Carbohydrate Research, 58 (1977) 65–72
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1,5-ANHYDRO-2-DEOXY-3-*O*-(2-DEOXY-β-D-*arabino*-HEXOPYRANOSYL)-D-*arabino*-HEX-1-ENITOL, THE BY-PRODUCT IN THE ENZYMIC HYDRATION OF D-GLUCAL BY β-D-GLUCOSIDASE FROM EMULSIN*

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(Received November 16th, 1976; accepted for publication, February 7th, 1977)

ABSTRACT

The by-product (**3**) in the hydration of D-glucal (**1**) catalyzed by emulsin β-D-glucosidase has been identified as 1,5-anhydro-2-deoxy-3-*O*-(2-deoxy-β-D-*arabino*-hexopyranosyl)-D-*arabino*-hex-1-enitol. Two models for the formation of **3** are discussed, involving transfer of a 2-deoxy-D-*arabino*-hexopyranosyl cation to HO-3 of D-glucal (glycon transfer) and transfer of an allylic D-pseudoglucal cation to HO-1 of 2-deoxy-D-*arabino*-hexopyranose (aglycon transfer). The enzymic production of **3** is highly regiospecific, which lends support to the second model and implies the presence of a specific binding-site for the aglycon moiety.

INTRODUCTION

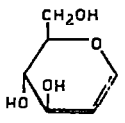
The conversion of D-glucal (**1**) and D-galactal into the corresponding 2-deoxy-D-hexoses by β-D-glucosidase and β-D-galactosidase, respectively, has been reported[1]. Also, **1** is hydrated by an α-D-glucosidase from *Candida tropicalis*[2]. The enzymic conversion of **1** by β-D-glucosidase yielded a by-product (**3**), in appreciable quantity, which was thought to be a rearrangement product, as its mobility in p.c. and t.l.c. was intermediate between those of **1** and 2-deoxy-D-*arabino*-hexose (**2**)[1]. We now report the identification of the by-product and comment on the relevance of its formation to the elucidation of the action of the enzyme.

RESULTS

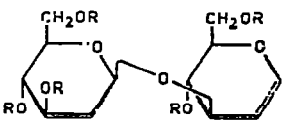
When **1** was incubated with β-D-glucosidase in 0.2M sodium phosphate buffer (pH 6.8), **3** was formed (up to 50%) in addition to **2**. The yield of **3** (which was determined[1] by scanning paper chromatograms of incubation mixtures containing ^{14}C-labelled **1**) depended on the concentration of **1**. In very dilute incubation mixtures

*Uncommon Results of Glycosidase Action, Part III. For Part II, see M. Brockhaus and J. Lehmann, *Carbohydr. Res.*, 53 (1977) 21–31.

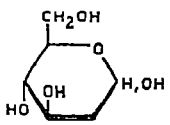
only **2** could be detected. These data indicated **3** to be a product of more than one molecule of **1**. Extraction of the freeze-dried incubation mixture, followed by chromatography of the extract, yielded crystalline **3** which possessed i.r. absorption indicative of a double bond. The n.m.r. spectrum of **3** was similar to a combined spectrum of **1** and **2**, and indicated a dimer formed from two molecules of **1** by the addition of a hydroxyl group of one molecule across the double bond of the other to give a glycosidic link. The presence of a glycosidic link in **3** was confirmed when mild hydrolysis gave **2** as the only detectable product. Moreover, the n.m.r. spectra of **3** and its acetate **4** indicated the β-anomeric configuration[3] and ruled out a glycosidic link to the hydroxymethyl group. Two possible structures fit these data, namely 1,5-anhydro-2-deoxy-3-*O*-(2-deoxy-β-D-*arabino*-hexopyranosyl)-D-*arabino*-hex-1-enitol (**3**) and the (1→4)-linked isomer **5**.



