# Phosphorylated Sugars. Part 25.[1] Synthesis and Behaviour in Acidic Media of 2-Acetamido-2-deoxy-D-glucose 4- and 6-Phosphates and of a 'Lipid A' Analogue

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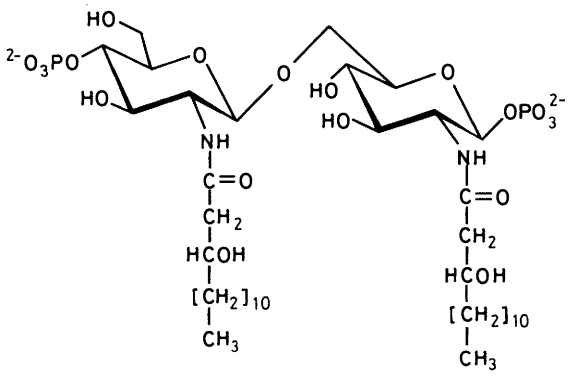
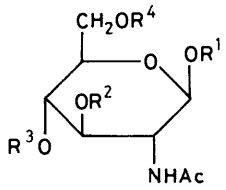
With the aid of 2-acetamido-2-deoxy-D-glucose 4- and 6-phosphates and of 2-amino-2-deoxy-D-glucose-6-phosphate, for all of which new and efficient syntheses are described, it has been shown that during treatment of 2-acetamido-2-deoxy-D-glucose 4-phosphate and of the model 2-hydroxyethyl 2-deoxy-2-[(3*R*)-3-hydroxytetradecanamido]-β-D-glucopyranoside 4-phosphate (which has also been synthesised) under conditions of acidic hydrolysis used in the determination of the structure of 'Lipid A' preparations from bacterial endotoxins, migration of the phosphate group from position 4 to position 6 takes place. Conditions are described in which the *N*-acetyl group is completely removed whilst the extent of phosphate migration is very small. Longer heating times in the same conditions are required to effect almost complete removal of the *N*-(3*R*)-3-hydroxytetradecanoyl group from the glucoside, but again very little phosphate migration occurs. The amount of inorganic phosphate formed after removal of the amide-bound substituent from the two 4-phosphates (7.5 and 15.4% respectively) was superior to that formed from *N*-acetylglucosamine 6-phosphate (2.4%).

The disaccharide 2-amino-6-*O*-(2′-amino-2′-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucopyranose substituted by various fatty acids [typically (3*R*)-3-hydroxytetradecanoic acid] in amide and ester linkages and phosphorylated in positions 1 and 4′ [*e.g.*, compound (**1**)] is frequently found as a constituent of the hydrophobic domain (Lipid A) of bacterial endotoxins.[2–8]



(**1**)

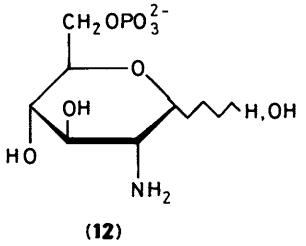
Determination of the hexosamine content of 'isolated Lipid A' preparations (a mixture of breakdown products of the hydrophobic domain) includes treatment of the material with strong acid. For the 'isolated Lipid A' preparation of *Bordetella pertussis* endotoxin, the highest values for hexosamine were observed after treatment with 4M-hydrochloric acid for 6 h at 100 °C.[9] Analysis of the hydrolysate by paper electrophoresis revealed, however, that a considerable amount of material containing esterified phosphate and free amino groups was present. The same phenomenon had previously been observed[10,11] with 'isolated Lipid A' preparations derived from enterobacterial endotoxins: the phosphate ester was thought to be 2-amino-2-deoxy-D-glucose (glucosamine) 6-phosphate arising from the 4-phosphate by phosphate migration during hydrolysis.[10] Since glucosamine derivatives phosphorylated in position 4 are not readily available, the alleged phosphate migration was not, to our knowledge, further investigated. As production of large amounts of novel structures under uncontrolled conditions considerably increases the difficulties encountered in the structural analysis of macromolecules, it was considered of interest to examine the behaviour, in acidic media, of synthetic phosphorylated derivatives of *N*-acylglucosamine.



