic acid (to 50 mg) on columns containing 3 ml of resin/mequiv. of sulphate, only 0.1–0.2% of the D-glucuronic acid was lost in the neutral effluent washed from the columns with 5 bed-volumes of water.

Calibration. — Calibration factors, representing figures for overall total recovery resulting from application to reference compounds of the sequence of operations shown in Fig. 1, are shown in Table I, along with the standard deviations found. Twelve mixtures, each containing 4 different aldoses, were used, and each mixture contained a total sugar content of 100–150 mg.

A number of trials were made to determine the accuracy of the method. Microgranular cellulose (Whatman Column Chromedia Grade CC-31) was analysed by using the general procedure, with the exception that 1 ml of 72% sulphuric acid/100 mg of polysaccharide was used for the primary acid digestion. Duplicate results showed a composition of anhydro glucose residues of 96%, with traces of xylose and arabinose also present.

A hemicellulose B fraction isolated from guinea grass (*Panicum maximum*) was

TABLE I

RECOVERY FROM THE TOTAL ANALYTICAL PROCEDURE

Starting material	*Recovery (%)*	*Standard deviation*
Rhamnose	106.8	2.8
Arabinose	93.6	5.6
Xylose	88.5	5.2
Mannose	89.9	2.7
Galactose	92.7	3.5
Glucose	95.3	2.2

analysed both by g.l.c. as above and by paper chromatography, sugars being separated with the solvent 8:2:1 ethyl acetate–pyridine–water and measured colorimetrically by the phenol–sulphuric procedure[17] after elution from the paper. A separate chromatogram was necessary to obtain a separation of galactose and glucose under these conditions, thus reflecting a difficulty in total analysis by this technique. The results were as follows:

Method	*Composition (anhydro sugar residue)*			
	Ara	*Xyl*	*Gal*	*Glu*
Paper chromatography	14.2	65.3	2.4	9.4[a]
G.l.c.	12.7	66.4	2.4	10.6

[a]Combined glucose and galactose by separate analysis amounted to 12.2.

A chromatogram showing the components of guinea grass, neutral and acidic, as determined by g.l.c., is shown in Fig. 4. A chromatogram showing the inadequate resolution of the acetates of galactitol and 4-*O*-methylglucitol is shown in Fig. 5.

DISCUSSION

The hydrolytic procedure is a modification of the Saeman procedure[20]. Because non-crystalline polysaccharides were being examined, it was felt that milder conditions could be used for the primary hydrolysis. Since Saeman points out that the degradation occurring during secondary hydrolysis is at least four times as much as that in primary hydrolysis, an investigation using hemicellulose B from spear grass (*Heteropogon contortus*) was made to find an optimal time. In an analysis[21] extending over 7 h, it was found that the heating period in the secondary stage was not critical in the range 3–5 h, and 3 h was therefore chosen as the standard time.

Crystalline polysaccharides hampered the studies of Albersheim and coworkers[22] who used 2M trifluoroacetic acid in attempts to hydrolyse plant cell-wall polysaccharides. They found difficulties in arriving at a suitable compromise between

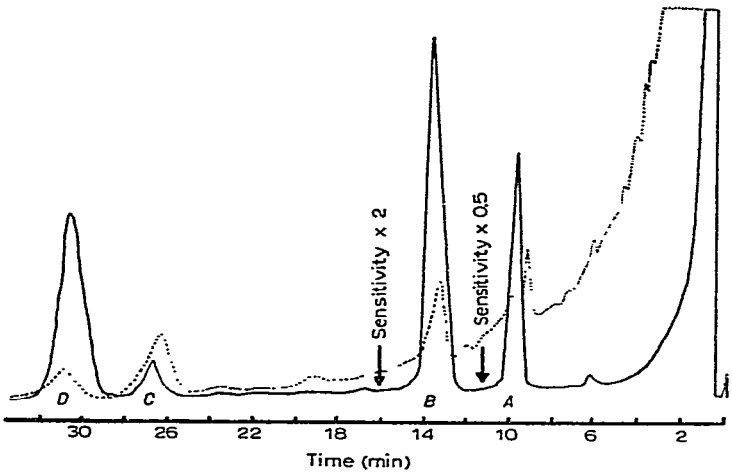


Fig. 4. Gas–liquid chromatograms showing products from (*a*) neutral and (*b*) acidic components of hemicellulose *B* from guinea grass (—, neutral sugars; – – – –, acids (1 × 32); parent sugar *A*, L-arabinose; *B*, D-xylose; *C*, D-galactose; *D*, D-glucose).

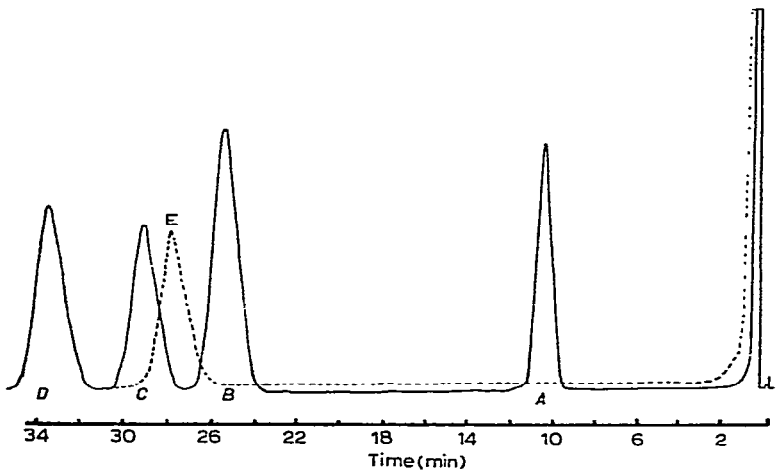


Fig. 5. The resolution of galactitol hexaacetate and 4-*O*-methylglucitol pentaacetate on 3% ECNSS-M in a 210 × 0.7-cm column at 185° at a carrier flow of 45 ml/min. *A*, arabinitol pentaacetate; *B*, mannitol hexaacetate; *C*, galactitol hexaacetate; *D*, glucitol hexaacetate; *E*, 4-*O*-methyl-D-glucitol pentaacetate).

degradation of components released from xylans and pectic substances and incomplete hydrolysis. Furthermore, recoveries varying from 11–60% of the total cell-wall vitiate this approach to a meaningful assessment of plant cell-wall character[23].

In most methods of chromatographic analysis of hydrolysates, the sugars are separated from the hydrolysing acid either by ion exchange or by precipitation of the acid as an insoluble salt. The Saeman procedure[20] uses ion-exchange removal of acid, and since this approach has been adopted by other workers[10,11], our findings on the use of resins are pertinent.

Weakly basic resins, such as Amberlite IR-45 (and probably the Amberlite IR-4B used by Saeman *et al.*) in the free base form, should be avoided, since they react with neutral sugars (especially pentoses) and release them only slowly on washing with water[24]. On the other hand, the hydroxyl form of a strongly basic resin, such as Amberlite IRA-400, must be avoided because of the alkaline degradation or rearrangement of reducing sugars which would result[25].

Our studies involving concomitant sorption of uronic acid components and sulphate suggest that sorption of the uronic components occurs over a broad zone in the resin, but it appears that the actual amount of uronic acid present in the hydrolysate of a typical hemicellulose is not critical to the separation. The incomplete removal of uronic acids, which appears probable in the batch treatment used in the Saeman procedure, introduces many problems in subsequent analytical steps. We have shown that lactonisation of uronic acids can be extensive, and such lactones would not be hydrolyzed under the conditions used. They would thus be isolated with the neutral sugars after ion exchange. The errors which arise from this situation are illustrated by our finding that D-glucurono-6,3-lactone is sufficiently resistant to hydrolysis of the lactone ring during reduction to undergo 75% conversion into glucitol on the addition of sodium borohydride[16]. In this case, therefore, the glucitol hexaacetate determined by g.l.c. would be derived from glucuronic acid and not glucose in the polysaccharide. Unremoved uronic acids not present in lactone form would be reduced to aldonic acids and give rise to acetylated aldonolactones which interfere with resolution of alditol derivatives (*cf.* Fig. 6).

Albersheim and co-workers[22] reduced sugar mixtures from hydrolysed polysaccharides to their respective alditols with sodium borohydride in M ammonium hydroxide to ensure that uronic acids were present as anions during reduction. We have noted, however, that D-glucurono-6,3-lactone undergoes alkaline rearrangements in 95mM ammonium hydroxide and introduces interfering substances into the reaction mixture[16].

Uronic acids are common constituents of plant cell-walls and should not be ignored in analytical schemes. The common uronic acid component of hemicelluloses is 4-*O*-methyl-D-glucuronic acid and has been isolated from some 30 different hemicelluloses of plants and trees. D-Glucuronic acid does not occur abundantly and, so far, has been observed more often in hemicelluloses from annual plants than from woods[26].

Perry and Hulyalkar[12] claimed successful analytical trimethylsilylation of lactonised aldonic acids. However, in our attempts to extend this approach to the direct acetylation of aldonic acid after lactonisation, we obtained irreproducible results as determined by g.l.c. of the products. This effect was possibly due to variability in the relative extent of formation of 1,4- and 1,5-lactones, although the irreproducibility could not be eliminated by lactonisation methods previously claimed to give only 1,4-lactones[12] (*cf.* Fig. 6). It is also possible that hydrolysis of lactone rings may become more significant in the (slower) acetylation than in trimethylsilylation.

In an effort to avoid the lactonisation problems inherent in analysis of uronic

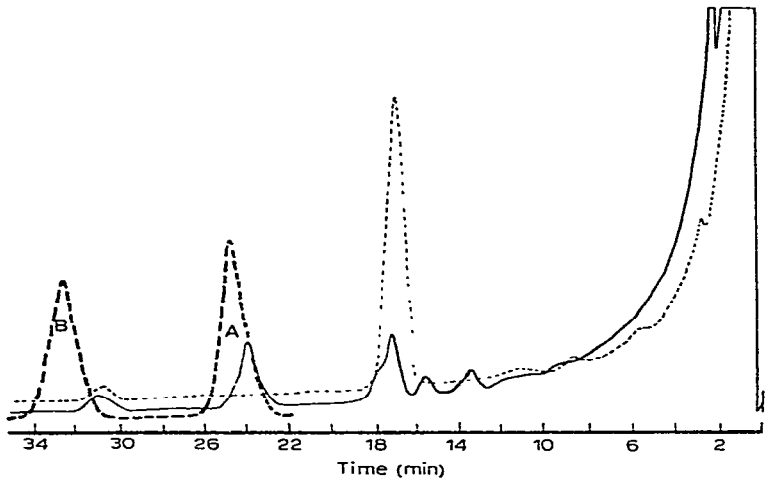


Fig. 6. Acetylated derivatives of D-gluconic acid after lactonisation (—, lactonisation without catalysis; – – –, acetylated D-glucono-1,5-lactone; ·····, (*A*) mannitol hexaacetate, (*B*) glucitol hexaacetate).

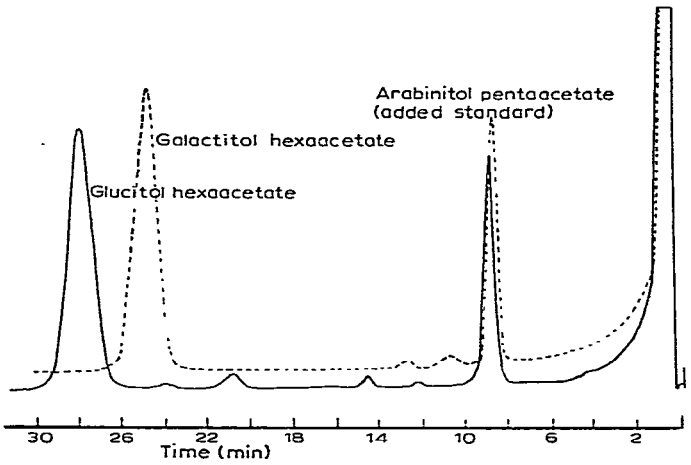


Fig. 7. Chromatograms obtained in the determination of calibration constants for D-glucuronic and D-galacturonic acids in the total hydrolytic analysis.

acids by reduction to aldonic acids, we have examined several methods for conversion of the uronic acids into alditols.

Our preliminary attempts to reduce hexuronic acids with lithium aluminium hydride by refluxing in *p*-dioxane[27], or by esterification with diazomethane[28] and subsequent reduction with sodium borohydride, resulted in variable and low yields of alditol (17–20%), as determined by g.l.c. of the acetylated products. It was decided, therefore, to attempt formation of the alditol by reduction of the corresponding aldonolactone in solutions buffered to maintain acidic pH during the initial stage of the reaction[19]. The Frush and Isbell procedure[19], using strongly acid ion-exchange

resin, proved less convenient, difficulties being experienced in adjustment of resin volumes and the manipulations required to separate resin and solution. The boric acid procedure has the advantage that the whole of the analysis can be carried out in one vessel. Results in Table II show that the reduction is inadequately reproducible when carried out by single-step addition. Chromatograms obtained in the determination of calibration constants for D-glucuronic and D-galacturonic acids in the overall hydrolytic analysis (Fig. 7), however, show the efficiency of the reduction achieved by the careful addition of reducing agent and the relative absence of volatile by-products. Sjostrom *et al.*[13] claimed that conversion into alditols was practically quantitative after three complete borohydride treatments with no special precautions to minimise lactone hydrolysis.

TABLE II

REDUCTION EFFICIENCIES FOR CONVERSION OF ALDONIC AND URONIC ACIDS INTO ALDITOL ACETATES

Sample	*Percentage conversion*[a]	
	No prior treatment	*Full analysis including simulated polysaccharide hydrolysis*
D-Glucono-1,5-lactone	$\overline{87}$ (85,85,85,91,87,93,84)	
D-Glucuronic acid	$\overline{76}$ (73,73,78,78)	$\overline{48}$ (49,46,50)
D-Galacturonic acid	$\overline{56}$ (58,53,51,60)	$\overline{37}$ 40,31,39)

[a]Mean values are given with individual results in brackets.

Apart from the extra manipulation involved in attempting such a procedure, a number of other difficulties arise in the analysis of hexuronic acids. Pectic substances (polygalacturonides) are frequently contaminants of hemicelluloses, and resolution of their constituent acidic sugars, as galactitol and 4-*O*-methylglucitol after reduction (*cf.* Fig. 5), is inadequate on the stationary phases which we have used. This makes even qualitative identification of hexuronic components difficult by g.l.c., and, in our experience, paper chromatography in the solvent 18:3:1:4 ethyl acetate–acetic acid–formic acid–water proved to be the more useful method of identification.

During the hydrolysis of 4-*O*-methylglucuronoxylans, an aldobiouronic acid, 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose, accumulates in the hydrolysate and is relatively resistant to further acid hydrolysis[29]. This accumulation of aldobiouronic acid is responsible for some apparent loss of xylose in the final analysis. The aldobiouronic acid is also incapable of forming a derivative of sufficient volatility or thermal stability to be detectable by g.l.c. Since 4-*O*-methylglucuronic acid can be reduced (*via* the aldonolactone) to the corresponding glucitol, it appears that the xylitol derivative of the aldobiouronic acid is sterically hindered in the formation of a stable lactone and so is not reduced to a neutral (non-acidic) product. In

our experience, the acid fractions isolated from hemicelluloses and treated according to the procedures outlined were viscous, and thermal degradation frequently led to blocking of the needle during injection into the chromatograph. We attribute this to the accumulation of non-volatile products such as those arising from aldobiouronic acids.