1



3 R = H
4 R = Ac



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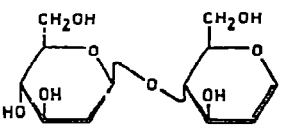
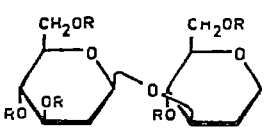
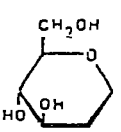


5

Catalytic hydrogenation of **3** gave one product (**6**), which could be hydrolysed by weak acid to yield two products having the same chromatographic behaviour as **2** and 1,5-anhydro-2-deoxy-D-*arabino*-hexitol (dihydro-D-glucal, **8**), respectively. Methylation of **6** with ^{14}C-methyl iodide and subsequent methanolysis gave two



6 R = H
7 R = CH_3



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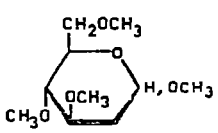

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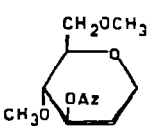

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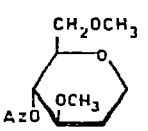

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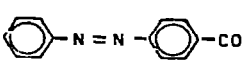
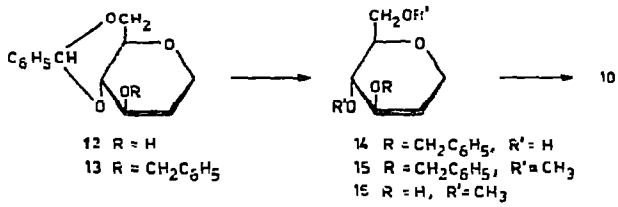
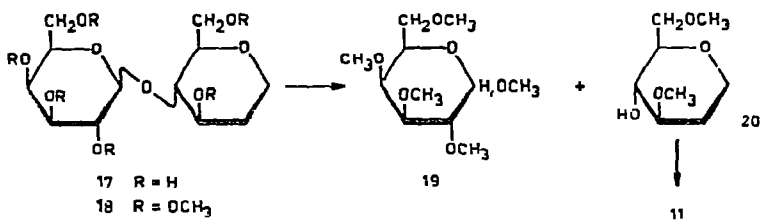
Az = 

products (t.l.c.). The faster moving was methyl 2-deoxy-3,4,6-tri-*O*-methyl-D-*arabino*-hexopyranoside (**9**), which was identified by acid hydrolysis, and by co-crystallization of the resulting 2-deoxy 3,4,6-tri-*O*-methyl-D-*arabino*-hexose with an authentic sample[10]. The slower moving compound reacted with 4-phenylazobenzoyl chloride in pyridine to yield a product (**10**) which was identical with 1,5-anhydro-2-deoxy-4,6-di-*O*-methyl-3-*O*-(4-phenylazobenzoyl)-D-*arabino*-hexitol, but not with 1,5-anhydro-2-deoxy-3,6-di-*O*-methyl-4-*O*-(4-phenylazobenzoyl)-D-*arabino*-hexitol (**11**).

Compound **10** was synthesized from 1,5-anhydro-4,6-*O*-benzylidene-2-deoxy-D-*arabino*-hexitol[4] (**12**), and **11** from 1,5-anhydro-2-deoxy-4-*O*-β-D-galactopyranosyl-D-*arabino*-hexitol[5] (**17**), as shown in the annexed schemes.



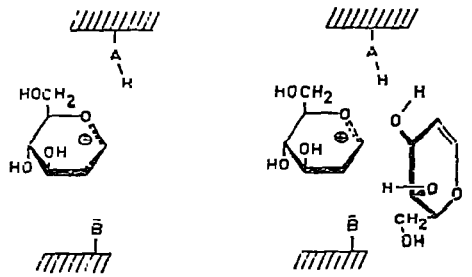


DISCUSSION

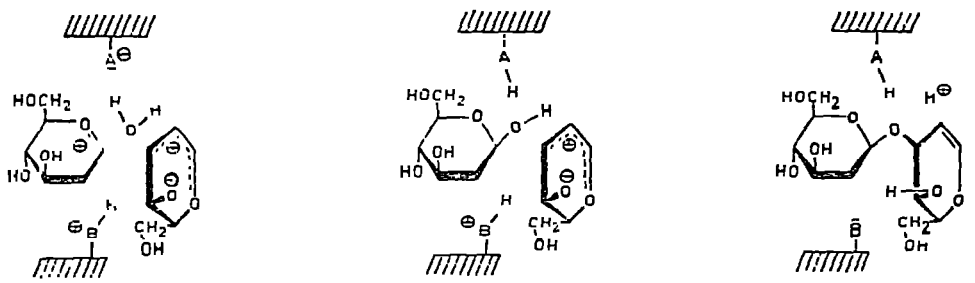
The formation of **3** is remarkable because of three facts: (*1*) D-glucal (**1**) is a much better acceptor than glycerol[6]; (*2*) the 2-deoxy-D-*arabino*-hexopyranosyl moiety becomes attached to position 3 of **1**, although the primary hydroxyl group, to which transfer normally takes place[7], is available; (*3*) there is no formation of ^{14}C-labelled glycoside when D-galactal-^{14}C is added to the incubation mixture. These data imply that a *trans*-diol grouping adjacent to a C=C bond is a good acceptor when a glycal is the substrate of the corresponding β-D-glycosidase.

