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Note

G.l.c.–m.s. analysis of methylated 2-acetamido-2-deoxy-D-glucitol phosphates*

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2-Amino-2-deoxy-D-glucose phosphates are characteristic components of lipopolysaccharides (LPS, endotoxins), the lipid A backbone of which is represented by a bis-phosphorylated 2-amino-6-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucopyranose [β-Glc*p*N-(1→6)-Glc*p*N]. Chemical and enzymic analysis[1], g.l.c.–m.s.[2], ^{1}H- and ^{31}P-n.m.r. spectroscopy[3–5], and f.a.b.-m.s.[6] have been used in order to determine the positions of the phosphate residues of the lipid A backbone. The reducing (GlcN I) and non-reducing (GlcN II) moieties are phosphorylated at positions 1 and 4′, respectively. Unless certain modifications[2] are applied, methylation analysis has not given information on the position of the lipid A phosphates.

We now report on the chemical behaviour of the 3-, 4-, and 6-phosphates of Glc*p*NAc and the 5-phosphate of the GlcNAc-ol during methylation analysis.

The hexosamines (or hexosamine phosphates) were *N*-acetylated[7], reduced, and methylated by the Hakomori procedure[8] and by the modification described by Ciucanu and Kerek[9]. The hexosamine phosphates could be permethylated by the NaOH–Me_2SO–MeI procedure[9] only if the free acid forms (**2**, **6**, and **7**) were first transformed into the dimethyl phosphate derivatives with diazomethane.

GlcNAc 6-P (**7**) gave the expected 2-deoxy-1,3,4,5-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol 6-(dimethyl phosphate) (**10**) in low yields (2–5%)

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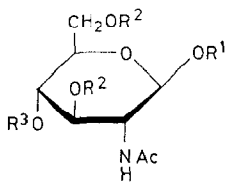
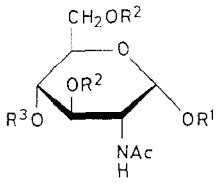


1 $R^1 = CH_2CH{=}CH_2$, $R^2 = Bn$, $R^3 = PO(OPh)_2$

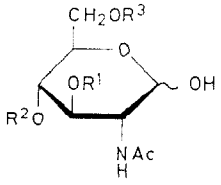
2 $R^1 = Pr$, $R^2 = H$, $R^3 = PO(OH)_2$

3 $R^1 = CH_2CH{=}CH_2$, $R^2 = Bn$, $R^3 = H$



4 $R^1 = R^2 = Bn$, $R^3 = H$

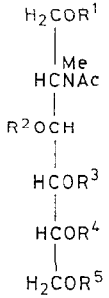
5 $R^1 = R^2 = Bn$, $R^3 = PO(OPh)_2$



6 $R^1 = R^3 = H$, $R^2 = PO(OH)_2$

7 $R^1 = R^2 = H$, $R^3 = PO(OH)_2$

8 $R^1 = PO(OH)_2$, $R^2 = R^3 = H$



9 $R^1 = R^2 = R^3 = R^4 = R^5 = Me$

10 $R^1 = R^2 = R^3 = R^4 = Me$, $R^5 = PO(OMe)_2$

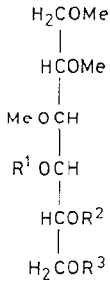
11 $R^1 = R^2 = R^3 = R^5 = Me$, $R^4 = PO(OMe)_2$