(**2**) $R^1$ = Bn, $R^2$ = $R^3$ = $R^4$ = H
(**3**) $R^1$ = Bn, $R^2$ = H, $R^3R^4$ = $CMe_2$
(**4**) $R^1$ = $R^2$ = Bn, $R^3R^4$ = $CMe_2$
(**5**) $R^1$ = $R^2$ = Bn, $R^3$ = $R^4$ = H
(**6**) $R^1$ = $R^2$ = $R^4$ = Bn, $R^3$ = H
(**7**) $R^1$ = $R^2$ = $R^4$ = Bn, $R^3$ = $P(O)(OBn)_2$
(**8**) $R^1$ = $R^2$ = $R^4$ = H, $R^3$ = $P(O)(OH)(ONH_4)$
(**9**) $R^1$ = Bn, $R^2$ = $R^3$ = H, $R^4$ = $P(O)(OPh)_2$
(**10**) $R^1$ = $R^2$ = $R^3$ = H, $R^4$ = $P(O)(OH)_2$
(**11**) $R^1$ = $R^2$ = $R^3$ = H, $R^4$ = $P(O)(OH)(ONH_4)$
Bn = $CH_2Ph$

With this purpose in mind, new and efficient syntheses for *N*-acetylglucosamine 4-phosphate (**8**), for *N*-acetylglucosamine 6-phosphate (**11**), and for glucosamine 6-phosphate (**12**) have been elaborated and a model compound related to the disaccharide of 'Lipid A,' *viz.*, 2-hydroxyethyl 2-deoxy-2-[(3*R*)-3-hydroxytetradecanamido]-β-D-glucopyranoside 4-(ammonium hydrogen phosphate) (**15**), has also been synthesized. By submitting these compounds to various acidic treatments used for structural studies in the endotoxin field, it has been confirmed that derivatives of *N*-acylglucosamine 4-phosphate undergo phosphate migration during acidic hydrolysis and give rise to glucosamine 6-phosphate which is known[12] to be relatively stable in acidic media.

Two small-scale, low yielding syntheses[13,14] (11 and 9% respectively) of 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine) 4-phosphate that start with suitably protected *N*-acetylglucosamine derivatives having only the 4-hydroxy group free have been reported. The main problem in these syntheses



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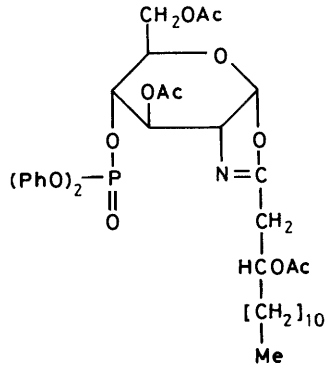

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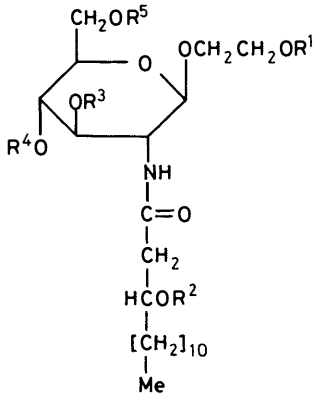

(14) $R^1$ = Bn, $R^2$ = $R^3$ = $R^5$ = Ac, $R^4$ = P(O)(OPh)$_2$
(15) $R^1$ = $R^2$ = $R^3$ = $R^5$ = H, $R^4$ = P(O)(OH)(ONH$_4$)

seems to reside in the choice of a sufficiently reactive phosphorylating agent which is, at the same time, readily amenable to deprotection. With *o*-phenylene phosphorochloridate, Bundle and Jennings[13] obtained good yields of a protected phosphorylated compound but difficulties encountered in the deprotection of the phosphate group led to low yields of the 4-phosphate. The phosphorylation step (diphenyl phosphorochloridate) used by the Russian authors[14] afforded only moderate yields of the triester (37%) and the deprotection step was inefficient (24%). For the synthesis reported herein, a suitably protected *N*-acetylglucosaminide was treated with lithium di-isopropylamide (LDA) and then with tetrabenzyl pyrophosphate according to a recently reported method,[15,16] giving the phosphotriester in reasonable yield (55%) (not optimised) and the deprotection step was rapid and efficient (80%).