Polyhydroxy compounds are well known to be good acceptors in transfer reactions catalysed by β-D-glucosidase and β-D-galactosidase. Moreover, the occurrence of acceptor specificity indicates that a polyhydroxy compound can bind to the acceptor site in several energetically different ways, thus producing variously linked *O*-glycosides[7]. The acceptor site of β-D-galactosidase binds prochiral acceptors such as glycerol stereospecifically[8]. Thus, a preferred transition state may be visualised for the transfer step leading to **3**.

It is probable that D-glucal (**1**) is fixed in a flattened conformation[1] (*E* or *H*), thus optimising resonance in the substrate as well as in the ionized substrate (**21**) activated by protonation. For steric reasons, the protonation cannot be effected by the functional group A–H on the enzyme which normally protonates the glycosidic oxygen[9]. In order to obtain **3**, the second D-glucal molecule (acceptor) must be fixed in a way to allow HO-3, which is allylic, to react with the glycosyl cation. This necessarily brings HO-3 in close contact with the proton donor A–H (**22**). The transfer step in **22** must be kinetically preferred and the reason for the comparatively low energy of activation could lie in the charge distribution indicated in **23–25**. If the stage shown in **24** is essential for the regioselective formation of **3**, the synthesis is a transfer of the allylic cation to the glycon rather than a 2-deoxyglucosyl transfer to an acceptor (aglycon). This possibility changes the usual role of the hydroxy compound as the acceptor, and it is more reasonable to call this molecule the aglyconic substrate.



21 22



23 24 25

If the preceding assumptions are correct, then it is necessary for the binding site of the aglyconic substrate of β-D-glucosidase to have at least two functional groups (A–H and B) situated on either side of the aglyconic substrate (see **23**). This arrangement ensures a conformation of the aglyconic substrate (D-glucal) in which HO-3 and HO-4 are *anti*.

In its protonated form, A–H would be the functional group which normally protonates the glycosidic oxygen[9], and in its deprotonated form accepts the proton

from a hydroxyl group of the acceptor molecule. D-Glucal is the first example of an acceptor molecule (aglyconic substrate) which perhaps, like the glyconic substrate, can be activated by a glycosidase through protonation.

EXPERIMENTAL

General methods. — T.l.c. was performed on silica gel F_{254} (Merck) with *A*, 4:1 benzene–methanol; *B*, 4:1 ether–light petroleum (b.p. 60–70°); or *C*, 25:14:7 ethyl acetate–2-propanol–water. P.c. was performed on Whatman No. 1 paper with 6:4:3 1-butanol–pyridine–water. Detection of unlabelled compounds was effected with aniline hydrogen phthalate or by charring with conc. sulphuric acid at 150°. Radioactive materials were detected autoradiographically (Kodak Medical Film, blue sensitive, single coated) or with a Packard 7200 Radiochromatogram scanner. Melting points are uncorrected. I.r. and n.m.r. data were obtained with Perkin–Elmer Infracord 137 and Varian A-60, HA-100, or Bruker 90 spectrometers. Where necessary, spin decoupling was performed.

*1,5-Anhydro-2-deoxy-3-O-(2-deoxy-β-*D*-arabino-hexopyranosyl)-*D*-arabino-hex-1-enitol* (**3**). — A solution of D-glucal (**1**, 6 g) in sodium phosphate buffer (pH 6.8, 50 ml) was incubated with β-D-glucosidase (5 mg; Boehringer, Mannheim) at 35° for 6 days. The reaction was monitored by t.l.c. (solvent *C*). The incubation mixture was diluted with water (300 ml), heated at 95° for 5 min, and then filtered and freeze-dried. The residue was extracted with acetone (3 × 100 ml), the extracts were concentrated *in vacuo*, and the syrupy residue was eluted from a column (150 × 2.5 cm) of silica gel (70–230 mesh, Merck) with solvent *C*. Fractions containing **3** were subjected to rechromatography to give pure material (520 mg), m.p. 170° (from methanol), $[\alpha]_{578}^{22}$ −57°, ν_{max}^{KBr} 3320 (OH) and 1660 cm^{-1} (C=C). N.m.r. data (100 MHz, D_2O): δ 1.76 (m, 1 H, $J_{1,2a}$ 9.5, $J_{2a,2e}$ 12, $J_{2a,3}$ 12 Hz, H-2a of glycon), 2.28 (m, 1 H, $J_{1,2e}$ 2, $J_{2e,3}$ 5 Hz, H-2e of glycon), 3.2–5.0 (m, 12 H), 6.48 (q, 1 H, $J_{1,2}$ 7, $J_{1,3}$ 2 Hz, H-1 of aglycon).