12 $R^1 = R^2 = R^4 = R^5 = Me$, $R^3 = PO(OMe)_2$

13 $R^1 = R^3 = R^4 = R^5 = Me$, $R^2 = PO(OMe)_2$

14 $R^1 = R^4 = CD_3$, $R^2 = R^5 = Me$, $R^3 = PO(OCD_3)_2$

15 $R^1 = R^3 = CD_3$, $R^2 = R^5 = Me$, $R^4 = PO(OCD_3)_2$



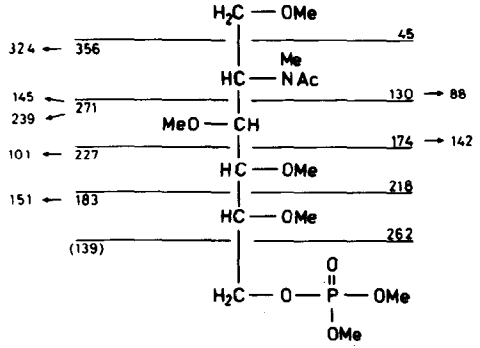
16 $R^1 = R^2 = Me$, $R^3 = PO(OMe)_2$

17 $R^1 = R^3 = Me$, $R^2 = PO(OMe)_2$

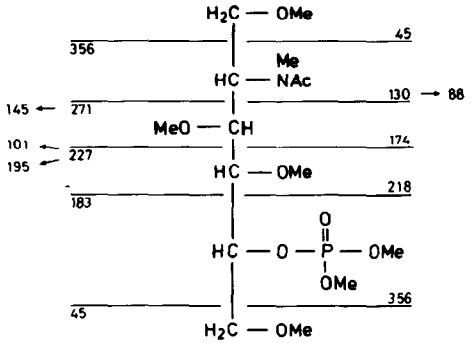
18 $R^2 = R^3 = Me$, $R^1 = PO(OMe)_2$

regardless of the methylation procedure used; GlcN (internal standard) gave methylated GlcNAc-ol (**9**) in quantitative yield.

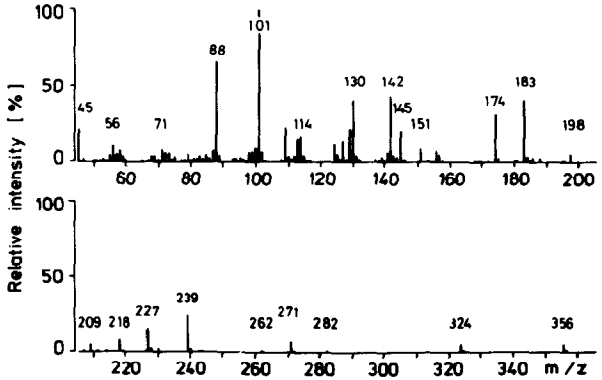
The NaOH–Me_2SO–MeI procedure[9] gave significantly reduced amounts of by-products, was easier and quicker to use, and was employed in all further experiments. However, prolonged treatment (>15 min) of GlcNAc-ol 6-P with NaOH–Me_2SO prior to methylation led to isomerization products (data not shown) and therefore this treatment should not exceed 3 min.

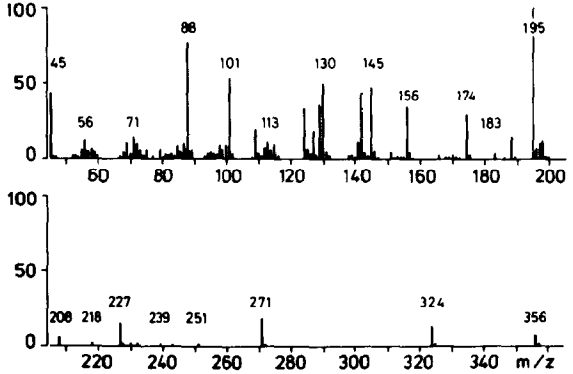


10 (mol. wt. 401)



11 (mol. wt. 401)