Automated acid–base titrimetry to date has proved to be our best means of estimating total acid composition. It must remain a crude measure of inherent hexuronic acid composition, since aldonic and saccharinic acids would be expected to be constituents, to some extent, of polysaccharides isolated from chlorite holocelluloses by alkaline extraction. The complexity of the acid fraction from hemicellulose of spear grass is illustrated by the components detectable by paper chromatography with the acidic solvent mentioned above. R_{Xyl} values of 1.65, 1.35, 1.13, 0.91, 0.66, 0.54, 0.49, 0.37, 0.32, 0.24, 0.14, and 0.08 were measured. When this same mixture was chromatographed in the basic, irrigating solvent 8:2:1 ethyl acetate–pyridine–water, five mobile components were detected, while the major spot remained near the point of application. Until acid degradation products are identified and accounted for, hexuronic acid content will remain a crude estimate.

Perry and Hulyalkar[12] could not resolve the problem of recovering bound hexuronic acids from polymeric materials in good yield, reporting that acid hydrolysis gave, at best, recoveries of about 70% and this decreased significantly for the more acid-labile uronic acids. Reduction of uronic acids *in situ* as they were released failed to stabilise the acids. Sjostrom and co-workers[10] also reported difficulties in extending their analytical method to pulp and wood samples, attributing spurious peaks to acidic fragments. Their alternative procedure supports our view that these should be removed from neutral sugars for separate analysis, but the presence of contaminants remains a serious analytical flaw. Although the nature of the samples studied was not stated clearly in the report, one might speculate that degraded lignin fragments may interfere in the method proposed for wood samples.

There is little doubt that borate adversely affects acetylation, but confusion exists as to the extent. Sawardeker *et al.*[9] and Sjostrom *et al.*[10] claimed quantitative conversion into acetylated alditols in the presence of borate after refluxing for 4 h with equal volumes of acetic anhydride and pyridine. Crowell and Burnett[22] studied the acetylation of D-glucitol in the presence of borate and found a minimum of 10 h was required for quantitative conversion. We confirm their observation that acetylation of alditols in the absence of borate requires about 1 h at room temperature but, contrary to their experience, found that galactitol is more resistant than glucitol to acetylation. Furthermore, when galactitol was acetylated at 95° under conditions similar to those used by Crowell and Burnett, we found less than 40% conversion occurred after 50 h. It was our experience that this method of acetylation produced very dark-coloured mixtures of uncertain composition, and we consider that removal of borate as its volatile methyl ester[30] is essential before acetylation. The findings of Albersheim *et al.*[22] support this approach. They did not remove cations, however, but preferred to use sodium acetate as the basic catalyst in the subsequent acetylation

in a sealed tube at 121° for 3 h, conditions which seem a rather drastic alternative to our acetylation procedure.

Oades[31] has reported difficulties in acetylation of alditols with acetic anhydride–pyridine in the microgram range. He ascribed this to incomplete removal of borate by exhaustive distillation *in vacuo*. It is conceivable that, at this level, traces of borate, perhaps retained by complexing with the alditol, can be significantly inhibiting, but the general reproducibility of our results discounts this as an important source of errors in the milligram range.

The calibration factors reported in Table I and reflecting absolute recoveries in terms of hydrolytic survival, reaction efficiencies, and experimental technique, are of the order reported by other workers. Detector responses determined with respect to mannitol hexaacetate showed variations similar to those observed by Sjostrom *et al.*[10], but were contrary to those of Sawardeker *et al.*[9] who found no variation for all alditols studied. In the absence of a detector response for rhamnitol pentaacetate which could not be isolated as a crystalline derivative, we are unable to account for the high figure for apparent recovery of this sugar.

The results obtained for cellulose indicate an accuracy of better than 5%, and the data (see Experimental) show that the results are comparable with those of quantitative paper chromatography for the determination of the neutral sugar constituents of hemicelluloses. Satisfactory, separate analysis of hexuronic acids has not been achieved.

ACKNOWLEDGMENTS

One of us (J.D.B.) wishes to thank the Australian Meat Research Committee for a scholarship, and we thank that body and also the Australian Research Grants Committee for a grant for equipment.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

THE REACTIONS OF ISOTHIOCYANATES WITH 2-AMINO SUGARS: ELECTROPHORESIS IN BORATE AND TUNGSTATE BUFFERS, AND SPECTROSCOPY OF THE PRODUCTS

J. E. SCOTT

Medical Research Council Rheumatism Unit, Canadian Red Cross Hospital, Taplow, Maidenhead, Berkshire (Great Britain)

(Received July 29th, 1969; in revised form, February 6th, 1970)

ABSTRACT

The reaction of 2-amino-2-deoxyaldoses with isothiocyanates goes through three stages, producing a thiourea (**1**), which cyclises to a 5-hydroxyimidazolidine-2-thione (**4**) with concomitant opening of the sugar ring. A molecule of water can then be lost, with the formation of a furano ring, the imidazolidine-2-thione remaining intact in a fused, bicyclic structure (**3**). Products (**3**) derived from 1-amino-1-deoxy-2-ketoses are spiro compounds. Compound **4** is stable in neutral or alkaline solution, but converts quickly into **3** in acid. The conversion into **4** is extremely rapid, and its existence is inferential.

Conversion of **4**, with loss of 1 mole of water per mole, into either an imidazole-2-thione (**6**) or a C-5 substituted imidazolidine-2-thione (**3**) is discussed, utilising the pseudo-basic properties of **4**.

Mixtures of products **4** and **3** are separable by electrophoresis in tungstate and borate buffers. Electrophoretic mobilities of a number of amino-sugar derivatives are given.

INTRODUCTION

In preliminary reports[1,2], it was shown that thioureido compounds initially formed in the reaction of 2-amino-2-deoxy-hexoses and -pentoses with organic isothiocyanates were converted quickly and quantitatively into hydroxyimidazolidine-2-thiones, in which the sugar rings had opened. From more vigorous conditions, crystalline products were isolated[3] which were thought to contain fused imidazolidine and pyranose rings, but which were later shown to possess furanoid structures[4]. The relationships between these compounds are considered in this investigation.

MATERIALS AND METHODS

Materials. — 2-Acetamido-2-deoxy-D-allose, 2-acetamido-2-deoxy-D-ribose, 2-acetamido-2-deoxy-D-arabinose, 2-acetamido-2-deoxy-D-altrose (Professor R. Kuhn *via* Dr. D. A. L. Davies), 2-amino-2-deoxy-D-fucose (Dr. D. A. L. Davies), 2-acetamido-2-deoxy-3-*O*-methyl-D-glucose (Dr. R. W. Jeanloz), 3-amino-3,6-dideoxy-L-talose

(Dr. A. C. Richardson), 1-amino-1-deoxy-D-fructose (Dr. G. Huber), 2-amino-2-deoxy-D-glucitol (Dr. P. W. Kent), phenyl 2-amino-2-deoxy-β-D-glucopyranoside (Dr. D. M. Leaback), and methyl 2-amino-2-deoxy-α-D-glucopyranoside (Dr. R. D. Marshall) were gifts, and 2-deoxy-2-methylamino-L-glucose was prepared by hydrolysing streptomycin.

Compounds originally formulated[3] as "2-deoxy-2-(*p*-tolylcarbamido)-β-D-glucose", "2-deoxy-2-(*p*-tolylthiocarbamido)-β-D-glucose", "2-oxo-3-(*p*-tolyl)-4,5-D-glucopyranotetrahydroimidazole", "2-thio-3(*p*-tolyl)-4,5-D-glucopyranotetrahydroimidazole" were gifts from Dr. C. Morell. 2-Mercapto-4-(D-*arabino*-tetrahydroxybutyl)-1-tolylimidazole was a gift from Dr. G. Huber.

N,N-Dimethyldodecylamine, distilled grade (DM12D) was a gift from Armour Hess Ltd.

Methods. — Ascending chromatography was carried out by using 20:3 isopropyl alcohol–water and Whatman No. 1 paper, with or without impregnation with 50mM sodium molybdate–H_2SO_4 (pH 5.0) and drying.

Electrophoresis was carried out on a horizontal strip of Whatman No. 1 paper, in a Kohn apparatus (Shandon Scientific Co. Ltd.) at 10 volts/cm, with a maximum current of 0.7 mamp/cm width. The paper was soaked in buffer and blotted dry, and aliquots were applied with the paper in position. After 1–2 h, the paper was removed and rapidly dried at 110°.

Borate, molybdate, and tungstate were 50mM as sodium salts, adjusted to the appropriate pH with hydrochloric, sulphuric, or acetic acid. McIlwaine's phosphate–citrate buffers were 50mM.

Thiourea, used as the origin marker, was easily detected by its u.v. absorbance, or with silver nitrate[5], or dichloroquinone–chloroimide[6]. Glycerol was also used as a non-migratory marker during electrophoresis in sodium tungstate.

D-Mannitol or D-glucose were reference compounds in tungstate or borate, respectively.

Detection. — Products of the reaction of isothiocyanates with amines appear as dark spots on a light-blue ground in light of 2537Å (Chromatolite, Shandon). Tungstate and molybdate absorb u.v. light, thus reducing the sensitivity. Alkaline silver nitrate[5] is particularly sensitive for compounds containing a divalent sulphur atom. Molybdate, tungstate, and borate do not interfere. Sulphur-containing products can be detected by passing the dried paper through a 0.1% solution of iodine in chloroform, and air-drying. The dark-brown spots on a pale ground fade in a matter of hours, but can be re-produced on subsequent dipping. Multiple dipping increases the sensitivity, at the expense of increase in the background. Dichloroquinone–chloroimide[6] reacts well with the amino sugar–isothiocyanate derivatives, giving red or orange spots which develop in an hour or two. Spots and background turn grey in a few days. Naphthyl derivatives fluoresce intensely yellow on the *dried* paper under a Chromatolite lamp. The strip is examined immediately after removing from the drying oven. The fluorescence intensity declines in minutes, but can be restored by reheating to 105° for a short time.

U.v. spectra were determined for aqueous solutions by using a Unicam SP500 spectrophotometer fitted with a synthetic quartz prism. Slightly soluble materials were dissolved to 1% in ethanol, and 0.1-ml aliquots were diluted rapidly to 100 ml with distilled water. Appropriate controls containing 0.1% of ethanol were used in these cases.

I.r. spectra were determined for KBr discs on a Perkin–Elmer 137 spectrometer, by Mrs. J. Chittenden and Mr. L. C. Thomas of the Chemical Defence Establishment, Porton.

Isothiocyanate derivatives of amino sugars. — Two volumes of a pyridine solution of the isothiocyanate (≮20% excess), also containing 5–10% of collidine, triethylamine, or *N*,*N*-dimethyldodecylamine, were added to one volume of the aqueous sugar solution. After incubation, usually at 55°, for 20–60 min, four volumes of benzene were added with thorough mixing followed by centrifugation. The upper, benzenoid layer was removed, and the extraction was repeated twice to remove pyridine, tertiary amine, and by-products. The following compounds were prepared thus.

(*a*) 5-Hydroxy-1-phenyl-4-(D-*arabino*-tetrahydroxybutyl)imidazolidine-2-thione (**4**, R = phenyl) was obtained by employing solutions of 2-amino-2-deoxy-D-glucose hydrochloride (10%), phenyl isothiocyanate, and triethylamine at 37°. After storage for 16 h *in vacuo* over P_2O_5, the crystals in the syrup were washed rapidly with cold water and dried over P_2O_5 to give material (6%) which "bubbled" at 90–100°, resolidified at 116–118°, and melted at 152–159°. It had λ_{max} (water) 238 nm (ε 20,000) (Found: C, 49.7; H, 5.9; N, 8.9; S, 10.4. $C_{13}H_{18}N_2O_5S$ calc.: C, 49.7; H, 5.7; N, 8.9; S, 10.2%).

(*b*) 5-Hydroxy-1-naphthyl-4-(D-*arabino*-tetrahydroxybutyl)imidazolidine-2-thione (**4**, R = 1-naphthyl) was obtained with solutions of 1-naphthyl isothiocyanate, collidine, and 2-amino-2-deoxy-D-glucose hydrochloride (10%). The aqueous layer set to a mass of fine crystals after a few min at room temperature. The crystals were shaken with water, filtered, and washed with water to give material (77%), m.p. 154–155° (dec.) (from water) (Found: C, 54.2; H, 5.6; N, 7.2; S, 8.3. $C_{17}H_{20}N_2O_5S{\cdot}H_2O$ calc.: C, 53.3; H, 5.8; N, 7.3; S, 8.4%.)

(*c*) 5-Hydroxy-1-naphthyl-4-(D-*lyxo*-tetrahydroxybutyl)imidazolidine-2-thione (**5**, R = 1-naphthyl) (60%) was prepared as in (*b*), using 2-amino-2-deoxy-D-galactose hydrochloride as a 1% solution (Found: C, 55.6; H, 5.5; N, 7.8. $C_{17}H_{20}N_2O_5S$ calc.: C, 56.0; H, 5.5; N, 7.7%.)

(*d*) 4,5-(2-Deoxy-D-glucofurano)-1-phenylimidazolidine-2-thione (**3**, R = phenyl) (43%) was prepared by adjusting an aliquot of an aqueous solution containing **4** (R = phenyl) (see above) to pH 3–4 with hydrochloric acid. After several hours at room temperature, the product separated and had m.p. 191°, λ_{max} (water) 238 nm (ε 19,000) (Found: C, 52.5; H, 5.5; N, 9.6; S, 10.9. $C_{13}H_{16}N_2O_4S$ calc.: C, 52.7; H, 5.4; N, 9.5; S, 10.8%).

Alternatively, an aliquot of the aqueous solution containing **4** (R = phenyl) was freeze-dried. The residue was crystallised from propan-2-ol to give material

TABLE I

SUBSTITUTED THIOUREAS

Amine	*Isothiocyanate*	*Melting point of the thiourea (degrees)*	*Analyses*	ε_{max}	λ_{max} (*nm*)
2-Aminoethanol (1)	phenyl	138[a] lit.[21] 138		14,000	243
2-Aminopropan-1-ol (2)	phenyl	137.5–139[a]	$C_{10}H_{14}N_2OS$ Calc.: C, 57.1; H, 6.8; N, 13.4; S, 15.3. Found: C, 57.4; H, 6.6; N, 13.3; S, 15.2.	13,920	243
3-Aminopropan-1-ol (3)	phenyl	85[a]	$C_{10}H_{14}N_2OS$ Calc.: C, 57.1; H, 6.8; N, 13.4; S, 15.3. Found: C, 56.9; H, 6.5; N, 13.6; S, 15.2.	15,450	242–243
2,2-Diethoxyethylamine (4)	phenyl	95[b] lit.[20] 96		13,325	243–244
2-Amino-2-methyl- (5) propan-1-ol	phenyl	122[a] lit.[7] 127-128		17,050	243–244
2-Amino-2-methyl-propane-1,3-diol (6)	phenyl	127[c]	$C_{11}H_{16}N_2O_2S$ Calc.: C, 55.0; H, 6.7; N, 11,7; S, 13.3. Found: C, 55.1; H, 6.9; N, 11.3; S, 13.0.	17,800	243–244
N-Methylaniline (7)	methyl	114[a]	$C_9H_{12}N_2S$ Calc.: C, 59.9; H, 6.7; N, 15.6; S, 17.8. Found: C, 60.0; H, 6.9; N, 15.6; S, 17.9.	19,250	235

[a]From 50% EtOH–H_2O. [b]From 60%EtOH–H_2O. [c]From 40% EtOH–H_2O.