Anal. Calc. for $C_{12}H_{20}O_8$: C, 49.31; H, 6.90. Found: C, 49.43; H, 6.89.

The syrupy penta-acetate (**4**) of **3**, prepared conventionally by using pyridine–acetic anhydride, was homogeneous in t.l.c. (solvent *B*), and had the following n.m.r. data (90 MHz, $CDCl_3$): δ 1.77 (m, 1 H, $J_{1,2a}$ 10, $J_{2a,2e}$ 12, $J_{2a,3}$ 11 Hz, H-2a of glycon), 2.02 (s, 3 H, AcO), 2.03 (s, 3 H, AcO), 2.08 (s, 6 H, 2 AcO), 2.09 (s, 3 H, AcO), 2.28 (m, 1 H, $J_{1,2e}$ 2, $J_{2e,3}$ 5 Hz, H-2e of glycon), 3.45–5.45 (m, 12 H), 6.44 (1 H, $J_{1,2}$ 6, $J_{1,3}$ 1.5 Hz, H-1 of aglycon).

*Methylation analysis of 1,5-anhydro-2-deoxy-3-O-(2-deoxy-β-*D*-arabino-hexopyranosyl)-*D*-arabino-hexitol.* — Conventional hydrogenation of a solution of **3** (40 mg) in water (2 ml) over Pt (from 20 mg of PtO_2) at room temperature and atmospheric pressure was complete (t.l.c., solvent *C*) within 20 h. The freeze-dried product **6** was methylated without further purification.

A solution of **6** (40 mg) in *N*,*N*-dimethylformamide (1 ml) was added to an ice-cold suspension of sodium hydride (300 mg) in *N*,*N*-dimethylformamide (4 ml). The

mixture was stirred vigorously under nitrogen for 30 min and then cooled with liquid nitrogen, and ^{14}C-methyl iodide (55 mCi, 258 μg, The Radiochemical Centre, Amersham) was condensed into the reaction flask. The mixture was allowed to thaw, stirred for 1 h, treated with unlabelled methyl iodide (100 μl), and stirred for 10 h. Unreacted sodium hydride was decomposed with methanol, and the mixture was concentrated to dryness under reduced pressure, and partitioned between water (10 ml) and chloroform (10 ml). The aqueous phase was extracted with chloroform (3 × 10 ml), and the combined chloroform solutions were washed with water (20 ml), dried ($CaSO_4$), and concentrated under reduced pressure to yield **7** as a pale-yellow syrup (48 mg).

The foregoing product **7** (2 mg) was treated with 2% methanolic hydrogen chloride (200 μl) at room temperature for 8 h. One drop of methanolic ammonia was then added, the solvent was evaporated, and the product was subjected to preparative t.l.c. (solvent *C*). The compound having lower mobility (R_F 0.62) was added to a sample of 1,5-anhydro-2-deoxy-4,6-di-*O*-methyl-D-*arabino*-hexitol (**16**), and the mixture was 4-phenylazobenzoylated as described below.

The faster moving compound (R_F 0.78) was slowly stirred with Amberlite IR-120(H^+) resin in water (2 ml) for 5 h. The resin was removed, authentic 2-deoxy-3,4,6-tri-*O*-methyl-D-*arabino*-hexose[10] (500 mg) was added, and the mixture was concentrated to dryness *in vacuo*. After recrystallization from ether, the specific radioactivity of the product was constant.