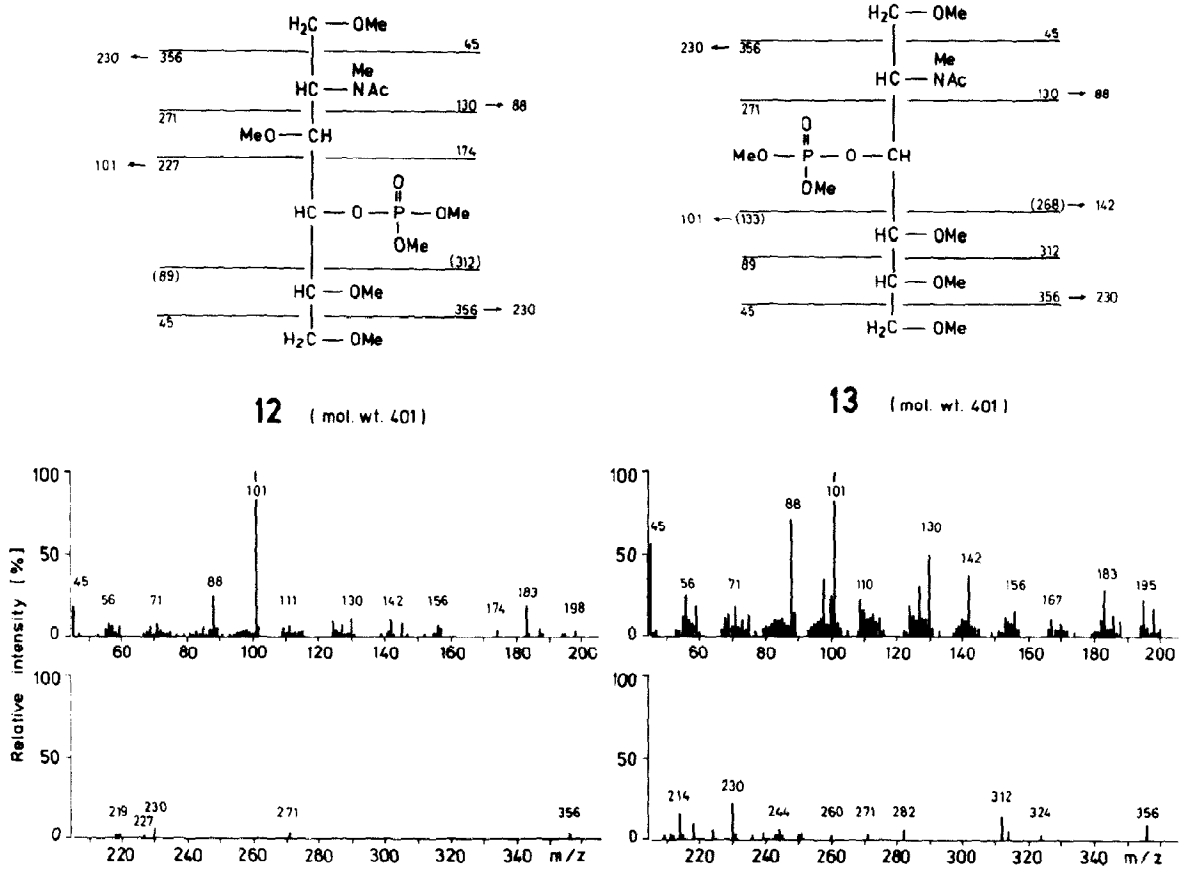


Fig. 1. E.i.-mass spectra (70 eV) and fragmentation pattern of 2-deoxy-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol (dimethyl phosphate) isomers **10–13**.

Methylation of α-GlcNAc 1-P did not result in any detectable sugar phosphate upon g.l.c. or g.l.c.–m.s. irrespective of which methylation procedure was used. However, the failure to detect methylated α-GlcNAc 1-P by g.l.c.–m.s. may also be due to the thermal instability of the substance.