(40%), m.p. 176–178°, λ_{max} 239 nm (ε 18,750) (Found: C, 53.0; H, 5.6; N, 9.5; S, 11.7%).

*"Anhydro-PTC" (acid-treated "PTC") derivatives**. — These compounds were prepared by acidifying to pH 3–4 aliquots of the aqueous layer containing the "*PTC*" derivative of the amino sugar from the general procedure. In most cases, concentrations were too low to encourage crystal formation, and the solution was used directly for electrophoresis after neutralisation with dilute, aqueous sodium hydroxide.

Substituted thioureas (Table I). — Isothiocyanates were treated in slight excess with amines, in ether where possible, from which the product usually separated quickly at room temperature. Acetone or aqueous acetone were used for amines which were not soluble in ether.

Anilinothiazolines. — 2-Anilinothiazoline (m.p. 162°, lit. m.p. 158–160°) and 2-anilino-4,4-dimethylthiazoline (m.p. 156–157°) were prepared from *N*-(2-hydroxyethyl)-*N'*-phenylthiourea and *N*-(1,1-dimethyl-2-hydroxyethyl)-*N'*-phenylthiourea (see Table I).

Imidazolidine-2-thiones. — 1-(1-Naphthyl)imidazolidine-2-thione and 1-phenylimidazolidine-2-thione (m.p. 164°, lit. m.p. 155°) were prepared from the appropriate 2-(arylamino)ethylamines, *via* the dithiocarbamic acid, by the general method given in Beilstein (System No. 3557).

2-Anilinoethylamine was prepared[8] from aniline and ethylene imine.

N-(1-Naphthyl)imidazolidine-2-thione had m.p. 208° (from acetone) (Found: C, 68.3; H, 5.1; N, 12.5; S, 14.3. $C_{13}H_{12}N_2S$ calc.: C, 68.4; H, 5.3; N, 12.3; S, 14.1%.)

Preparation of a substituted phenyl-2-thiohydantion from 2-amino-2-deoxy-D-gluconic acid, and its reduction with sodium borohydride. — The phenylthiocarbamoyl (PTC) derivative was made from 2-amino-2-deoxy-D-gluconic acid and phenyl isothiocyanate by the general procedure, using triethylamine as the buffer, for 3 h at 37°.

The spectrum of the aqueous layer showed a characteristic phenylthioureide absorption λ_{max} 244 nm (ε 14,220, on the assumption that reaction was quantitative and no other absorbing species was present). Electrophoresis in sodium tungstate at pH 7.0 showed a single u.v.-absorbing component that travelled slightly *faster* than D-mannitol and reacted very sensitively to the silver nitrate reagent.

The solution was freeze-dried and the product (245 mg) was dissolved in 10 ml of M trichloroacetic acid and left for 16 h at room temperature. A few crystals deposited. The supernatant was thrice extracted with ether to remove trichloroacetic acid. The solution showed a characteristic phenylthiohydantoin absorption (λ_{max} 265 nm with a minimum at 245 nm). Electrophoresis in tungstate buffer at pH 7 showed two components, the minor one being immobile, the other moving just *behind* D-mannitol (Table II).

To 3 ml of the solution was added 50 mg of sodium borohydride. After 1 h, the

*PTC = Phenylthiocarbamoyl, used when the structure is not in doubt. "PTC" describes derivatives of amines with isothiocyanates in which the PTC formulation is in doubt.

TABLE II

ELECTROPHORETIC MOBILITIES OF AMINO SUGARS AND THEIR DERIVATIVES IN 50mM TUNGSTATE BUFFER

	$R_{Mannitol}$ (pH 7.0)		$R_{Glucitol}$ (pH 5.0)
	N-*Acetyl*	"*PTC*"	*2-Deoxyhexitols*[10]
2-Amino-2-deoxy-D-glucose	0	0.8	
2-Deoxy-2-methylamino-L-glucose		0.8	
2-Amino-2-deoxy-D-glucitol		0.8	1.0
2-Amino-2-deoxy-D-mannose	0	0.7	
1-Amino-1-deoxy-D-fructose		0.8	
2-Amino-2-deoxy-D-galactose		0.4	0.6
2-Amino-2-deoxy-D-altrose	0	0.7[a]	0.57[b]
2-Amino-2-deoxy-D-allose	0	0.7[b]	
2-Amino-2-deoxy-D-fucose		0.0	
2-Amino-2-deoxy-D-ribose		0.0	
2-Amino-2-deoxy-D-arabinose		0.0	0.0[c]

Other derivatives	
3-Phenyl-5-(D-*arabino*-tetrahydroxybutyl)-2-thiohydantoin (Phenyl-2-thiohydantoin from 2-amino-2-deoxy-D-gluconic acid)	0.8
1-*p*-Tolyl-4-(D-*arabino*-tetrahydroxybutyl)-imidazole-2-thione	
Compound 6 (R = *p*-tolyl)	
"Anhydro-PTC"-2-amino-2-deoxy-D-glucose (3, R = phenyl)	0.0
"Anhydro-PTC"-1-amino-1-deoxy-D-fructose	0.0
"2-deoxy-2-(*p*-tolylthiocarbamido)-D-glucose"	0.8
"2-deoxy-2-(methylthiocarbamido)-D-glucose"	0.8
"2-deoxy-2-(1-naphthylthiocarbamido)-D-glucose"	0.8
4,5-(2-Deoxy-D-glucofurano)-1-*p*-tolylimidazolidine-2-thione[4]	0.0
PTC-3-amino-3,6 dideoxy-L-talose	0.0
PTC-1-amino-1-deoxy-D-lyxose	0.0

[a]Streak. [b]Elongated spot. [c]2-Deoxy-D-*erythro*-pentitol.

thiohydantoin-absorption at 265 nm had disappeared, to be replaced by a somewhat higher peak at 238 nm (see Fig. 1). On electrophoresis in tungstate buffer at pH 7, two u.v.- absorbing components were observed, in the same positions and with the same relative intensities as those present in the solution prior to reduction. The faster spot migrated at the same rate as the reaction product from 2-amino-2-deoxy-D-glucose and phenyl isothiocyanate, prepared by the general procedure.

RESULTS

U.v. spectra (in water). — Spectra of *N'*-substituted *N*-phenylthioureas show maxima at 242–244 nm (Table I), and minima around 225 nm. The long-wave edge of this absorption band declines relatively slowly (Fig. 2). The absorption intensity is more sensitive than the frequency to changes in the nature of the non-phenyl substituent. A secondary or tertiary carbon substituent at the non-phenyl nitrogen e.g., *1–4*, Table I) is associated with an ε_{max} of $13–15 \times 10^3$; ε_{max} for a quaternary carbon substituent (*5* and *6*) increases to $17–18 \times 10^3$.

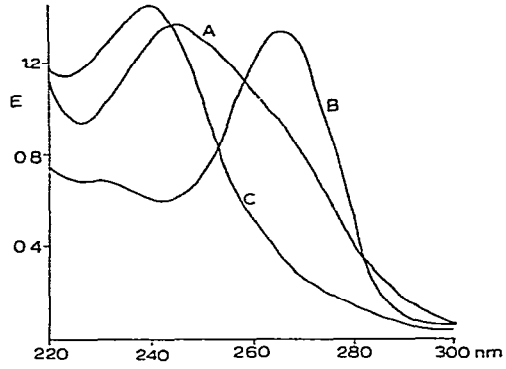


Fig. 1. Conversion of 2-deoxy-2-(3-phenylthioureido)-D-gluconic acid (Curve *A*) into the phenylthiohydantoin derivative (Curve *B*), and reduction with aqueous sodium borohydride to the 5-hydroxyimidazolidine-2-thione (Curve *C*).

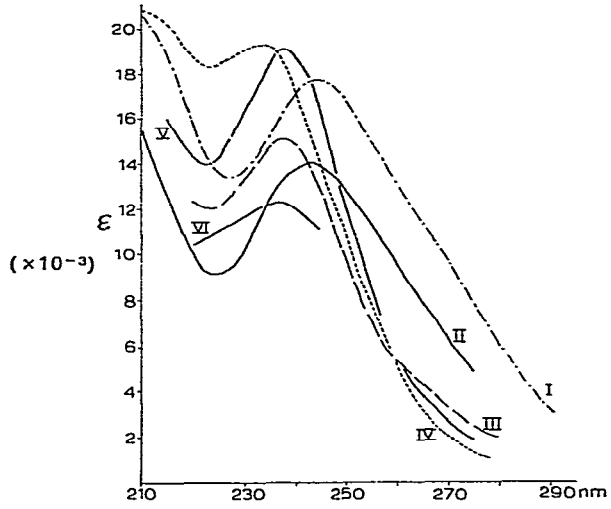


Fig. 2. U.v. spectra (in water) of: I (—·—) *N*-(1,3-dihydroxy-2-methyl)-*N'*-phenylthiourea, II (———) *N'*-(2-hydroxyethyl)-*N'*-phenylthiourea, III (– – –) 5-hydroxy-4,4-dimethyl-1-phenylimidazolidine-2-thione, IV (------) *N*-methyl-*N*-phenyl-*N'*-methylthiourea, V (———) "PTC"-2-amino-2-deoxy-D-glucose, and VI (———) 2-anilino-4,4-dimethylthiazoline.

The spectra of phenylthioureas in which the "anilino" nitrogen atom carries a second substituent show maxima at 238 nm or less (*7*, Table I). In addition to compound *7*, there are[9] 1-phenylimidazolidine-2-thione (ε_{max} 14,400) and 5-hydroxy-4-methyl-1-phenylimidazolidine-2-thione (λ_{max} 237, ε_{max} 17,500). The long-wave edge falls much more sharply than in the symmetrically *NN'*-disubstituted compounds.

2-Anilinothiazoline (ε_{max} 10,350) and 2-anilino-4,4-dimethylthiazoline (ε_{max} 12,250) both absorb maximally at about 238 nm with rather broad peaks.

The spectra of 5-hydroxy-1-phenyl-4-(D-*arabino*-tetrahydroxybutyl)imidazolidine-2-thione (**4**, R = phenyl), its 2-amino-2-deoxy-D-galactose analogue (**5**, R =

phenyl), and 4,5-(2-deoxy-D-glucofurano)-1-phenylimidazolidine-2-thione (3, R = phenyl) were identical, within experimental error, in water, 10mM borate buffer pH 8.95, and 10mM hydrogen carbonate buffer pH 8.95 (λ_{max} 238 nm, ε_{max} 19,000 ±500). The spectrum of "*p*-tolylthiocarbamoyl"-2-amino-2-deoxy-D-glucose showed λ_{max} 238 nm, ε_{max} 18,250.

Electrophoresis. — (*a*) *Citrate–phosphate buffers.* "PTC"-2-amino-2-deoxy-D-glucose and its "anhydro" derivative did not migrate on electrophoresis in McIlwaine citrate–phosphate buffers at pH 7, 5, and 3.2. The substituted phenylthioureas in Table I were also immobile, whereas 2-anilinothiazoline and 2-anilino-4,4-dimethylthiazoline moved rapidly and compactly as cations, at all three pH values.

(*b*) *Borate buffers.* Table III shows mobilities of a series of "PTC" amino sugars, compared with those of the analogous *N*-acetyl derivatives. Mobilities (R_G) are expressed relative to that of D-glucose. All of the "PTC" derivatives move much more rapidly than the *N*-acetyl compounds. Also shown are R_G values of the acid-treated "anhydro-PTC" derivatives of a number of amino sugars, most of which (except PTC-2-amino-2-deoxy-D-glucitol) are drastically reduced following the acid treatment. The R_G of acid-treated "PTC"-2-amino-2-deoxy-D-glucose is the same as that of the product of dissolution of "PTC"-2-amino-2-deoxy-D-glucose in ethanol (see preparative section). The D-glucofurano-imidazolidine derivatives of Morell migrated identically to the "anhydro PTC" derivative of 2-amino-2-deoxy-D-glucose (Table III).

TABLE III

ELECTROPHORETIC MOBILITIES OF AMINO SUGAR DERIVATIVES IN 50mM BORATE BUFFER

	R_G *in borate buffer (pH 9.2)*		
	N-*Acetyl*	*"PTC"*	*Acid-treated "PTC"*
2-Amino-2-deoxy-D-glucose	0.20	0.73	0.65
2-Amino-2-deoxy-D-galactose	0.35	0.71	0.21
2-Amino-2-deoxy-D-mannose	0.6	0.65	0.17
2-Amino-2-deoxy-D-allose	0.37	0.71	
2-Amino-2-deoxy-D-altrose	0.48	0.69	
2-Amino-2-deoxy-D-ribose	0.21	0.49	
2-Amino-2-deoxy-D-arabinose	0.21	0.47	
2-Amino-2-deoxy-3-*O*-methyl-D-glucose		0.4	0.27
1-Amino-1-deoxy-D-fructose		0.73	0.0
2-Amino-2-deoxy-D-glucitol		0.75	0.75
2-Amino-2-deoxy-D-fucose		0.60	
3-Amino-3,6-dideoxy-L-talose		0.80	
4,5-(2-Deoxy-D-glucofurano)-1-*p*-tolylimidazolidine-2-thione[4]			0.63
4,5-(2-Deoxy-D-glucofurano)-1-*p*-tolylimidazolidine-2-one[4]			0.63

"PTC" derivatives of 2-amino-2-deoxy-D-glucose, -D-mannose and -D-galactose migrate at very similar rates, so that mixtures of them could not be resolved by electrophoresis in borate buffer. There is, however, no difficulty in separating acid-

treated "PTC"-2-amino-2-deoxy-D-glucose from analogous derivatives of D-galactose and D-mannose.

The substituted phenylthioureas in Table I were all immobile in borate buffer.

(*c*) *Tungstate and molybdate buffers.* The structural features required of polyhydroxy compounds in order to complex with both molybdate and tungstate are identical[10]. Tungstate has been used most in this investigation, because it is effective at pH 7.0 (compared with pH 5.0 for molybdate), it does not absorb u.v. light as strongly, and electrode decomposition was not so noticeable. Whenever comparisons were made, results in molybdate and tungstate were similar.

In complete contrast to the behaviour of "PTC"-2-amino-2-deoxyhexoses, *N*-acetyl derivatives did not migrate in tungstate buffers (Table II). The mobilities (R_M) of "PTC"-2-amino-2-deoxyhexoses, relative to that of D-mannitol, fall into three very distinct groups, *i.e.*, *ca.* 0.8, 0.4, or 0.0. Migration is generally rapid and compact.