1,5-Anhydro-3-O-benzyl-4,6-O-benzylidene-2-deoxy-D-arabino-*hexitol* (**13**). — A mixture of 1,5-anhydro-4,6-*O*-benzylidene-2-deoxy-D-*arabino*-hexitol[4] (**12**, 1.5 g), *N*,*N*-dimethylformamide (15 ml), benzyl bromide (6 ml), and silver oxide (7 g) was stirred vigorously at room temperature for 7 h. After the addition of benzene (50 ml), inorganic material was collected, and washed with benzene (50 ml) and then chloroform (50 ml). The combined filtrates and washings were washed with water (3 × 50 ml), dried ($CaSO_4$), and concentrated to an oil which still contained benzyl bromide. On treatment with light petroleum (b.p. 60–70°) at −20°, the product crystallized, and recrystallization from ether–light petroleum (b.p. 30–50°) gave **13** (1.3 g, 63%), m.p. 87°, $[\alpha]_{578}^{22}$ −6° (*c* 1, chloroform). N.m.r. data (60 MHz, $CDCl_3$): δ 1.90–2.15 (m, 2 H, H-2a,2e), 4.86 (d, 2 H, *J* 2.5 Hz, PhCH_2), and 5.70 (s, 1 H, PhC*H*).

Anal. Calc. for $C_{20}H_{22}O_4$: C, 73.60; H, 6.79. Found: C, 73.47; H, 6.82.

1,5-Anhydro-3-O-benzyl-2-deoxy-D-arabino-*hexitol* (**14**). — A solution of **13** (0.9 g) in 50% acetic acid (25 ml) was boiled under reflux for 1.5 h, and then concentrated under reduced pressure. The residue was crystallized from ether and light petroleum (b.p. 30–50°) at −20° to give **13** as the hydrate (0.55 g, 83%). After drying *in vacuo* over phosphorus pentaoxide for 16 h, **13** had m.p. 54°, $[\alpha]_{578}^{22}$ −43° (*c* 1, chloroform). N.m.r. data (60 MHz, $CDCl_3$): δ 1.9–2.1 (m, 2 H, H-2a,2e), 4.70 (d, 2 H, PhCH_2), and 7.45 (s, 5 H, Ph).

Anal. Calc. for $C_{13}H_{18}O_4$: C, 65.52; H, 7.61. Found: C, 65.43; H, 7.46.

1,5-Anhydro-3-O-benzyl-2-deoxy-4,6-di-O-methyl-D-arabino-*hexitol* (**15**). — A mixture of **14** (0.5 g), *N*,*N*-dimethylformamide (10 ml), methyl iodide (5 ml), and

silver oxide (3 g) was stirred vigorously for 5 h. Ether (50 ml) was then added, and inorganic material was collected, and washed thoroughly with ether. The combined filtrate and washings were concentrated at 14 mmHg, and the residue was distilled at 140°(bath)/0.005 mmHg to yield **15** as a colourless syrup (480 mg, 79%), $[\alpha]_{578}^{22}$ +9° (*c* 1.04, chloroform). N.m.r. data (60 MHz, $CDCl_3$): δ 1.6–2.1 (m, 2 H, H-2a,2e), 3.45 and 3.63 (2 s, 6 H, 2 OMe), and 4.73 (s, 2 H, PhCH_2).

Anal. Calc. for $C_{15}H_{22}O_4$: C, 67.70; H, 8.33. Found: C, 67.76; H, 8.33.

1,5-Anhydro-2-deoxy-4,6-di-O-methyl-3-O-(4-phenylazobenzoyl)-D-arabino-*hexitol* (**10**). — To a solution of **15** (532 mg) in dry benzene (25 ml), bromine (320 mg) was added, followed by irradiation with a commercial 60-VA lamp for 7 h at 20–25°. Treatment with bromine and irradiation was repeated after successive intervals of 3 h and 2 h. The mixture was then diluted with chloroform (100 ml), shaken with saturated, aqueous sodium hydrogen carbonate (2 × 50 ml), dried ($CaSO_4$), and concentrated at 50°(bath)/13 mmHg. The oily residue (**16**, 350 mg) was homogeneous in t.l.c. (solvent *C*).

A solution of **16** (350 mg) and 4-phenylazobenzoyl chloride (750 mg) in dry pyridine (10 ml) was boiled under reflux for 2 h. Water (2 ml) was added, and the mixture was kept at 100° for 20 min, cooled, poured into saturated, aqueous sodium hydrogencarbonate (200 ml), and extracted with dichloromethane (3 × 100 ml). The combined extracts were dried ($CaCl_2$), added to a short column (30 ml) of aluminium oxide, and eluted with dichloromethane. Concentration of the eluate and recrystallization of the product (1.18 g, 76%) from ether gave **10**, m.p. 95°, ν_{max}^{KBr} 1710 (C=O) and 1595 cm^{-1} (N=N). N.m.r. data (100 MHz, $CDCl_3$): δ 1.86 and 2.25 (2 m, 2 H, H-2,2'), 3.44 and 3.51 (2 s, 6 H, 2 Me), 3.67 (d, 2 H, H-6,6'), 3.45–3.80 (m, 3 H, H-1a,4,5), 4.04 (m, 1 H, H-1e), 5.16 (m, 1 H, H-3), and 7.48–8.28 (m, 9 H, aromatic protons).