N-Acetylation (acetic anhydride–NaOH)[7], borohydride reduction, and methylation of GlcN 6-P (**7**) gave a single peak (**10**) in g.l.c. (*T* 18.3 min). However, if reduction preceded *N*-acetylation, an additional compound (*T* 16.9 min) was formed in about the same amount and was identified (g.l.c.–m.s.) as 2-deoxy-1,3,4,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol 5-(dimethyl phosphate) (**11**). On c.i. (ammonia)-m.s., **10** and **11** gave $(M + H)^+$ and $(M + NH_4)^+$ ions at *m/z* 402 and 419, respectively. Also, the e.i.-mass spectra (Fig. 1) differed only in the intensities of the fragments *m/z* 195 and 183 which were the (dimethyl phosphate)-containing C-4,5,6 moiety in **11** and the C-5,6 moiety in **10**, respectively. Fission between C-3/4 in **11** gives *m/z* 227, and loss of methanol resulted in an intense secondary fragment at *m/z* 195. For **10**, fission between C-4/5, each of which carries an OMe group, is more likely[10], and resulted in an intense primary fragment at *m/z* 183. Thus, when borohydride reduction of GlcNAc 6-P (**7**) preceded *N*-acetylation, 6→5 phosphate migration occurred.

That acetic anhydride in the presence of NaOH caused phosphate migration was demonstrated when GlcNAc-ol 6-P was treated with acetic anhydride–NaOH and then methylated; **10** and **11** were formed in almost identical amounts. When the treatment with acetic anhydride–NaOH was omitted, only **10** was formed. Furthermore, when 0.2M NaOH was used in the absence of acetic anhydride, no 5→6 phosphate migration occurred. However, treatment of GlcNAc-ol 6-P with M NaOH for 1 h at 37° gave a 3:1 mixture (g.l.c.–m.s.) of the 6- and 5-phosphates. It is concluded that phosphate migration occurs in the acyclic (reduced) form.

Similar phosphate migration was observed with Gal 6-P. After reduction, treatment with acetic anhydride–NaOH, and methylation, g.l.c.–m.s. (c.i.- and e.i.-mode) revealed comparable amounts of 1,2,3,4,5-penta-*O*-methyl-D-galactitol (6-dimethyl phosphate) (**16**), 1,2,3,4,6-penta-*O*-methyl-D-galactitol (5-dimethyl phosphate) (**17**), and 1,2,3,5,6-penta-*O*-methyl-D-galactitol (4-dimethyl phosphate) (**18**).

In order to avoid phosphate migration, the treatment with acetic anhydride–NaOH should be monitored.

Borohydride reduction of GlcNAc 4-P (**6**) and GlcNAc 3-P (**8**) followed by methylation gave 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol 3-(dimethyl phosphate) (**13**) (*T* 17.6 min) and 2-deoxy-1,3,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol 4-(dimethyl phosphate) (**12**, *T* 17.5 min). Although the retention times of **12** and **13** were similar, their behaviour in e.i.-m.s. was quite different. Thus, **12** gave a simple mass spectrum (Fig. 1c). The base peak at *m/z* 101 probably originated from fission between C-3/4, leading to a primary fragment of *m/z* 227 followed by a characteristic loss (125 m.u.) of $-OPO(OMe)_2$ and subsequent proton abstraction. This interpretation was supported by the mass spectrum of trideuteriomethyl-labelled GlcNAc-ol 4-P (**14**, Fig. 2a), which gave a