Chromatography with a variety of solvents, using untreated paper or paper previously soaked in borate buffer, did not result in any useful separations of the various "PTC"-hexosamines. However, chromatography on paper previously treated with 1% sodium molybdate solution, at pH 5.0, resulted in very clear resolution of 2-amino-2-deoxy-D-glucose–2-amino-2-deoxy-D-galactose mixtures. The "PTC"-derivatives move more rapidly than the parent amino sugars in all solvent systems.

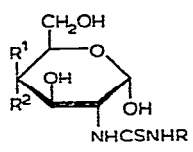
DISCUSSION

The evidence for the structure of "PTC"-2-amino-2-deoxyaldoses is of three kinds: (1) elemental analysis of the isolated product, (2) behaviour on complexing with borate and tungstate, (3) u.v. and i.r. spectroscopy.

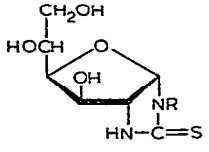
The isolation of "PTC"-2-amino-2-deoxy-D-glucose is not easy. A rapid change takes place on dissolution in many organic solvents, with the appearance of new compounds characterised by completely different electrophoretic mobility, both in tungstate and borate buffers. It is therefore necessary to work in aqueous solution, in which the compound is very soluble. Crystals are obtained from concentrated syrups, but in rather low yield. Their analysis corresponds to 2-deoxy-2-(3-phenylthioureido)-D-glucose (**1**, R = phenyl). The isolation and recrystallisation of 1-naphthyl derivatives in high yield is easy, because of a lower solubility in water. Elemental analysis of the 2-amino-2-deoxy-D-galactose derivative corresponds to a naphthylthioureido compound (**2**, R = naphthyl), whereas the D-glucose analogue is apparently a monohydrate. The melting points of both "crude" and purified products are not very meaningful. Vigorous evolution of a gas occurs at quite low temperatures, and although crystals are reformed, the subsequent melting ranges are not well defined.

Elemental analysis suggests that 2-(3-arylthioureido)-2-deoxyaldoses have been obtained, but this formulation is incompatible with the pattern of complexing in tungstate and borate buffers. The comparison with 2-acetamido-2-deoxyhexoses

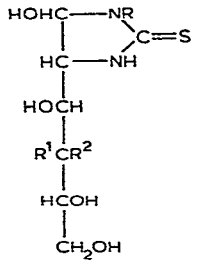
is particularly interesting, in that they are totally immobile in tungstate buffer, whereas the "PTC" derivatives migrate almost as rapidly as D-mannitol. It is pertinent that pyranosides of D-glucose, D-galactose, and D-mannose do not complex with tungstate[10].



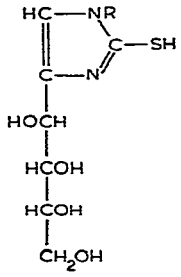
1 R^1=H, R^2=OH
2 R^1=OH, R^2=H



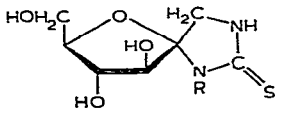
3



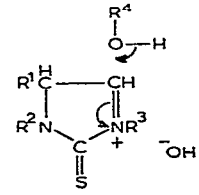
4 R^1=H R^2=OH
5 R^1=OH R^2=H



6 R=*p*-tolyl



7



8

The arylthioureido moiety is not responsible for complex formation, since none of the model compounds (Table I) showed any mobility in tungstate buffer. Alternatively, the thiocarbamoyl group could take part in a complex involving hydroxyl groups or some other part of the sugar moiety. This seems unlikely, since the PTC derivatives of phenyl 2-amino-2-deoxy-α-D-glucopyranoside and methyl 2-amino-2-deoxy-α-D-glucopyranoside do not complex with tungstate, nor do the "PTC" derivatives of 2-amino-2-deoxypentoses, 3-amino-3,6-dideoxy- and 2-amino-2,6-dideoxyhexoses, or 2-amino-2-deoxy-3-*O*-methyl-D-glucose shown in Tables II and III. If the sugar ring remains intact, then every position in the hexosamine molecule is somehow involved in the tungstate complex, and yet such a complex occurs with seven different reducing amino-sugars listed in Table II. A more reasonable alternative is that the sugar ring has opened, producing a polyhydroxy chain, since at least three suitably placed (usually vicinal) hydroxyl groups are required for formation of a tungstate complex[10]. As a first assumption, since *N*-aryl-*N'*-substituted thioureas do not complex with tungstate, the "PTC" grouping may be replaced by *O*-methyl, or hydrogen, without materially affecting the complexing ability of the rest of the molecule. When this is done, the behaviour of the appropriately 2-substituted hexitols exactly parallels those of the "PTC" derivatives of 2-amino-2-deoxyhexoses (Table II).

Furthermore, the electrophoretic behaviour of acyclic derivatives of 2-amino-2-deoxy-D-glucose ("PTC"-2-amino-2-deoxy-D-glucitol, the phenylthiohydantoin from 2-amino-2-deoxy-D-gluconic acid, and compound **6**, Table II) is identical with that of the "PTC"-2-amino-2-deoxy-D-glucose.

To reconcile a cyclic sugar derivative with the behaviour of the "PTC" compounds in borate buffer is equally difficult. The increase in electrophoretic mobility of "PTC" derivatives as compared with *N*-acetyl derivatives is unexpected, because of the larger size of the PTC substituent. Somewhat similar arguments to those used in the tungstate series are applicable. The arylthioureas of Table I do not complex with borate, so the great increase in complexing ability, shown throughout the series of aminodeoxy-hexoses and -pentoses, and *O*-methyl and deoxy derivatives can only be explained on the basis of a cyclic sugar by postulating novel and varied structures for the borate complex. With the assumption that the sugar ring has opened, the results are easily explicable. New glycol groups thus produced offer sites for additional types of complex with borate. It is concluded that an acyclic sugar derivative is present during electrophoresis in borate, if not before. In contrast, *N*-acetyl derivatives migrate as expected for 2-substituted pyranoses (with the single exception of 2-amino-2-deoxy-D-mannose, Table III).

A series of phenylthiocarbamoyl compounds containing various structural features of the "PTC"-aminodeoxyaldoses were prepared and examined spectroscopically (Table I). Remarkable constancy of λ_{max} (242–244 nm) and of the spectrum shape is observed, despite great changes in the nature of the non-phenyl substituent. It is therefore pertinent that λ_{max} of "PTC"-2-amino-2-deoxy-D-glucose is 5–6 nm lower, at 238 nm, and that the spectrum is somewhat dissimilar in shape (Fig. 2). Particularly significant is the difference in λ_{max} between "PTC"-2-amino-2-deoxy-D-glucose and PTC-2-amino-2-deoxy-D-glucitol, PTC-2-amino-2-deoxy-D-gluconic acid, and PTC-(methyl 2-amino-2-deoxy-D-glucopyranoside); the last three compounds, for which the PTC formulation seems beyond doubt, have characteristic PTC maxima at 243 nm. Moreover, λ_{max} of "PTC"-2-amino-2-deoxy-D-glucose is *ca.* 30% higher than for all of the relevant PTC model compounds. The u.v. spectrum is therefore inconsistent with a simple PTC group in "PTC"-2-amino-2-deoxy-D-glucose, and, by implication, also with an acyclic PTC aldehyde form.

On the other hand, phenylthioureas having a second substituent on the "anilino" nitrogen atom (including the *N*-phenylimidazolidine-2-thiones) have identical λ_{max}, and the spectral curves are of a very similar shape, to those of the "PTC" compounds.

The reaction of C-1 with the thioureido sulphur atom (*cf.* ref. 11), giving anilinothiazolines with the required λ_{max}, can be excluded, since "PTC"-2-amino-2-deoxy-D-glucose and its analogues are not cationic on electrophoresis in citrate–phosphate buffer at pH 3–7. Anilinothiazolines are basic by virtue of their "isothiouronium" structure.

The interpretation of i.r. spectra of compounds containing the N–C = S system is not completely clear[12]. Nevertheless, in the region 1500–1625 cm^{-1}, there is a strong band which is present in all of the relevant model compounds. The

N-phenyl, symmetrically disubstituted thioureas of Table I, and 18 other symmetrically disubstituted thioureas (not all *N*-phenyl) from the literature (Sadtler spectra) have this band at 1500–1550 cm^{-1}. Since it occurs at 1610 cm^{-1} in the spectrum of "PTC"-2-amino-2-deoxy-D-glucose, the PTC formulation (**1**, R = phenyl) is contra-indicated. 1-Phenylimidazolidine-2-thione, 5-hydroxy-4,4-dimethyl-1-phenylimidazolidine-2-thione and 5-hydroxy-4-methyl-1-phenylimidazolidine-2-thione show the band at 1560–1590 cm^{-1}, and so does 1-naphthylimidazolidine-2-thione. The occurrence of this band at 1560–6125 cm^{-1} in all of the sugar derivatives (naphthyl, phenyl, or *p*-tolyl, both "PTC" and "anhydro-PTC") is compatible with the imidazolidine-2-thione formulation.

U.v. spectra and elemental analyses are incompatible with a phenylimidazole-2-thione (**6**, R = phenyl, Neuberg and Wolf[13]), which lacks a molecule of water, and has a λ_{max} at *ca.* 265 nm (see, *e.g.*, Ref. 9). Since the spectrum is unchanged in borate at pH 8.9, the state of combination of the "PTC" group is unlikely to be affected by borate-complex formation.

In summary, u.v. and i.r. spectral evidence is against a simple PTC formulation, with or without a cyclic sugar. The electrophoretic data are most easily interpreted on the basis of an acyclic sugar. The u.v. spectra suggest that the "anilino" nitrogen atom has acquired a second substituent, *before* contact with the complexing anions. Analyses indicate that no water is lost or gained, compared with the PTC form (**1**). The structure which satisfies these considerations is **4**, R = phenyl.

Structure **4**, a 4(5)-hydroxyimidazolidine-2-thione, is the reduced form of a 2-thiohydantoin. This reduction is easily affected by borohydrides in mild conditions[9] (Scheme 1). The phenylthiohydantoin of 2-amino-2-deoxy-D-gluconic acid (Scheme 1, R′ = D-*arabino*-tetrahydroxybutyl, R^2 = H, R^3 = phenyl), characterised by the method of preparation, ultraviolet spectrum, and mobility in tungstate buffer, was reduced by sodium borohydride in aqueous solution at room temperature to a compound having the spectral and electrophoretic properties of "PTC"-2-amino-2-deoxy-D-glucose. The spectral change on reduction should be compared with the practically identical result of the reduction[9] of 5-methyl-4-phenyl-2-thiohydantoin (Fig. 1). This evidence strongly supports the imidazolidine-2-thione formulation of "PTC"-2-amino-2-deoxy-D-glucose.

It is concluded that **4** (R = phenyl) is the first stable, isolable product of reaction of isothiocyanates with 2-amino-2-deoxyaldoses from aqueous solution. I.r. spectra suggest that **4** is also the structure in the solid state.

Compounds having analyses less by one molecule of water as compared with **4** were investigated by García González and co-workers[14] who, with Morell[3], concluded that these were fused-ring compounds of imidazolidine-2-thione and a pyranose. Recently, a re-investigation[4] by the n.m.r. method has shown that the sugar ring is furanoid (**3**). The compounds derived from **4**, by treatment with dilute acid or organic solvents, are also formulated as 4,5-furanoimidazolidine-2-thiones (**3**), since (*a*) a molecule of water was lost on formation from **4**; (*b*) the u.v. spectrum is identical in detail with that of **4** (R = phenyl); (*c*) the i.r. spectrum band at 1610 cm^{-1}

supports an imidazolidine-2-thione ring formulation; (*d*) the products are *immobile* in tungstate buffer, indicating a change of structure at C-3, C-4, C-5, or C-6; (*e*) the PTC derivative of 2-amino-2-deoxy-D-glucitol does not convert, suggesting that another part of the molecule must be involved in addition to the tetrahydroxybutyl chain; and (*f*) since "PTC"-2-amino-2-deoxy-3-*O*-methyl-D-glucose converts, HO-3 is not involved in the change.

Points (*a*)–(*e*) establish that a molecule of water was lost, not solely from the tetrahydroxybutyl chain, without affecting the imidazolidine-2-thione ring. There remains ring formation involving C-1 of the sugar chain (*i.e.*, HO-5 of the imidazolidine-2-thione). The electrophoretic properties of the "anhydro-PTC" derivatives of 2-amino-2-deoxy-D-glucose, 2-amino-2-deoxy-D-galactose, and 2-amino-2-deoxy-3-*O*-methyl-D-glucose, assuming that a similar reaction has taken place in each case, tend to rule out the pyranoid configuration, since (*a*) the 3-*O*-methyl derivative (R_G 0.27) would not be expected to migrate (*cf.* 2,3-di-*O*-methyl-D-glucopyranose[15], R_G 0.12); (*b*) the mobility of the 2-amino-2-deoxy-D-glucose derivative (R_G 0.65) should be much lower (*cf.* 2-*O*-methyl-D-glucose[15], R_G 0.23; and (*c*) the 2-amino-2-deoxy-D-galactose derivative (R_G 0.21) should have a higher R_G than the 2-amino-2-deoxy-D-glucose derivative (*cf.* 2-acetamido-2-deoxy-D-galactose, R_G 0.35, with 2-acetamido-2-deoxy-D-glucose, R_G 0.20).

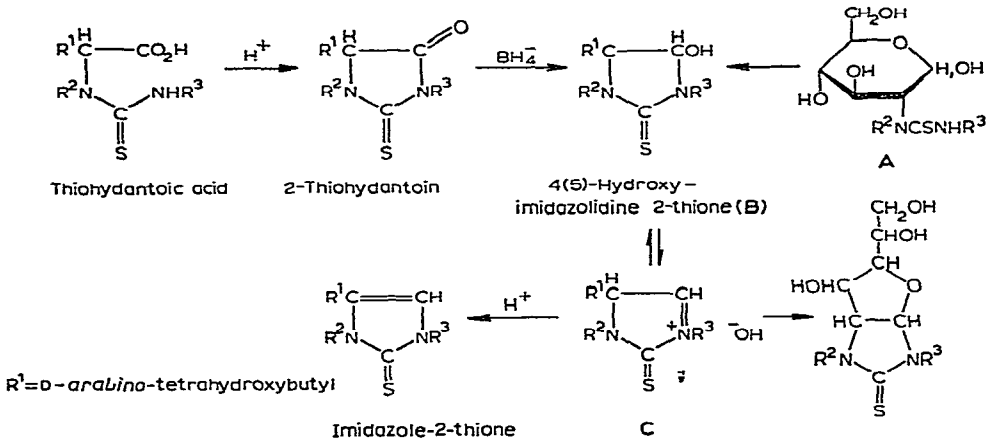
A furanoid structure fits the observed properties. The comparable 1,2-*O*-isopropylidene-α-D-glucofuranose, to which the borate is assumed to be attached at C-3, C-5, and C-6, migrates rapidly[15] (R_G 0.73). Significantly, the "PTC"-3-*O*-methyl derivative, in which this complex could not form, has a low R_G value. Their electrophoretic behaviour in both tungstate and borate buffers is identical (Tables II and III) with thio and oxo analogues (kindly given by Dr. C. Morell) which were shown by n.m.r. to be glucofurano-imidazolidine derivatives (**3**, R = *p*-tolyl). The evidence therefore strongly supports **3** as a typical structure of the acid- or solvent-treated **4**.