Anal. Calc. for $C_{21}H_{24}N_2O_5$: C, 65.60; H, 6.29; N, 7.29. Found: C, 65.55; H, 6.32; N, 7.53.

When **16** was mixed with the slower migrating product of methanolysis of **7** before starting the azoylation, **10** could be recrystallized to constant specific radioactivity.

*1,5-Anhydro-2-deoxy-3,6-di-O-methyl-4-O-(2,3,4,6-tetra-O-methyl-β-*D-*galacto-pyranosyl)*-D-arabino-*hexitol* (**18**). — A solution of dihydrolactal[5] (**17**, 2 g) in *N,N*-dimethylformamide (30 ml) was added to a suspension of sodium hydride (3 g) in *N,N*-dimethylformamide (60 ml). The mixture was stirred in an ice bath under nitrogen for 30 min, and methyl iodide (15 g) was added. The mixture was allowed to attain room temperature and stirring was continued for 48 h. After decomposition of excess of sodium hydride with methanol, the mixture was concentrated under reduced pressure, chloroform (100 ml) was added, and the suspension was shaken vigorously before being washed with water (2 × 50 ml). The dried ($CaSO_4$) organic layer was concentrated, and the colourless residue (2.8 g) was distilled at 180°(bath)/0.005 mmHg, to give **18**, $[\alpha]_{578}^{22}$ +4° (*c* 0.75, chloroform).

Anal. Calc. for $C_{18}H_{34}O_9$: C, 54.81; H, 8.69. Found: C, 54.87; H, 8.67.

Methanolysis of 1,5-anhydro-2-deoxy-3,6-di-O-methyl-4-O-(2,3,4,6-tetra-O-methyl-β-D-galactopyranosyl)-D-arabino-hexitol (**18**). — A solution of **18** (0.6 g) in 2% methanolic hydrochloric acid was boiled under reflux for 24 h, and then concentrated to 2 ml and co-concentrated with pyridine (2 × 20 ml) *in vacuo*. Two products (**19** and **20**) could be detected by t.l.c. (solvent *C*).

A solution of the foregoing mixture and 4-phenylazobenzoyl chloride (750 mg) in dry pyridine (10 ml) was boiled under reflux for 7 h. Water (1 ml) was added, and the mixture was kept at 100° for 30 min, cooled, and poured into saturated, aqueous sodium hydrogen carbonate (400 ml). The solution was extracted with chloroform (3 × 50 ml), and the combined extracts were washed with water (3 × 100 ml), dried ($CaCl_2$), added to a short column (30 ml) of aluminium oxide, and eluted with chloroform. Concentration of the eluate gave a syrup containing (t.l.c., solvent *A*) **19** (colourless) and **11** (orange coloured). Trituration of the mixture with 1:1 methanol–water at 4° and recrystallization of the product from light petroleum (b.p. 30–60°) gave 1,5-anhydro-2-deoxy-3,6-di-*O*-methyl-4-*O*-(4-phenylazobenzoyl)-D-*arabino*-hexitol (**11**, 1.10 g), m.p. 89°, ν_{max}^{KBr} 1710 (C=O) and 1595 cm^{-1} (N=N). N.m.r. data (100 MHz, $CDCl_3$): δ 1.74 and 2.18 (2 m, 2 H, H-2,2'), 3.34 and 3.37 (2 s, 6 H, 2 Me), 3.50 (s, 2 H, H-6,6'), 3.17–3.37 (m, 3 H, H-1a,3,5), 4.12 (m, 1 H, H-1e), 5.10 (t, 1 H, H-4), and 7.48–8.28 (m, 9 H, aromatic protons).

Anal. Calc. for $C_{21}H_{24}N_2O_5$: C, 65.60; H, 6.29; N, 7.29. Found: C, 65.49; H, 6.35; N, 7.42.

ACKNOWLEDGMENTS

The authors thank Dr. E. Stengele, Institut für Makromolekulare Chemie der Universität Freiburg, for spin-decoupling experiments, and the Deutsche Forschungsgemeinschaft for financial support.

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