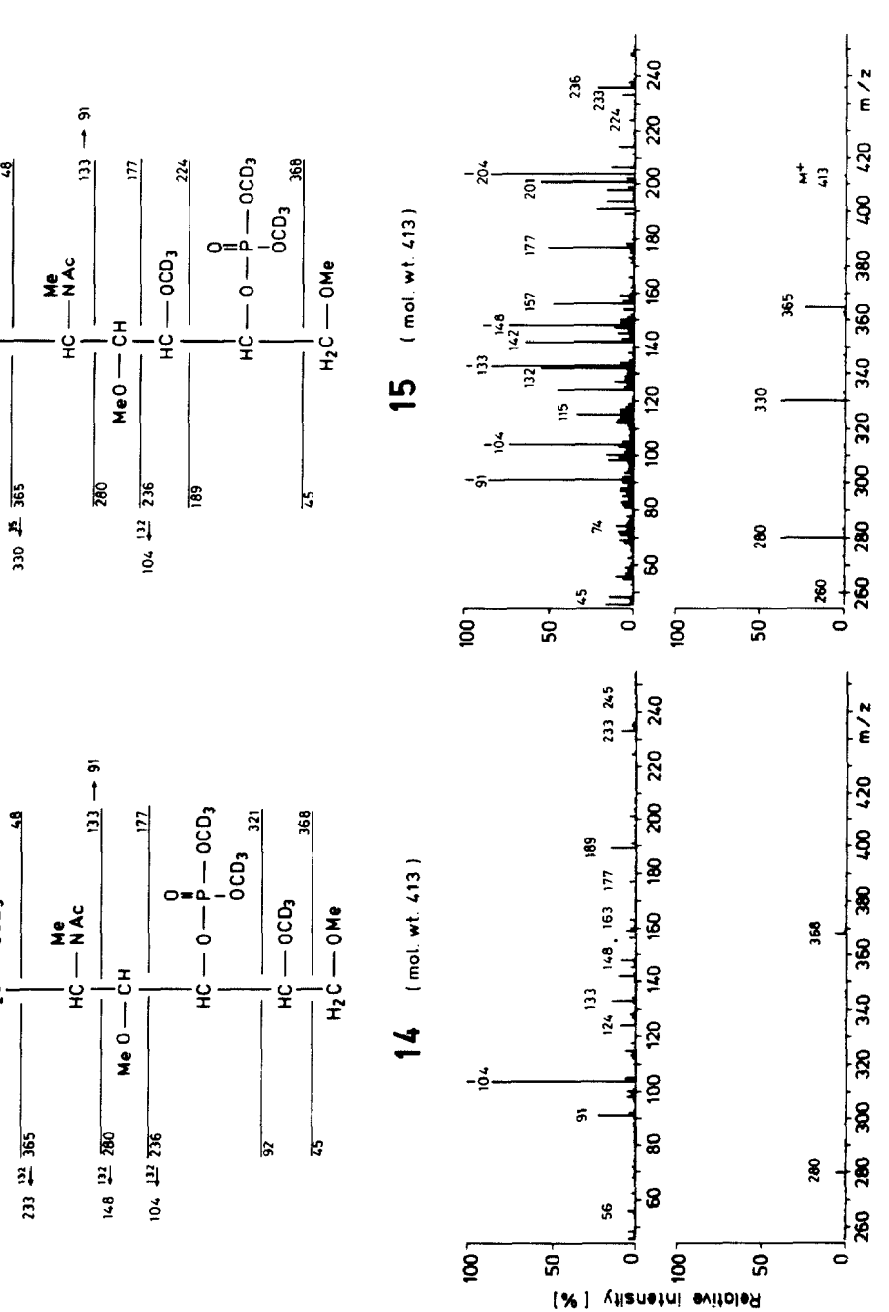


Fig. 2. E.i.-mass spectra (70 eV) and fragmentation pattern of the products (**14** and **15**) obtained from **2** after methylation (MeI), acid hydrolysis (acetolysis), reduction ($NaBH_4$), and remethylation (CD_3I).

base peak at *m/z* 104 (Fig. 1c), and that from **12** obtained by reduction with NaB^2H_4 (data not shown). The mass spectrum of **13** contained a diagnostic fragment at *m/z* 312 which was not present in the spectra of **12** and **13**, reflecting the more ready cleavage[10] of C(OMe)–C(OMe) bonds. This empirical rule is also obeyed by 3-deoxy-2-ketoaldonic acid phosphates[11].