The anhydro-compound derived from "PTC"-1-amino-1-deoxy-D-fructose has particular interest as a spiro compound (**7**). It was not isolated. It also is represented as a furano derivative, since, in the pyrano form, there is an available *cis*-glycol group at C-4–C-5 which, since $R_G = 0$ in borate, is unlikely to be present [*cf.* comparable methyl α- or β-D-arabinofuranosides (R_G 0.04), whereas the alternative methyl-D-arabinopyranosides have R_G 0.38 (Ref. 15)].

The anhydro-PTC compounds of this investigation are therefore similar to, if not identical with, those described by Morell. Since C-5 of the hydroxyimidazolidine-2-thione (C-1 of the sugar chain) is asymmetric, there should be two epimers of each compound. It is not possible to state which form has been isolated; in some cases, mixtures of both may have been prepared. This could explain the variability of some melting points.

The reaction of isothiocyanates with 2-amino-2-deoxyaldoses therefore goes through at least 3 stages (Scheme 1). Attempts to demonstrate that PTC-2-amino-2-deoxyaldoses exist in tungstate buffer failed, and the formation of **1** (R = phenyl) is,

therefore, inferential. It is assumed that the conversion **1**→**4** is very rapid. Since neither methyl 2-amino-2-deoxy-α- nor phenyl 2-amino-2-deoxy-β-D-glucopyranosides showed any tendency to change from PTC forms to imidazolidine-2-thiones, it is suggested that the open-chain form is the reaction intermediate.



Scheme 1.

It was pointed out[9] that 4(5)-hydroxyimidazolidine-2-thiones are pseudo-bases, in which three tautomeric forms (an amino-aldehyde, a carbinolamine, and a mesomeric quaternary ammonium cation (Scheme 1) are in equilibrium. Ether-formation, and ether-exchange reactions, in which alkoxy groups are interchanged freely with alcohols, are characteristic of pseudo-bases (see, *e.g.*, ref. 16). The formation of the furano-ring (an internal ether) from **4** in mild conditions is exemplary. It is suggested that ether formation from **4** is a reaction of the quaternary ammonium cation (Scheme 1) derived from the pseudo-bases, proceeding by the mechanism shown (**8**). This mechanism accounts for the pH dependence of the formation of furanose rings, which are formed in weak acid. At low concentrations of OH^-, the equilibrium would be displaced (Scheme 1) from forms **A** and **B** into the mesomeric ammonium cation **C**, and thence into the ether. Alkaline conditions would suppress ionisation of the mesomeric ammonium hydroxide, thus stabilising 4(5)-hydroxyimidazolidine-2-thiones, as found in this investigation.

A second reaction of the ammonium cation is its ready conversion into imidazole-2-thiones by the loss[9] of a proton (which is finally derived from C-4). The ratio of imidazole-2-thione to 4(5)-alkoxyimidazolidine-2-thione obtained must then be decided by the relative rates of proton loss and alkoxy gain. Presumably because of steric factors, HO-2 of the tetrahydroxybutyl chain reacts at the greater rate, so that imidazole-2-thione is formed in small proportions, if at all. This is compared with the reaction of 1-arylamino-1-deoxyfructoses with ammonium thiocyanate to form imidazole-2-thiones[17]. Presumably, this reaction went *via* a thiourea, and an intermediate 4-hydroxyimidazolidine-2-thione[9]. In contrast to the present investigation, the aryl substituent was not at the mesomeric ammonium nitrogen, which instead

carried a proton (Scheme 1; **C**, $R^3 = H$) likely to dissociate at a much greater rate than H-4. Thus, when the pseudo-basic hydroxyl group and the *N* substituent (Scheme 1; $R' =$ tetrahydroxybutyl, $R^3 = H$) are vicinal, cyclic ethers should be formed faster than the deprotonation from C-4 to an imidazole-2-thione, but when the pseudo-basic hydroxyl group is vicinal to an unsubstituted $\succ NH$ ($R^3 = H$), the faster deprotonation from $\gtrless N^+H$ should produce an imidazole-2-thione. In agreement with this hypothesis, the "PTC"-1-amino-1-deoxy-D-fructose derivative (*N*-substituent vicinal to the pseudobasic hydroxyl group), on treatment with acid, produced an anhydro derivative having the electrophoretic behaviour of a cyclic (furanose) compound (**7**, R = phenyl), quite different from the imidazole-2-thione prepared from the corresponding non-vicinal derivative (**6**, R = phenyl), kindly supplied by Dr. Huber (see Tables II and III). It appears likely, therefore, that Neuberg and Wolf[2] prepared the "anhydro-PTC" [*i.e.*, glucofuranoimidazolidine-2-thione (**3**)] derivative, since they started from hexosamine and isothiocyanate. The analyses would be identical with those of the hoped-for imidazole-2-thione. Their melting point (208°) differs considerably from that (179–181°) of the authentic phenylimidazole-2-thione[17] (**6**), but is identical with that of the compound formulated as an imidazolidine-2-thione having λ_{max} 238 nm. Thus, the reaction analogy by which Neuberg and Wolff assigned an acyclic sugar ring and an imidazole-2-thione structure to the 2-amino-2-deoxy-D-glucose–isothiocyanate product was unsound.

The conversion in alcohols of "PTC"-2-amino-2-deoxy-D-glucose into a cyclic ether is interesting, since Morrell's "p-tolyl thiocarbamoyl" compounds survived prolonged treatment in methanolic ammonia without splitting off water. Two explanations are possible. (*1*) In the completely non-aqueous synthesis of Morell's compounds, the aldehydo sugar (which is suggested to be necessary for the conversion of **1** into **4**) was not present. If so, Morell's compounds would be "thiocarbamoyl" derivatives similar to **1**. The i.r. evidence is against this; Morell's "2-deoxy-2-*p*-tolylthiocarbamido-D-glucose" absorbs at the imidazolidine-2-thione frequency of 1560 cm^{-1}. (*2*) It is more likely that the alkalinity of methanolic ammonia suppressed the ionisation of the mesomeric ammonium hydroxide, so that no reactive ammonium cation was formed (by analogy with the situation in water).

It can, nevertheless, be predicted that if true PTC-2-amino-2-deoxyaldoses could be made, they would be converted into hydroxyimidazolidine-2-thiones on contact with any solvent in which mutarotation (and hence the aldehydo form) occurred.

Although this paper has dealt exclusively with thio compounds, the conclusions probably also hold for oxo compounds (*e.g.*, naphthyl isocyanate reacts with 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose to give compounds migrating in molybdate buffer[18]).

Resolution of mixtures of amino sugars. — 2-Amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose are the two naturally occurring amino sugars which it is most commonly necessary to separate and measure. Fortunately, the difference in configuration of the respective tetrahydroxybutyl chains is such that the complex

of the former with tungstate has only half the mobility of the latter. Equally clear separations would be expected of the derivatives of any *threo–erythro* pair of hexosamines, *e.g.*, 2-amino-2-deoxy-D-talose and 2-amino-2-deoxy-D-mannose. On the other hand, electrophoretic separation of PTC-aminodeoxyhexoses differing only at C-2 would not be expected. The resolving power is similar to that of the ninhydrin degradation-to-pentose procedure[19] in which C-1 is removed. However, on further treatment of the "PTC" derivative with dilute acid, the anhydro "PTC" derivative is formed, and pairs previously inseparable in tungstate or borate (*e.g.*, 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-mannose) become easily separable in borate buffer.

Clear distinctions are possible between the "PTC"-2-amino-2-deoxypentoses (which do not migrate) and the 2-amino-2-deoxyhexoses, and, in principle, between 2-amino-2-deoxyglycosides (which would not be expected to migrate) and the parent PTC-2-deoxy-2-aminoglycose. The presence at C-3, C-4, C-5, or C-6 of a substituent at a hydroxyl group would be expected to greatly decrease or eliminate the electrophoretic mobility in tungstate (*cf.* ref. 10).

The combination of the isothiocyanate reaction with electrophoresis of the product is very advantageous in the assay and resolution of mixtures of naturally occurring amino sugars, in speed, convenience, and sensitivity. This aspect will be dealt with in a later paper.

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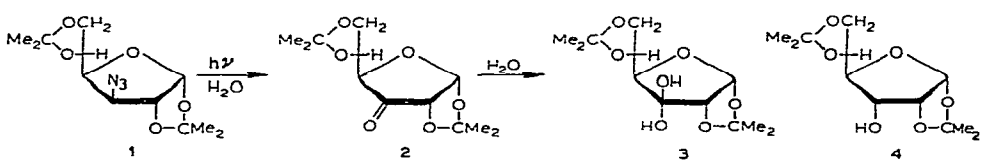
Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

Note

Photolysis of secondary azides of sugars*,†

D. M. CLODE AND D. HORTON**
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210 (U. S. A.)
(Received March 16th, 1970)

The photolysis of primary azides ($R\text{-}CH_2N_3$) in sugar systems leads to non-monomeric products, having elemental compositions corresponding to that of $R\text{-}CH{=}NH$, which, on treatment with water and a trace of acid, give the corresponding aldehydes, R–CHO, in high yield[1–3]. The procedure provides an excellent synthetic route for a wide range of *ω-aldehydo* derivatives of protected and unprotected glycosides[1–3], and for analogous derivatives of such polysaccharides as amylose[4], starch, and cellulose[5]. The reaction presumably takes place by way of an intermediate nitrene ($R\text{-}CH_2N$:) which rearranges by a 1,2-hydrogen migration to give the initial product isolated from the reaction[6–8]. It was considered that a similar process operating with secondary azides might provide a useful route to ketones in the sugar field, allowing preparation of keto derivatives without exposure of the precursor molecule to conventional oxidizing agents. The reaction was evaluated with 3-azido-3-deoxy-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose[9] (**1**), a secondary azide obtainable in high yield from 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose by the sequence: oxidation, reduction, *p*-toluenesulfonylation, and displacement by azide.



Irradiation of a solution of the azide **1** in benzene (or cyclohexane) with unfiltered light from a mercury arc led to decomposition of the starting material and the formation of a syrup that, by t.l.c. on silica gel, contained a nonmigrating component and a fast-moving component. The latter was isolated by column chromatography, and was identified as the anticipated 3-ketone[10] **2**, characterized as its crystalline hydrate, **3**. Presumably, the initial product of photolysis underwent hydrolysis during the exposure to silica gel, to give the ketone **2**. The yield of **2** was

*For preliminary reports, see Refs. 1 and 2.
†Supported, in part, by the Agricultural Research Service, U. S. Department of Agriculture, Grant No. 12-14-100-9201 (71) (O. S. U. R. F. Project 2573), administered by the Northern Utilization Research and Development Division, Peoria, Illinois.
**To whom inquiries should be addressed.

low (15%) and a considerable proportion of the reaction product, apparently polymeric material, remained on the column. As the ketone **2** is somewhat unstable, an experiment was performed in which the product of photolysis of **1** in moist benzene was immediately treated with sodium borohydride, which reduces **2** stereospecifically to 1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose[11] (**4**), a stable compound. The alcohol **4** was isolated crystalline in 18% yield.

Irradiation of methyl 2-azido-4,6-*O*-benzylidene-2-deoxy-α-D-altropyranoside[12] in cyclohexane gave a solid product whose elemental composition indicated an empirical formula of $C_{14}H_{17}NO_5$, corresponding to that of an imino derivative (RR'C=NH), and the i.r. absorption at 6.0 μm indicated the presence of C=N bonds. However, n.m.r. spectra of the product in various solvents were complex and were clearly inconsistent with a monomeric structure. The exact molecular formula was not determined. Very similar results were observed when methyl 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-altropyranoside was irradiated. Irradiation of methyl 2-azido-2-deoxy-α-D-altropyranoside[12] led to a product that had only about half the nitrogen content calculated for the polymeric imine structure; some hydrolysis had evidently occurred, as indicated also by an i.r. absorption at 5.8 μm (carbonyl). These products were not further characterized.

It is evident from the foregoing results that photolysis of secondary azides of sugars does, indeed, lead to carbonyl derivatives, probably by way of intermediate, polymeric imines, but the yield of ketone is low. Although the yields in such conversions as **1**→**2** might be improved somewhat by alteration of the experimental conditions, it is doubtful that this photochemical method will be so attractive a synthetic procedure for keto sugars as is the photolysis of primary azides for the synthesis of aldehydo sugars. Presumably, the 1,2-hydrogen migration, postulated for conversion of a nitrene intermediate into an imine, is a favored process with the primary azides. With the secondary azides, there are probably competing processes, such as δ-hydrogen abstraction[8], that divert a large proportion of the initial intermediate into products that do not give the ketone upon treatment with water.

EXPERIMENTAL

General methods. — Melting points were determined with a Thomas–Hoover "Unimelt" apparatus and are uncorrected. Specific rotations were determined in a 2-dm polarimeter tube. I.r. spectra were recorded with a Perkin–Elmer Model 137 "Infracord" i.r. spectrophotometer. Elemental analyses were performed by W. N. Rond. T.l.c. was conducted on Silica Gel G (E. Merck, Darmstadt, Germany) activated at 110°, with 4:1 dichloromethane–ethyl acetate as developer, and indication was effected with sulfuric acid. Irradiations were conducted at room temperature, under nitrogen, with unfiltered light from a mercury arc[13], and the reactions were monitored by periodic removal of a small sample of the solution and examination for disappearance of the starting material by t.l.c. and i.r. spectroscopy (4.75 μm, N_3).

Photochemical conversion of 3-azido-3-deoxy-1,2:5,6-di-O-isopropylidene-α-D-

glucofuranose (**1**) *into 1,2:5,6-di-*O*-isopropylidene-α-*D*-ribo-hexofuranos-3-ulose* (**2**). — A solution of the azide[9] **1** (528 mg) in benzene (180 ml) was irradiated for 1 h and then evaporated under diminished pressure to a syrup (412 mg). T.l.c. of the product showed a component at the base line and a component that showed migration characteristics indistinguishable from those of the ketone **2**; the starting azide **1** was absent. The product was placed on a column (1 × 20 cm) containing 25 g of silica gel (No. 7734, Merck) and the column was eluted with 9:1 dichloromethane–ethyl acetate. The faster-moving component was eluted in effluent fractions between 175 and 300 ml. Evaporation of these fractions gave a syrup that crystallized from a mixture of water-saturated ether and pentane to give the crystalline hydrate (**3**) of 1,2:5,6-di-*O*-isopropylidene-α-D-*ribo*-hexofuranos-3-ulose; yield 76 mg (15%), m.p. 110–112°, mixed m.p. (with authentic **3**) 110–112°, $[\alpha]_D^{20}$ +44° (*c* 1, chloroform) (lit.[10] m.p. 112–114°, $[\alpha]_D$ +44.5° in chloroform).