The starting material for this synthesis was the known[17–19] benzyl 2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (**6**) obtained by a more straightforward synthesis than those previously described. Benzyl 2-acetamido-2-deoxy-4,6-*O*-isopropylidene-β-D-glucopyranoside[19] (**3**), prepared by the method of Charon *et al.*,[20] was benzylated and the 3-*O*-benzyl ether (**4**) was isolated without chromatography in 78% yield. The isopropylidene group was removed by hydrolysis with trifluoroacetic acid (TFA) and the stannylene derivative of the 4,6-diol[17,18,21] (**5**) was selectively benzylated in position 6 using tetrabutylammonium iodide as catalyst according to the procedure of David *et al.*[22] Benzylation of the corresponding α anomer with tetrabutylammonium bromide as catalyst has been described.[23] Despite slight difficulty encountered in the isolation of the dibenzyl ether due to the presence of free iodine, it was found to be preferable to use the iodide, known to be a more efficient catalyst,[24] rather than the bromide. The 4-hydroxy derivative (**6**) was treated successively with LDA and tetrabenzyl pyrophosphate. Hydrogenolysis of the resulting dibenzyl phosphate (**7**) in the presence of palladium–charcoal catalyst afforded *N*-acetylglucosamine 4-phosphate, isolated as the monoammonium salt (**8**), which was identical with the compound previously described.[13,14] Attempted preparation of the 3,6-dibenzyl ether (**6**) by displacement of the tosyl group in benzyl 2-acetamido-3-*O*-benzyl-2-deoxy-6-*O*-(*p*-tolylsulphonyl)-β-D-glucopyranoside with sodium benzylate as described for the corresponding α anomer[25] failed in our hands. Nor was it possible to effect regioselective reductive cleavage of the benzylidene group in benzyl 2-acetamido-3-*O*-benzyl-4,6-benzylidene-2-deoxy-β-D-glucopyranoside[17,18,21] with sodium cyanoborohydride, as described for the α anomer;[26] the failure of this reaction could probably be attributed to the extreme insolubility of the β anomer compared with that of the α anomer.

*N*-Acetylglucosamine 6-phosphate (**10**) has hitherto been obtained by chemical[27,28] and enzymatic[29,30] acetylation of glucosamine 6-phosphate. In the convenient synthesis described below, selective phosphorylation of the primary hydroxy group of benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**2**) gave a 75% yield of the 6-diphenyl phosphate (**9**). Hydrogenolysis of this compound, first in the presence of a palladium–charcoal catalyst and then in the presence of Adams platinum catalyst, removed the benzyl and phenyl groups, and afforded an 83% yield of *N*-acetylglucosamine 6-phosphate. Gram quantities of the compound can easily be prepared in one batch.

Glucosamine 6-phosphate has been obtained by enzymatic phosphorylation of glucosamine[31] and several chemical syntheses have been reported.[12,27,28] Crystalline glucosamine 6-phosphate (**12**) was easily prepared by acidic hydrolysis of the above *N*-acetyl derivative in the conditions described for the hydrolysis of the peracetylated 6-phosphate.[12] In order to obtain a crystalline compound, we found that it was imperative to adjust the pH of the sulphuric acid-containing hydrolysis mixture to 2 with barium hydroxide and not to 4 as described.[12]

In the course of studies, described below, on the hydrolysis of *N*-acetylglucosamine 6-phosphate with M-hydrochloric acid at 100 °C, t.l.c. showed that removal of the *N*-acetyl group was practically complete after 1 h, whereas t.l.c. of the 0.5M-sulphuric acid hydrolysate showed that after 2 or 3 h at 100 °C there remained an appreciable quantity of the starting material. Indeed, if the mother liquors were re-hydrolysed, a further amount of the phosphate ester could be obtained, raising the total yield from 45 to 62%. We therefore attempted to prepare glucosamine 6-phosphate by hydrolysis of its *N*-acetyl derivative with molar hydrochloric acid at 100 °C for 1.5 h. Although a clean reaction mixture resulted, isolation of the 6-phosphate was not as convenient as when sulphuric acid was used, due to the difficulty of removing hydrochloric acid. Nevertheless, a 62% yield of the desired phosphate was obtained in one operation by this method.