As a model compound for the methylation analysis of the lipid A backbone, propyl 2-acetamido-2-deoxy-β-D-glucopyranoside 4-phosphate (**2**) was methylated (MeI), hydrolyzed (acetolysis)[12], reduced ($NaBH_4$), and methylated with (CD_3I). G.l.c.–c.i.m.s. then revealed two isomers [*m/z* 414, $(M + H)^+$; and 431, $(M + NH_4)^+$] in almost identical yields that were identified as 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-1,5-di-*O*-trideuteriomethyl-D-glucitol 4-[bis(trideuteriomethyl)phosphate] (**14**, *T* 16.9 min) and 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-1,4-di-*O*-trideuteriomethyl-D-glucitol 5-[bis(trideuteriomethyl) phosphate] (**15**, *T* 17.6 min). The retention time of **14** was identical to that of **12** but its molecular weight was 12 higher, indicating the presence of four trideuteriomethyl groups. The retention time of **15** was the same as that of **11** but the molecular weight was 12 higher. E.i.-m.s. (Fig. 2) showed that 4→5 phosphate migration had occurred. Since MeO-1 and the two phosphate methyl ester groups are lost during acetolysis[12] of partially methylated 2-acetamido-2-deoxyhexitols[13], the formation of the two isomers **14** and **15** can be explained by a phosphate migration.

Thus, methylation of hexosamine phosphates gave low yields of products (2–6% as compared with the yields from GlcN), and the only by-products detected by g.l.c.–m.s. were cyclic phosphates (yield: 0.5–1%). The low yields may reflect the susceptibility of the phosphate ester toward strong alkaline conditions. Furthermore, treatment with acetic anhydride–NaOH, strong alkaline conditions (M NaOH), or acetolysis can cause phosphate migration and the formation of isomers which may complicate the identification of the parent molecule. Derivatization reactions using conditions that avoid phosphate migration (*e.g.*, methanolysis) have been described for the g.l.c.–m.s. of sugar phosphates[2]. However, they have the disadvantage that the position of the phosphate groups cannot be deduced unequivocally from the mass-spectral fragmentation pattern alone, and synthetic reference substances are necessary.

EXPERIMENTAL

General. — The instrumentation was the same as described[14]. GlcNAc 3-P (**8**) was synthesized as described[15]. GlcN 6-P, GlcNAc 6-P, αGlcNAc 1-P, and Gal 6-P were commercial products (Sigma).

Propyl 2-acetamido-2-deoxy-β-D-glucopyranoside 4-phosphate (**2**). — To a solution of allyl 2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside[16] (**3**; 221 mg, 0.5 mmol) in dichloromethane (5 mL) were added diphenyl phosphorochloridate (269 mg, 1.0 mmol), 4-dimethylaminopyridine (122 mg, 1.0 mmol), and pyridine (79 mg, 1.0 mmol). After stirring for 4 h at room temperature, the mixture was washed with dil. HCl, water, aq. $NaHCO_3$, and water, dried ($MgSO_4$), and

concentrated *in vacuo*. Column chromatography (4:1 chloroform–acetone) of the residue on silica gel gave syrupy allyl 2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside 4-(diphenyl phosphate) (**1**; 305 mg, 91%). ^{1}H-N.m.r. data (100 MHz, $CDCl_3$): δ 1.76 (s, 3 H, Ac), 4.42 (s, 2 H, PhCH_2), 4.95 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), ~5.15 (m, 2 H, -CH=CH_2), ~5.8 (m, 2 H, -CH=CH_2 and NH), 7.0–7.3 (20 H, 4 Ph).

Anal. Calc. for $C_{37}H_{40}NO_9P$: C, 65.97; H, 5.98; N, 2.08. Found: C, 65.75; H, 6.02; N, 2.04.