Similar results were observed when cyclohexane or *p*-dioxane–water was used as the solvent for photolysis.

In a second experiment, the azide **1** (392 mg) in water-saturated benzene (180 ml) was irradiated as before for 1 h, and the mixture was then evaporated to a syrup. Sodium borohydride (400 mg) was added to a solution of the syrup in 70% aqueous ethanol, and the mixture was stirred for 1 h. Water (40 ml) was then added, and the solution was extracted with six 20-ml portions of ethyl acetate. The extract was dried (magnesium sulfate) and evaporated to a syrup (136 mg) that, by t.l.c., showed a nonmigrating component and a component that migrated as the allose derivative **4**. The product was resolved by chromatography on a column containing 10 g of silica gel (No. 7734, Merck) by elution with 9:1 dichloromethane–ethyl acetate. Fractions (70–130 ml) containing the faster-moving component (by t.l.c.) were evaporated to a solid. Recrystallization from benzene–petroleum ether (b.p. 30–50°) gave 1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose (**4**) (65 mg, 18% from the azide **1**), m.p. 76–77°, mixed m.p. 76–77°, $[\alpha]_D^{20}$ +37.4° (*c* 0.5, water); lit.[11] m.p. 76–77°, $[\alpha]_D^{21}$ +37.7° (water).

*Methyl 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-*D*-altropyranoside.* — Acetylation of methyl 2-azido-2-deoxy-α-D-altropyranoside[12] with acetic anhydride in pyridine in the usual way gave the title compound as a distillable syrup, b.p. 150°/0.2 torr, $[\alpha]_D^{20}$ +60.2° (*c* 1.2, chloroform).

Anal. Calc. for $C_{13}H_{19}N_3O_8$: C, 45.22; H, 5.51; N, 12.17. Found: C, 45.29; H, 5.53; N, 12.10.

*Photolysis of derivatives of methyl 2-azido-2-deoxy-α-*D*-altropyranoside.* — *A.* Methyl 2-azido-4,6-*O*-benzylidene-2-deoxy-α-D-altropyranoside[12] (500 mg) in cyclohexane (180 ml) was irradiated until the starting material disappeared (85 min), and the solution was evaporated. The resultant solid was dissolved in the minimal volume of chloroform, and hexane was added, to precipitate an amorphous solid (295 mg), λ_{max}^{KBr} 6.0 μm (C=N) that did not migrate in t.l.c.

Anal. Calc. for $C_{14}H_{17}NO_5$: C, 60.22; H, 6.09; N, 5.02. Found: C, 59.96; H, 6.13; N, 4.87.

B. Methyl 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-altropyranoside (575 mg) in cyclohexane (180 ml) was irradiated as before. The starting material had all decomposed after 100 min. The product was isolated by the procedure used for *A*, to give an amorphous solid (213 mg), λ_{max}^{KBr} 6.0 (C=N) and 5.7 μm (C=O), that did not migrate in t.l.c. (Found: C, 50.57; H, 6.12; N, 5.18. $C_{13}H_{19}NO_8$ requires C, 49.21; H, 6.00; N, 4.42.)

C. A solution of methyl 2-azido-2-deoxy-α-D-altropyranoside[12] (200 mg) in 1:1 *p*-dioxane–benzene (180 ml) was irradiated until the starting material had all decomposed (105 min). The solution was evaporated to a syrup that was dissolved in ethanol. Hexane was added to precipitate an amorphous solid, yield 94 mg; λ_{max}^{KBr} 5.8 (C=O) and 6.0 μm (C=N). (Found: C, 51.58; H, 6.81; N, 4.64.)

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

Note

Methylation of carbohydrates bearing base-labile substituents, with diazomethane–boron trifluoride etherate
Part IV*. An improved procedure for the synthesis of 6-*O*-methyl-D-mannose

E. G. CROS** AND E. M. GRUÑEIRO

Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Perú 222, Buenos Aires (Argentina)

(Received March 16th, 1970)

In connection with our work on the hydrolysis products from the antibiotic Curamycin[2], a sample of 6-*O*-methyl-D-mannose was needed. This compound had originally been prepared by Hudson *et al.*[3] by methylation of methyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside with Purdie's reagents, followed by hydrolysis. From present knowledge[4], it is obvious that this procedure is unreliable, because of the acetyl migration that must occur in the basic medium used in the methylation. Dutton and Tanaka[5] could not obtain a good yield of 6-*O*-methyl-D-mannose by use of the method, and they correctly assumed that the very poor yield of desired product was due to acetyl migration. The title compound has recently been prepared[6] by direct methylation of methyl α-D-mannopyranoside, but the method does not afford the desired product in good yield.

We now report that, by use of diazomethane–boron trifluoride etherate as the methylating agent, no acetyl migration occurs, and the expected intermediate product is obtained in good yield.

Methyl α-D-mannopyranoside[7] (**1**) was tritylated and then acetylated in one step, to give methyl 2,3,4-tri-*O*-acetyl-6-*O*-trityl-α-D-mannopyranoside (**2**), and this was detritylated, by use of a column of silica gel as the acidic catalyst[8], to methyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (**3**). Compound **3** was then methylated with diazomethane–boron trifluoride etherate as previously described[1], affording methyl 2,3,4-tri-*O*-acetyl-6-*O*-methyl-α-D-mannopyranoside (**4**). Deacetylation of compound **4** with sodium methoxide in methanol yielded methyl 6-*O*-methyl-α-D-mannopyranoside (**5**) which, on acid hydrolysis, gave 6-*O*-methyl-D-mannose (**6**) as a syrup, $[\alpha]_D^{25}$ +34.9 (10 min)→ +32.9° (8 h), having spectral properties in agreement with the structure assigned. The m.p. of the phenylosazone of **6** was in accord with reported values[3,5,6].

*For Part III, see ref. 1.
**Research member of the Consejo Nacional de Investigaciones Científicas y Técnicas.

EXPERIMENTAL

Melting points were determined with a Fisher–Johns apparatus and are uncorrected. I.r. spectra were recorded with a Perkin–Elmer "Infracord" spectrophotometer. N.m.r. spectra were recorded with a Varian A-60 spectrometer for solutions in $CDCl_3$ or D_2O. Microanalyses were performed by A. Bernhardt, W. Germany. Solvents were removed under diminished pressure below 50°.

Methyl 2,3,4-tri-O-acetyl-6-O-trityl-α-D-mannopyranoside (**2**). — A solution of methyl α-D-mannopyranoside[7] (**1**, 3.0 g) and chlorotriphenylmethane (4.65 g) in pyridine (15 ml) was heated for 3 h at 100°, and then kept overnight at room temperature. To the solution, acetic anhydride (12 ml) was added, and the mixture was kept for 24 h at room temperature. It was then poured onto ice–water, and the solid was filtered off, washed with water, and dried. The product was recrystallized from ether–petroleum ether and from ethanol, to give 7.3 g of **2**, m.p. 128–129°, $[\alpha]_D^{26}$ +44.6° (*c* 1.0, chloroform)[3]; its i.r. and n.m.r. spectra were in agreement with the structure assigned.

Methyl 2,3,4-tri-O-acetyl-α-D-mannopyranoside (**3**). — To a column of silica gel (Davison, grade 12, activity I) (100 g) was added a solution of compound **2** (1 g) in benzene (20 ml); the column was developed to its full length with benzene (100 ml), and kept for 24 h at room temperature. The column was then eluted with 1:9 (v/v) ethyl acetate–benzene (600 ml). The eluate was found (by t.l.c.) to contain triphenylmethanol and a trace of the starting material. The column was then eluted with 1:4 (v/v) methanol–ethyl acetate (800 ml). On evaporation of the solvent, the residue obtained crystallized on drying; it was recrystallized from ether–petroleum ether, yielding **3** (428 mg), m.p. 97–98°, $[\alpha]_D^{25}$ +54.9° (*c* 1.1, chloroform)[3]; its i.r. and n.m.r. spectra were in accordance with the structure assigned.

Methyl 2,3,4-tri-O-acetyl-6-O-methyl-α-D-mannopyranoside (**4**). — A solution of compound **3** (1.0 g) in dichloromethane (10 ml) was cooled to 0°; boron trifluoride etherate (0.05 ml) was added, and, while the temperature was kept at 0°, a solution of diazomethane in dichloromethane was added until a yellow color persisted in the solution. After 30 min at 0°, the precipitate of polymethylene was filtered off, and the filtrate was evaporated. The residue failed to crystallize, but it was purified by distillation at 70°/10^{-3} torr; the compound (850 mg) showed only one component by t.l.c., and had $[\alpha]_D^{26}$ +56.8° (*c* 1.2, chloroform); its i.r. spectrum showed no hydroxyl band; n.m.r. data: τ 8.02, 7.97, 7.87 (3-proton singlets, acetyl groups); τ 6.65 (2-proton multiplet, H-6); τ 6.62 (OMe at C-1) and 6.59 (OMe at C-6)[9]; τ 6.28 (broad signal, H-5); τ 5.29 (1-proton doublet, $J_{1,2}$ 1.5 Hz, H-1); and τ 4.85 (3-proton multiplet, H-2, H-3, H-4).

Anal. Calc. for $C_{14}H_{22}O_9$: C, 50.29; H, 6.63. Found: C, 50.51; H, 6.73.

Methyl 6-O-methyl-α-D-mannopyranoside (**5**). — To a solution of compound **4** (700 mg) in methanol (10 ml) was added M methanolic sodium methoxide (1 ml), and the solution was kept for 12 h at room temperature. The base was neutralized with Dowex 50W ion-exchange resin, the suspension was filtered off, and the filtrate was

evaporated. The gummy residue was purified by distillation at 75–80°/10^{-3} torr; the product (260 mg) showed only one component by t.l.c. and had $[\alpha]_D^{25}$ +78.8° (*c* 1.2, chloroform); n.m.r. data: τ 5.20 (1-proton doublet, $J_{1,2}$ 1.5 Hz, H-1); τ 6.56 (OMe at C-1) and 6.58 (OMe at C-6)[9].

Anal. Calc. for $C_8H_{16}O_6$: C, 46.15; H, 7.75. Found: C, 46.06; H, 7.73.

6-O-Methyl-D-mannose (**6**). — A solution of compound **5** (410 mg) in M hydrochloric acid (8 ml) was kept for 2 h at 100°, cooled, and made neutral with Dowex 1 ion-exchange resin; the suspension was filtered, and the filtrate was lyophilized. The gummy residue (342 mg) was homogeneous by t.l.c.; it had $[\alpha]_D^{25}$ +34.9 (10 min)→ +32.9° (8 h) (*c* 1.1, water). N.m.r. data: τ 4.77 (1-proton doublet, $J_{1,2}$ 1.0 Hz, H-1 of the β-D anomer); τ 5.20 (1-proton doublet, $J_{1,2}$ 1.5 Hz, H-1 of the α-D anomer); τ 6.58 (OMe). Its phenylosazone had m.p. 173–174°, undepressed on admixture with an authentic sample; lit.[3,5,6] m.p. 173–174°.

ACKNOWLEDGMENTS

We thank Mr. J. J. Ferrer for the i.r. and n.m.r. spectra, the Universidad de Buenos Aires for a graduate fellowship to E. M. G., and the Consejo Nacional de Investigaciones Científicas y Técnicas for financial support.

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Note

A 1:1 adduct of methyl 4,6-*O*-benzylidene-β-D-glucopyranoside and pyridinium chloride

JACOB LEHRFELD AND JAMES C. GOODWIN

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

(Received March 9th, 1970)

Many complexes have been reported between carbohydrates and both inorganic and organic substances. Berry *et al.*[1] produced a 1:1 adduct between methyl α-D-glucopyranoside and potassium hydrogen carbonate. An extensive list of complexes formed between sugars and inorganic salts has been compiled by Rendleman[2]. Strain[3] isolated a 1:1 adduct of D-glucitol and pyridine. Other complexes between various sugars and amides have been noted[4].

A 1:1 adduct of methyl 4,6-*O*-benzylidene-β-D-glucopyranoside (**1**) and pyridinium chloride (**2**) has now been isolated. It is formed when solutions of **1** and **2** in acetone are mixed and cooled, or when acetyl chloride is added to a solution of **1** in acetone containing some pyridine and the solution is cooled.

The isolated adduct is a white, nonhygroscopic, crystalline solid that decomposes sharply at 154°. This decomposed material was separated into four major components by t.l.c. With a 23:2 butanone and water azeotrope–ethanol solvent system three of the spots coincided with known compounds **1**, methyl β-D-glucopyranoside, and D-glucose. A thin-layer chromatogram of the adduct with a 2:1 (v/v) ethyl acetate–hexane solvent system revealed only **1** and pyridine. Methyl 2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-glucopyranoside was formed when the complex was dissolved in pyridine and an excess of acetyl chloride was added. Elemental analysis of the adduct for C, H, Cl, and N agrees with a 1:1 molecular ratio. The n.m.r. spectrum also clearly shows this 1:1 relationship. When the adduct is dissolved in methyl sulfoxide-d_6, the signal for the phenyl ring protons (a 5-proton multiplet centered at τ 2.62) is separated from the signal of the pyridine-ring protons (a series of 3 multiplets centered at τ 1.96, 1.42, and 1.08). Integrals of the benzene protons and the pyridine protons are equal.

Raymond and Schroeder[5] separated a mixture of methyl α- and β-D-glucopyranoside by selective precipitation of a methyl β-D-glucopyranoside–potassium acetate complex. We found that an equimolar mixture (**3**) of methyl 4,6-*O*-benzylidene-α- and β-D-glucopyranoside in acetone reacted with pyridinium chloride to give a white crystalline precipitate, decomposing at 152°, whose i.r. and n.m.r. spectra were identical with those of the adduct. None of the α-D anomer was present in the pre-

cipitate. This procedure allows selective removal of **1** from the mixture **3** in about 90% yield. No attempt was made to maximize the yield.

EXPERIMENTAL

General. — Ascending t.l.c. was performed on Silica Gel G* (E. Merck Co., Darmstadt, Germany; plates were unactivated). Spots were located by iodine vapors and by charring of plates sprayed with 5% sulfuric acid in ethanol. Solvents for t.l.c. were (*A*) 2:1 (v/v) ethyl acetate–hexane and (*B*) 23:2 (v/v) butanone and water azeotrope–ethanol. Melting points were determined in capillary tubes in a Mel-temp apparatus and are uncorrected. N.m.r. spectra were determined on solutions in methyl sulfoxide-d_6 containing tetramethylsilane as an internal standard and were recorded at 100 MHz on a Varian HA-100 spectrometer. I.r. spectra were recorded from Nujol or Fluorolube mulls with a Perkin–Elmer Model 137 spectrometer.

Formation of the adduct. — *A.* Acetyl chloride (0.15 ml, 2.1 mmoles) was added dropwise to a stirred solution of **1** (0.78 g, 2.8 mmoles) in 30 ml of acetone and 1 ml of pyridine**. The solution was refrigerated for 24 h and then filtered. The precipitate, after drying in a desiccator, weighed 0.61 g and decomposed sharply at 154°.