For the synthesis of the *N*-acylglucosaminide 4-phosphate (**15**), 2′-[(2*R*)-2-acetoxytridecyl]-3,6-di-*O*-acetyl-1,2-dideoxy-4-*O*-(diphenoxyphosphoryl)-1,2-dihydro-α-D-glucopyranoso-[2,1-*d*]oxazole (**13**), prepared by the method described for the 2*RS* derivative,[32] was condensed with ethylene glycol monobenzyl ether in the presence of toluene-*p*-sulphonic acid (PTSA) under conditions described by Kiso *et al.*[33] The resulting diphenyl phosphate (**14**) was hydrogenated successively in the presence of palladium–charcoal catalyst and of Adams platinum catalyst to remove the benzyl and phenyl groups, and the resulting phosphomonoester was then de-*O*-acetylated with ammonia. The 2-hydroxyethyl glycoside 4-phosphate (**15**) was isolated as its ammonium salt in 84% yield from the fully

protected diphenyl phosphate. It should be noted that all of the deprotected *N*-acyl or free amino sugar phosphates described here should be stored in the cold (4 °C): at room temperature, slow decomposition with the formation of inorganic phosphate occurs.

For qualitative experiments on the hydrolysis of *N*-acetylglucosamine 4- and 6-phosphates with 4M-hydrochloric acid under conditions used for the removal of amide-bound fatty acids in 'lipid A' preparations[34] (4 h; 100 °C), a solvent system which gave complete separation on t.l.c. of all compounds likely to be present in the hydrolysate, *viz.* *N*-acetylglucosamine 4- and 6-phosphates, glucosamine 4- and 6-phosphates, and glucosamine, was used. Chromatograms showed that after 2 h the *N*-acetyl group was completely removed from both phosphates. While glucosamine 6-phosphate and traces of glucosamine were the only products found in the hydrolysate of *N*-acetylglucosamine 6-phosphate, in that of *N*-acetylglucosamine 4-phosphate, besides the expected de-acetylated product, a fair amount (visual estimation *ca.* 10%) of glucosamine 6-phosphate and about the same amount of glucosamine were present. That migration of the phosphate group from position 4 to position 6 had occurred was confirmed by treating the hydrolysates of both *N*-acetylglucosamine 4- and 6-phosphates successively with sodium periodate and sodium borohydride and then submitting the resulting mixtures to analysis by paper electrophoresis. Whereas the 6-phosphate gave a single spot corresponding to ethylene glycol dihydrogen phosphate, the 4-phosphate gave two spots: ethylene glycol dihydrogen phosphate, indicating the presence of glucosamine 6-phosphate and thus demonstrating the occurrence of phosphate migration, and glycerol dihydrogen phosphate[35] arising from glucosamine 4-phosphate. However, the presence of glucosamine 5-phosphate, which would also give a three-carbon fragment, could not be excluded. The absence of detectable amounts of this phosphate was demonstrated when, after borohydride reduction, re-*N*-acetylation, periodate oxidation, and a second borohydride reduction of the hydrolysate, analysis by electrophoresis showed the only phosphate-containing product present, besides ethylene glycol dihydrogen phosphate, to be a five-carbon fragment (arising from the 4-phosphate); no three-carbon fragment could be detected.

In a search for conditions of acidic hydrolysis which would effect removal of the *N*-acetyl group without causing phosphate migration, the hydrolyses of *N*-acetylglucosamine 4- and 6-phosphate (as their free acids) in 1M-hydrochloric acid at 100 °C were followed quantitatively by estimation of free amino group formation;[36] the quantity of inorganic phosphate produced simultaneously was also determined.[37] The results, summarised in the Table, showed that, for both compounds, hydrolysis of the *N*-acetyl group was almost complete after 1 h. At this time, the amount of glucosamine 6-phosphate detectable by t.l.c. was so small that it could be considered as being negligible from the point of view of structural analysis. Even after 0.5 h, traces of glucosamine 6-phosphate were, however, already present in the hydrolysate of the 4-phosphate.