A solution of **1** (190 mg) in tetrahydrofuran (5 mL) and AcOH (0.5 mL) was hydrogenolyzed over 10% Pd/C under 0.7 MPa of H_2. After 12 h, the catalyst was removed, platinum(IV) oxide was added to the filtrate, and the mixture was stirred for 1 h under hydrogen, filtered, diluted with water, and lyophilized to yield **2** (100 mg), $[\alpha]_D$ −11° (*c* 1.2, water). N.m.r. data (D_2O): ^{1}H (90 MHz), δ 0.72 (t, 3 H, $CH_2CH_2CH_3$), 1.42 (2 H, $CH_2CH_2CH_3$), 1.89 (s, 3 H, OMe); ^{13}C (90.6 MHz), δ 10.4 ($OCH_2CH_2CH_3$), 22.9 ($COCH_3$ and $OCH_2CH_2CH_3$ overlapped), 56.1 (C-2), 62.3 (C-6), 73.1 ($OCH_2CH_2CH_3$), 74.0 (b, $^2J_{C,P}$ ~1 Hz, C-3), 74.9 (d, $^3J_{C,P}$ 5.7 Hz, C-4), 75.8 (d, $^3J_{C,P}$ 5.7 Hz, C-5), 101.6 (C-1), 175.3 ($COCH_3$).

*2-Acetamido-2-deoxy-*D*-glucose 4-phosphate* (**6**). — To a solution of benzyl 2-acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside[17] (**4**; 246 mg, 0.50 mmol) in chloroform (5 mL) were added 4-dimethylaminopyridine (122 mg, 1.0 mmol) and diphenyl phosphorochloridate (207 μL, 1.0 mmol). After stirring for 4 h at room temperature, the mixture was washed with M HCl, water, and aq. $NaHCO_3$, dried ($MgSO_4$), and concentrated *in vacuo*. Column chromatography (chloroform–acetone, 25:1) of the residue on silica gel (25 g) and recrystallization from chloroform–ether–hexane gave benzyl 2-acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside 4-(diphenyl phosphate) (**5**; 241 mg, 67%), m.p. 91–93°, $[\alpha]_D$ +85° (*c* 0.7, chloroform). ^{1}H-N.m.r. data ($CDCl_3$, 100 MHz): δ 1.72 (s, 3 H, Ac), 5.26 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), and 7.0–7.4 (m, 25 H, Ph).

Anal. Calc. for $C_{41}H_{42}NO_9P$: C, 68.04; H, 5.85; N, 1.94. Found: C, 68.07; H, 5.83; N, 2.01.

A solution of **5** (150 mg, 0.207 mmol) in methanol (5 mL) and acetic acid (1 mL) was hydrogenolyzed for 36 h at room temperature in the presence of 10% Pd–C (150 mg) under 0.7 MPa of H_2 and then filtered. Platinum(IV) oxide (100 mg) was added, and the mixture was stirred for 1 h at room temperature under hydrogen as above, then filtered, and concentrated *in vacuo*. A solution of the residue in water was filtered through a membrane filter (0.45 μm) and lyophilized to give **6** (65 mg), $[\alpha]_D$ +39.1° (*c* 1.09, water). ^{13}C-N.m.r. data (D_2O, 90.6 MHz): δ 22.7 and 22.9 (α and β $NHCOCH_3$), 54.7 and 57.2 (C-2α and C-2β), 61.2 and 61.4 (C-6α and C-6β), 70.8 and 74.0 (C-3α and C-3β, $^3J_{C,P}$ ~1 Hz), 71.3 and 75.9 (C-5α and C-5β, $^3J_{C,P}$ 6.1 Hz), 75.3 and 74.9 (C-4α and C-4β, $^2J_{C,P}$ 6.1 Hz), 91.4 and 95.8 (C-1α and C-1β), and 176.4 and 177.1 (α and β $COCH_3$).

Derivatization of hexosamine phosphates for g.l.c. — Hexosamine phosphates were *N*-acetylated with acetic anhydride–NaOH[7], reduced ($NaBH_4$ or NaB^2H_4),

and methylated with MeI using (*a*) methylsulfinylmethanide[8] and (*b*) NaOH–Me_2SO–MeI[9].

G.l.c. was performed at 180° on a fused-silica capillary column (25 m × 0.22 mm i.d., Weeke, Mühlheim) with chemically bonded SE-54 (film thickness, 0.35 μm) with H_2 (2 mL/min) as carrier gas (0.08 MPa). G.l.c.–m.s. was performed as described[14].

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