B. A saturated solution of pyridine hydrochloride in acetone (30 ml) was added to **1** (0.7 g) in 30 ml of acetone. After the solution had been kept in a freezer for 1 h, a precipitate formed, which was filtered and air-dried. It decomposed at 154° and weighed 813 mg.

Anal. Calc. for $C_{19}H_{23}ClNO_6$: C, 57.36; H, 5.84; Cl, 8.91. N, 3.52. Found: C, 57.43; H, 6.15; Cl, 8.96; N, 3.32.

Selective precipitation. — Acetyl chloride (0.35 ml, 4.9 mmoles) was added dropwise to a stirred solution containing **1** (0.79 g) and methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (0.79 g) in 30 ml of acetone and 1 ml of pyridine. The flask was kept overnight in a refrigerator, and the precipitate that formed was filtered and dried in a vacuum desiccator; yield 1.0 g, m.p. 152°. The n.m.r. and i.r. spectra were the same as those of the adduct. None of the α-D anomer could be detected in the precipitate by either t.l.c. or n.m.r. (H-1 for the α anomer gives a doublet centered at τ 5.34).

ACKNOWLEDGMENTS

Gratitude is expressed to Mrs. Clara McGrew, Mrs. Karen Jones, and Mrs. Bonita Heaton for microanalyses and to D. Weisleder for n.m.r. spectra.

*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.

**For generation of pyridinium chloride *in situ* advantage is taken of the fact that an opened bottle of acetone normally contains ~1% water.

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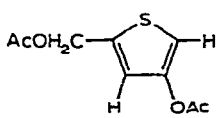
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Note

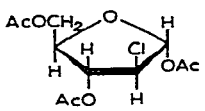
Conversion of 1,3,5-tri-*O*-acetyl-2-chloro-2-deoxy-D-arabinofuranose into a furan derivative

J. KUSZMANN AND P. SOHÁR
Research Institute for Pharmaceutical Chemistry, Budapest (Hungary)
(Received January 19th, 1970)

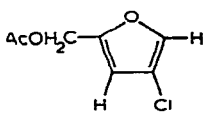
Recently, Whistler and Hoffman[1] described the decomposition of 1,2,3,5-tetra-*O*-acetyl-4-thio-D-ribofuranose to yield the thiophene derivative **1**, and noted that "No comparable reaction is observed for a related oxygen analogue." Some years ago, we synthesised[2] 1,3,5-tri-*O*-acetyl-2-chloro-2-deoxy-D-arabinose **2**, which could be purified by distillation at 135°/0.2 torr. At 0.5 torr, the boiling point rose to 150° and, in addition to compound **2**, a decomposition product **3** and acetic acid were obtained. On the basis of i.r. [ν_{max} 1750 (C=O) and 1605 cm^{-1} (conjugated C=C)] and analytical data, **3** is considered to be 2-acetoxymethyl-4-chlorofuran. This structure was confirmed by the n.m.r. spectrum which was similar to that of the thiophene derivative[1] **1**, in that it contained singlets at τ 7.97 (acetyl methyl) and 5.05 (methylene). The signals for the aromatic protons were separated singlets at τ 3.65 (H-3) and 2.65 (H-5), in contrast to those for compound **1**, which were doublets.



1



2



3

EXPERIMENTAL

General methods. — The i.r. spectrum (liquid film) was recorded with a Zeiss UR-10 (Jena) instrument. The n.m.r. spectrum (CCl_4) was obtained by using a Varian A-60D spectrometer, with tetramethylsilane as the internal standard.

2-Acetoxymethyl-3-chlorofuran (**3**). — The crude 1,3,5-tri-*O*-acetyl-2-chloro-2-deoxy-D-arabinofuranose[2] **2** was distilled at 150°/0.5 torr. The distillate was dissolved in ether, and the solution was washed with 5% aqueous sodium hydrogen carbonate and water, dried (Na_2SO_4), and evaporated. On distillation at 0.2 torr, two fractions, **3** (20%), b.p. 53–54°, and **2** (80%), b.p. 135–137°, were obtained as colourless liquids. Compound **3** could be stored without decomposition only in the refrigerator.

Anal. Calc. for $C_7H_7ClO_3$: C, 48.16; H, 4.04; Cl, 20.31. Found: C, 48.32; H, 4.15; Cl, 20.15%.

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Note

On the formation of malonaldehyde and deoxy compounds by photolysis of aqueous solutions of carbohydrates and related compounds

HEIMO SCHERZ

Institut für Strahlentechnologie, Bundesforschungsanstalt für Lebensmittelfrischhaltung, Karlsruhe (Germany)

(Received October 3rd, 1969; in revised form, January 30th, 1970)

In the u.v.-photolysis of neutral and alkaline, aqueous solutions of carbohydrates, a hitherto unidentified substance with absorption at 265 nm was found[1–3]. By analogy with studies of the gamma-radiolysis of carbohydrates[4, 5], we have shown that this compound is malonaldehyde. Generally, it is produced by the action of shortwave u.v.-radiation upon solutions of vicinal, polyhydroxy compounds having aliphatic or carbohydrate-like structures.

After u.v.-irradiation (185 nm) in the absence of oxygen, 0.5% solutions of D-glucose, D-fructose, D-glucono-1,5-lactone, D-mannitol (0.028M), and sucrose (0.014M) in 0.1M sodium hydroxide showed absorption peaks near 266 nm. The shape of the curves and the peak correspond to those of free malonaldehyde in 0.1M sodium hydroxide. The specific colour reaction with 2-methylindole[6] was strongly positive in each case. Thin-layer chromatography of the dye on Silica Gel G (butyl alcohol–acetic acid–ethyl ether–water, 45:30:15:5) gave R_F values which were identical with those obtained with malonaldehyde.

Fig. 1 shows the dependency of malonaldehyde formation on irradiation time, and the final concentration of malonaldehyde depended on the structure of the individual compound and ranged between 1 and 6 μmoles/ml. Fig. 2 shows the relation between the yield of malonaldehyde and the initial concentration of D-glucose. Up to 0.1 mmole/ml, the amount of malonaldehyde is proportional to the initial concentration.

Irradiation of neutral solutions of the above-mentioned carbohydrates produces only small yields of malondialdehyde. However, its high extinction coefficient[7] (ε_{molar} 30,000) makes it still clearly detectable in the u.v.-spectrum.

Deoxy compounds of the same type as encountered in radiolysis[8, 9] are produced by u.v.-irradiation of neutral, aqueous solutions of these vicinal, polyhydroxy compounds. Paper chromatography of the irradiated solutions, after concentration, showed, in all cases, the presence of more than one deoxy compound; the R_F values are listed in Table I. It is possible that the formation of deoxy compounds occurs by the same reaction mechanism[10, 11] as that described for the irradiation of dilute,

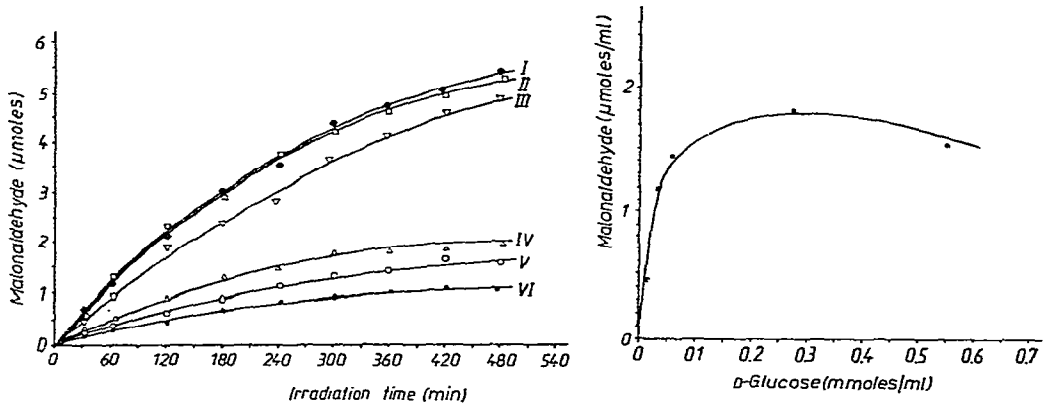
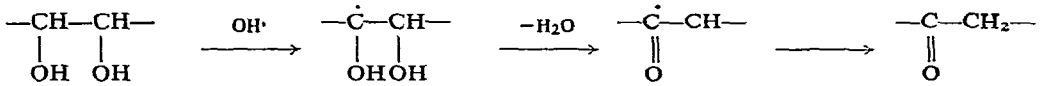


Fig. 1. Dependency of the yield of malonaldehyde (μmoles/ml) on irradiation time (40-watt lamp, 25°). I (◆), D-Mannitol; II (□), D-glucose; III (▽), D-glucono-1,5-lactone; IV (△), sucrose; V (○), D-fructose; VI (●), D-xylose.

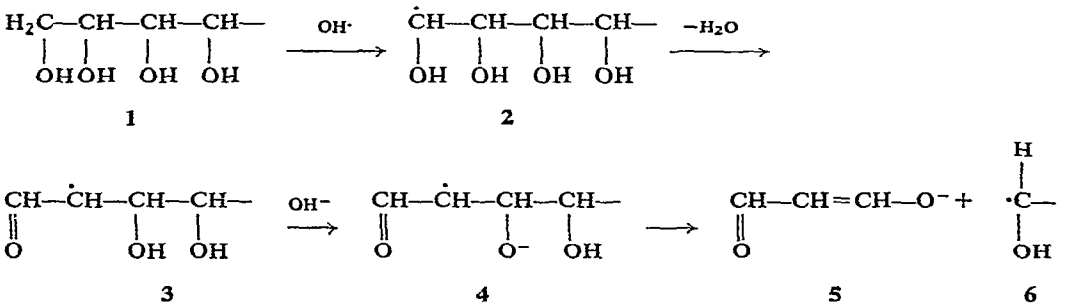
Fig. 2. Dependency on the initial concentrations (mmoles of D-glucose/ml) of the yield of malonaldehyde (μmoles/ml) (40-watt lamp; 25°; irradiation time, 60 min).

aqueous solutions of polyhydroxy compounds with beta and gamma rays (Scheme 1); the $OH^{\cdot}$ radicals were shown to be the reactive species derived from the primary radiolysis and photolysis[1,2] of water.



Scheme 1

The formation of malonaldehyde may be explained by interaction of OH^{-} ions with the deoxyradical, similar to the well-known β-elimination reaction (Scheme 2).



Scheme 2

Compound **1**, having more than three vicinal hydroxyl groups, reacts with an $OH^{\cdot}$ radical to give the radical **2** which is converted into the deoxyradical **3** as in Scheme 1. Under the influence of the planar O=CH–ĊH group, the adjacent OH becomes slightly acid, and it is now proposed that the resulting enolate **4** fragments into the

enolate of malonaldehyde and a further radical **6** which can react further by abstraction or disproportionation.

TABLE I

R_F VALUES OF THE DEOXY COMPOUNDS[a]

D-Glucose: 0.36, 0.44, 0.60.	
D-Fructose: 0.43, 0.49, 0.62.	
D-Xylose: 0.34, 0.40, 0.49, 0.60.	
Sucrose: 0.30, 0.36, 0.43, 0.60.	
D-Mannitol: 0.27, 0.36, 0.45, 0.64.	
D-Glucono-1,5-lactone: 0.36, 0.46, 0.58, 0.66.	

[a]Schleicher Schuell 2043b washed paper, developed with the organic phase of 4:1:5 butyl alcohol–ethanol–water; detection with periodic acid–thiobarbituric acid[14].

EXPERIMENTAL

The irradiation was performed with a low-pressure, mercury, plunger-type lamp (Gräntzel, Karlsruhe; 40 watts), the wall of which was permeable to u.v. light of 185 nm. During exposure, the solutions were maintained at a constant temperature, and agitated and de-oxygenated by vigorous flushing with argon. The qualitative detection and quantitative determination of malonaldehyde was effected by the 2-methylindole reaction[6]. The deep-red colour produced at room temperature with an absorption peak at 555 nm was evaluated photometrically after 20 min.

Deoxy compounds in the irradiated solutions were detected by treatment with periodic acid. Since the deoxy group is located between two –CH–CH– groups or

OH OH

between one such group and a –CHO group, malonaldehyde is produced in this reaction and is detected by the thiobarbituric acid reaction[13].

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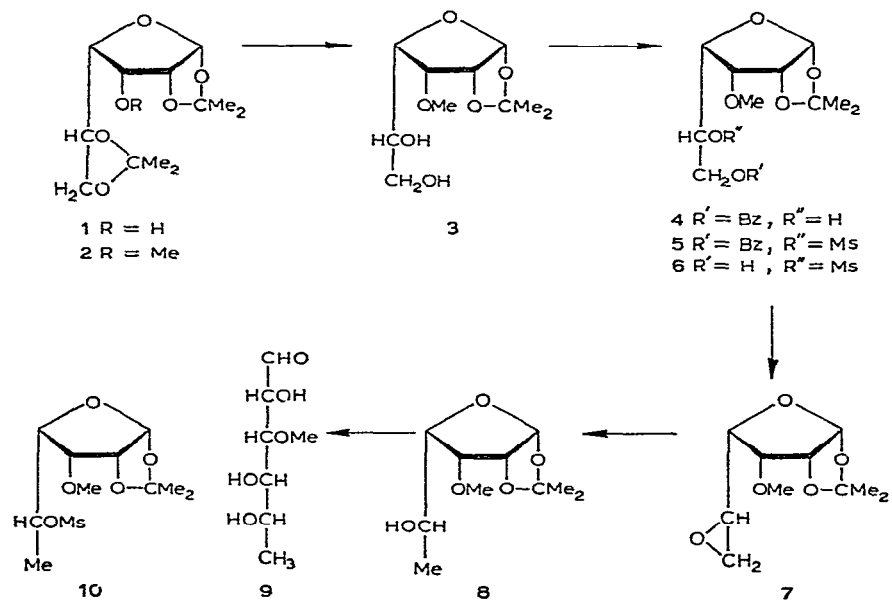
Preliminary communication

A synthesis of L-acofriose (6-deoxy-3-*O*-methyl-L-mannose)

J. S. BRIMACOMBE,
Chemistry Department, The University, Dundee DD1 4HN (Great Britain)
N. ROBINSON, and J. M. WEBBER
Chemistry Department, The University, P.O. Box 363, Birmingham 15 (Great Britain)

(Received June 1st, 1970; accepted for publication, June 8th, 1970)

6-Deoxy-3-*O*-methyl-L-mannose (L-acofriose) (9) is a component of certain cardiac glycosides isolated from such plants as *Acokanthera friesiorum* and *Antiaris toxicaria*, and the sugar has also been obtained from hydrolysates of the wood of *Picea nigra*[1]. There is no direct synthesis of L-acofriose, although it has been obtained, *inter alia*, by methylation and hydrolysis of polysaccharides isolated from the latex of *Cochlospermum gossypium*[2], *Brachychiton diversifolium*[3], and *Khaya grandifolia*[4]. The synthesis of L-acofriose outlined below was based on 1,2:5,6-di-*O*-isopropylidene-α-D-gulofuranose[5] (1), which has become available through recent oxidative procedures[6].



Methylation[7] of the diacetal **1** gave the 3-methyl ether **2** {m.p. 72–73.5°, $[\alpha]_D$ +32.5° (*c* 1.1, chloroform)} which, on partial hydrolysis with 70% acetic acid at room temperature, yielded 1,2-*O*-isopropylidene-3-*O*-methyl-α-D-gulofuranose (**3**) {b.p. 60–65° (bath)/0.1–0.2 mm, $[\alpha]_D$ +37° (*c* 0.7, chloroform)}. Benzoylation and methanesulphonylation, in sequence, of the latter diol gave the 6-benzoate **4** {m.p. 152–153°, $[\alpha]_D$ +25° (*c* 1, chloroform)}, and the 6-benzoate-5-methanesulphonate **5** {m.p. 124–125° (decomp.), $[\alpha]_D$ −5° (*c* 1, chloroform)}.

Treatment of **5** with M sodium methoxide in methanol–chloroform for 30 min at 0° removed the benzoate group to yield 1,2-*O*-isopropylidene-5-*O*-methanesulphonyl-3-*O*-methyl-α-D-gulofuranose (**6**) {m.p. 83–84°, $[\alpha]_D$ −24.5° (*c* 1, chloroform)} which, on further treatment with the base at room temperature for 16 h, was converted into the epoxide **7**, b.p. 60–65° (bath)/15 mm, $[\alpha]_D$ −37.5° (*c* 1, chloroform). Alternatively, **7** could be obtained directly from **5** by treatment with M sodium methoxide in methanol–chloroform for 16 h at room temperature.

Ring-opening of the epoxide with lithium aluminium hydride in ether at room temperature gave 6-deoxy-1,2-*O*-isopropylidene-3-*O*-methyl-β-L-mannofuranose (**8**) {m.p. 51–53° (after sublimation), $[\alpha]_D$ +51° (*c* 1, chloroform)} which afforded L-acofriose (**9**) {m.p. 118–119°, $[\alpha]_D$ +9 (6 min) → +37 ±2° (equil.; *c* 1, water)} on acid hydrolysis. The natural sugar has[8] m.p. 116–118°, $[\alpha]_D$ +39° (equil.; *c* 1, water), and it was chromatographically indistinguishable from the synthetic compound.

An attempt to prepare **8** by a more-direct route involving a benzoate-displacement reaction on 6-deoxy-1,2-*O*-isopropylidene-5-*O*-methanesulphonyl-3-*O*-methyl-α-D-gulofuranose (**10**), m.p. 97–98°, $[\alpha]_D$ −10° (*c* 1, chloroform), was abandoned, since the displacement, performed in boiling *N*,*N*-dimethylformamide, gave two unidentified products in extremely low yield. This necessitated the use of the epoxide route described, in order to achieve the requisite inversion of configuration at C-5 in the D-gulose series.

New compounds gave satisfactory microanalyses, and their n.m.r. spectra were in agreement with the structures assigned.

The authors thank Professor M. Stacey, F.R.S., for his interest, Professor Dr. T. Reichstein for a comparison of the synthetic and natural sugars, and the University of Birmingham for a studentship (to N.R.).

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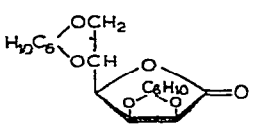
Preliminary communication

The synthesis of an ethyl 2-deoxy-3-octulosonate by the Reformatsky reaction

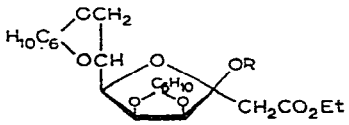
Yu. A. ZHDANOV, Yu. E. ALEXEEV, and Ch. A. KHOURDANOV
Department of Chemistry, Rostov-on-Don State University, Rostov-on-Don (U.S.S.R.)

(Received May 22nd, 1970; accepted for publication, June 22nd, 1970)

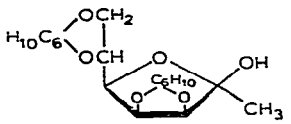
Apparently, carbohydrate lactones have not been used as carbonyl components in the Reformatsky reaction. We now report that 2,3:5,6-di-*O*-cyclohexylidene-D-mannono-1,4-lactone (**1**) reacts with the Reformatsky reagent obtained from ethyl bromoacetate and zinc with the formation of the protected 2-deoxy-3-octulosonic acid ethyl ester **2a**.



1



2a R = H
2b R = Ac



3

Lactone **1** (obtained by oxidation[1] of 2,3:5,6-di-*O*-cyclohexylidene-D-mannofuranose[2]), when heated in dry *p*-dioxane in the presence of a threefold excess of ethyl bromoacetate and zinc dust, gave a 69% yield of ethyl 4,5:7,8-di-*O*-cyclohexylidene-2-deoxy-D-*manno*-3-octulosonate (**2a**) as colourless plates, m.p. 67–68° (from light petroleum), $[\alpha]_D^{18}$ +2° (*c* 5.9, chloroform), ν_{max} 1743 cm^{-1} (strong) (Found: C, 61.72; H, 8.22. $C_{22}H_{34}O_8$ calc.: C, 61.97; H, 7.98%).

The presence of a hydroxyl group in compound **2a** was established by its conversion (acetic anhydride–pyridine) into the syrupy acetate **2b** (47%), $[\alpha]_D^{18}$ +24° (*c* 4.3, chloroform), ν_{max} 1748 and 1738 cm^{-1} (strong doublet) (Found: C, 60.78; H, 7.31. $C_{24}H_{36}O_9$ calc.: C, 61.53; H, 7.37%).

Structure **2a** was finally confirmed by "ketonic hydrolysis". Thus, storage of a mixture containing **2a** and an equimolar amount of potassium hydroxide in aqueous methanol for 2 days at room temperature, followed by acidification, evaporation, and heating at 100°, gave 3,4:6,7-di-*O*-cyclohexylidene-1-deoxy-D-*manno*-heptulose (**3**). Compound **3** (85% yield) had m.p. 106–106.5° (from light petroleum), $[\alpha]_D^{18}$ –29° (*c* 0.9, chloroform), and showed no absorption in the 1800–1500 cm^{-1} region (Found: C, 64.42; H, 8.49. $C_{19}H_{30}O_6$ calc.: C, 64.40; H, 8.47%).

The ester **2a** may be of interest in heterocyclisation reactions analogous to those

of ethyl acetoacetate, and the decarboxylation reaction provides a new route to 1-deoxy-2-ketoses.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

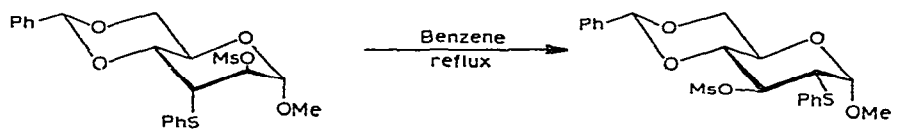
Preliminary communication

The diaxial–diequatorial rearrangement in the carbohydrate series

S. HANESSIAN and A. P. A. STAUB
Department of Chemistry, University of Montreal, Montreal, Quebec (Canada)

(Received July 2nd, 1970; accepted July 28th, 1970)

We report the rearrangement in quantitative yield of methyl 4,6-*O*-benzylidene-2-*O*-(methylsulfonyl)-3-*S*-phenyl-3-thio-α-D-altropyranoside (**1**), into methyl 4,6-*O*-benzylidene-3-*O*-(methylsulfonyl)-2-*S*-phenyl-2-thio-α-D-glucopyranoside (**2**). The transformation is irreversible in benzene and represents a case of diaxial–diequatorial rearrangement involving double transposition and configurational inversion.



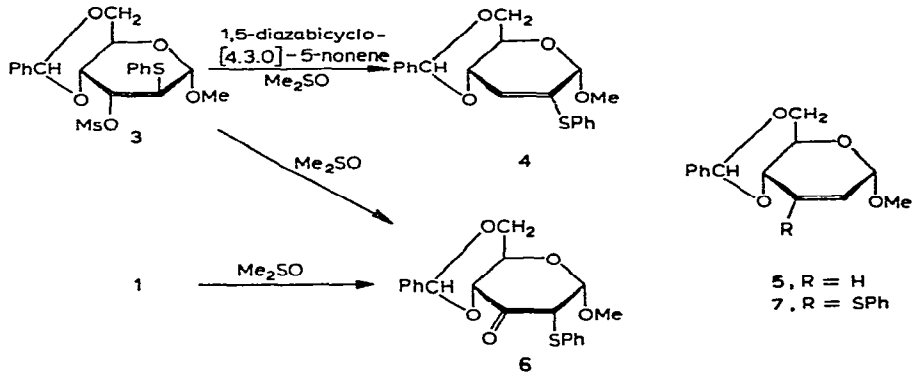
1 **2**

Base-catalyzed elimination reactions[1,2] in the methyl 4,6-*O*-benzylidene-α-D-altropyranoside derivatives **1** and **3** lead to olefinic products, the nature of which is markedly dependent on the solvent used and the disposition of the phenylthio and mesyloxy groups. Whereas **3** affords the 2-phenylthio vinyl ether derivative[1,2] **4** in the presence of 1,5-diazabicyclo[4.3.0]-5-nonene [3] in methyl sulfoxide at 85°, the isomeric **1** gives the product of double elimination **5**. Compound **4** is formed from **3** even in refluxing benzene, thus demonstrating the marked tendency for elimination.

Refluxing a solution containing **1** [m.p. 101–102° $[\alpha]_D^{26}$ −7.10° (*c* 3.4, $CHCl_3$)] in dry benzene for 18 h led to complete conversion into the rearranged product **2**; m.p. 142–143°, $[\alpha]_D^{26}$ −66.4° (*c* 1.78, $CHCl_3$)★. The structure of this product was proved by detailed n.m.r. studies at 100 MHz with appropriate model compounds having the D-*gluco* configuration★★, and by chemical means.

★All crystalline compounds gave acceptable analyses and afforded i.r. and 100-MHz n.m.r. spectra that were in accord with the assigned structures. Melting points are uncorrected. Optical rotations were measured with a Rudolph Model 80 photoelectric polarimeter (Caldwell, N.J., U.S.A.)
★★Model compounds used were methyl 4,6-*O*-benzylidene-2,3-di-*O*-(methylsulfonyl)-α-D-glucopyranoside and the corresponding 2-methanesulfonate.

In the presence of 1,5-diazabicyclo[4.3.0]-5-nonene in Me_2SO at 80° the rearranged product 2 afforded the known[1,2] 2-phenylthio vinyl ether 4 in 72% yield, thus establishing the position of the phenylthio group.



The comparative behavior of 1 and 3 under a variety of conditions led to interesting observations, particularly in Me_2SO. Thus, simulating the conditions of the elimination reaction, but without the addition of a base, compound 1 was heated for 3 h at 80° in Me_2SO. A complex mixture of products* was formed (t.l.c.), from which the highly insoluble 3-ulose derivative 6 was isolated in approximately 10% yield. Oxidation of 3 also afforded 6 in low yield. Although not of preparative significance, these reactions exemplify the relatively facile oxidation of sugar sulfonates having a phenylthio group in the alpha position to give the corresponding uloses, and represent rare examples of such Kornblum types of oxidations[4] in carbohydrate chemistry. Oxidations of this type are under further investigation in this laboratory. The formation of the 3-ulose 6 in the reaction of 1 with Me_2SO necessitates a preliminary rearrangement to 2, followed by oxidation of the latter, or possibly, oxidation of an intermediate species involved in the rearrangement process. To examine this point, 2 was heated in Me_2SO for 20 h at 80°. The ulose 6 was formed in very small amount, but unlike the situation where benzene was the solvent, a considerable proportion (40–50%) of the D-*altro* isomer 1 was formed, indicating a "reversible rearrangement" in Me_2SO. In *N,N*-dimethylformamide and acetonitrile at 80° compound 1 underwent rearrangement (30–40%) and degradation, whereas in nitromethane or ethanol the main reaction was degradation and the formation of benzaldehyde.

Although well documented in the literature[5], the diaxial–diequatorial rearrangement has few precedents involving the phenylthio group[6,7], and none to our knowledge in which the anionic species is a group of low nucleophilicity such as methanesulfonate and the assisting group is phenylthio**. A general mechanism cannot be suggested at this time

*The products were identified by isolation and direct comparison with authentic samples. These consisted of compounds 2, 4, 5, 6, and 7.

**An indirect analogy can be seen in the attempted mesylation of vicinal, *trans*-oriented alkylthio and hydroxyl groups in carbohydrate derivatives. The reaction leads to the corresponding *trans*-alkylthio chlorides *via* intermediate formation of an alkylsulfonium ion followed by attack by the strongest nucleophile in the medium, namely chloride ion. This type of transposition of groups however, is non-selective, except in the case of primary alkylthio ethers, which give the corresponding primary chlorides; see ref. 8.

for the various transformations of the conformationally and configurationally related compounds 1 and 3. Concerning the diaxial–diequatorial rearrangement however, a mechanism involving a dissociated or solvated ion-pair is unlikely in view of the low polarity of the medium. The mechanism is most probably intramolecular, proceeding *via* an intermediate or a transition state in which negligible charge-separation is involved, as in the case of an intimate ion-pair or a cyclic rearrangement[9,10]. Further studies are in progress to gain information relevant to these aspects.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

Book review

Carbohydrate Chemistry — Volume 2; A Specialist Periodical Report: The Chemical Society, London, 1969, ix + 309 pp., $8\frac{1}{2} \times 5\frac{1}{2}$", cloth, £3.10*s* (£2—Fellows).

The second volume in this series of Specialist Periodical Reports of the Chemical Society has been compiled by Dr. R. D. Guthrie (University of Sussex) with the assistance of Dr. R. J. Ferrier (Birkbeck College, University of London) and Drs. M. J. How and P. J. Somers (University of Birmingham), and the Reporters are to be complimented on maintaining the high standard of the first volume.

The organisation of Volume 2 deliberately follows closely that used in Volume 1, in order to aid information retrieval. The major part of the Report (213 pp.) is devoted to mono-, di-, and tri-saccharides, and their derivatives, variously classified in terms of structure and functionality, and physical and analytical methods. The remainder is devoted to macromolecules. As in Volume 1, some 1200 literature references are cited, and the harassed reader of primary journals will thereby hope that a nearly steady state of publication exists in the field of carbohydrate chemistry.

In the review of Volume 1 [*Carbohyd. Res.*, 11 (1969) 163], a plea was made for more-rapid publication of the Specialist Reports on Carbohydrate Chemistry. This was certainly achieved with Volume 2, for which the preface was dated March 1969 and publication was effected in September. It is to be hoped that future volumes will be published as quickly.

The Reporters note in the preface that they have not included work described in *Abstracts of the American Chemical Society Meetings*, *Dissertation Abstracts*, or in the patent literature. Many will disagree with this decision, since data published in these various sources are very much a reality. However, this Reviewer sympathises with the Reporters in what would be a problem of selection from these literature sources.

Chester Beatty Research Institute, London A. B. FOSTER