Owing to its very low solubility in water, the hydrolysis of 2-hydroxyethyl 2-deoxy-2-[(3*R*)-3-hydroxytetradecanamido]-β-D-glucopyranoside 4-(ammonium hydrogen phosphate) (**15**) could not be followed in the conditions described above. It was, however, possible to perform the hydrolyses in 1:3 methanol–water solutions. Complete hydrolysis of the *N*-acyl group required more than 4 h (see Table); at that time, 15% of the total amount of phosphate was present as inorganic phosphate. As judged by t.l.c., a corresponding amount of glucosamine was formed but only a small quantity of glucosamine 6-phosphate was detectable (*ca.* 5–10%). The considerably decreased rate of de-*N*-acylation observed for the glycose (**15**) that carries a long-chain fatty acid compared with that of the analogous *N*-acetyl derivative (**8**) is probably due to the formation of aggregates by the former in the aqueous methanolic environment, a situation prevailing in aqueous 'solutions' of endotoxins.[38]

**Table.** Hydrolysis in 1M-HCl, 100 °C (values are % analyses of the free $NH_2$ groups and of inorganic P formed)

| | | *t* (h) | | | | |
|---|---|---|---|---|---|---|
| Cpd | | 1 | 1.5 | 2 | 3 | 4 |
| (**8**) | $NH_2$ | 90 | 100 | 100 | | |
| | $P_i$ | 5.8 | 7.5 | 9 | | |
| (**10**) | $NH_2$ | 90 | 100 | 100 | | |
| | $P_i$ | 1.6 | 2.4 | 3.5 | | |
| (**15**) | $NH_2$ | 41 | | 72 | 83 | 90 |
| | $P_i$ | | | 6.5 | 12.5 | 15.4 |

## Experimental

Evaporations were carried out under reduced pressure at 40 °C. M.p.s were determined on a Kofler hot-plate and are uncorrected. Optical rotations were determined with a Perkin-Elmer 141 polarimeter. T.l.c. was performed on silica gel (60 $F_{254}$ on aluminium foil, Merck); all compounds were revealed by spraying with sulphuric acid (10%) in ethanol and heating on a hot-plate. Column chromatography was performed on silica gel (Merck 60, 70—230 mesh). Paper electrophoresis was carried out on Whatman 3MM paper with a flat-plate electrophoresis apparatus in 0.2M-pyridine–acetic acid buffer (pH 5) at 70 V $cm^{-1}$ for 1 h. Phosphate esters were detected according to the method of Hanes and Isherwood.[39] Free amino groups were estimated by a modification, described below, of the method of Moore and Stein,[36] and total and inorganic phosphate by the method of Chen *et al.*[37]

*Estimation of Free Amino Groups.*—The reagent consists of a mixture of freshly prepared solutions of ninhydrin (0.5 g) in ethylene glycol monomethyl ether (methyl cellosolve) (11 ml) (2.75 ml) and of tin(II) chloride (24 mg) in a pH 5.5 sodium acetate buffer (15 ml) [sodium acetate trihydrate (54.4 g) and acetic acid (10 g) made up to 100 ml with water] (3.75 ml). Reagent (0.3 ml) was added to an aqueous solution (0.3 ml) of the mixture to be analysed, containing not more than 9 μg of nitrogen per ml, in screw-capped tubes which were heated for 15 min at 100 °C. The cooled tubes, to which (1:1) ethanol–water (0.6 ml) was added, were kept in the dark for 0.5 h and the absorbancies of the solutions were read at 570 nm against a reagent blank. Glucosamine hydrochloride was used as standard.

*Benzyl 2-Acetamido-3-O-benzyl-2-deoxy-4,6-O-isopropylidene-β-D-glucopyranoside* (**4**).—A solution of benzyl 2-acetamido-2-deoxy-4,6-*O*-isopropylidene-β-D-glucopyranoside[20] (**3**) (8.726 g, 24.86 mmol) in *N*,*N*-dimethylformamide (41.4 ml) was stirred with barium oxide (15.27 g, 99.6 mmol), barium hydroxide octahydrate (3.93 g, 12.46 mmol), and benzyl bromide (5.92 ml, 49.4 mmol) for 16 h. The mixture was diluted with dichloromethane (400 ml) and cooled, and cold 50% aqueous acetic acid was added with rapid stirring to dissolve the solids. The organic phase was then washed successively with ice–water, saturated aqueous sodium hydrogen carbonate, and ice–water and dried ($Na_2SO_4$). Solvents were removed and the residue was crystallised (ethanol–hexane) to yield pure [t.l.c. (8:4:1) ethyl acetate–hexane–ethanol] compound (**4**) (8.56 g, 78%), m.p. (144—145 °C, loss of crystalline form) 155—157 °C; $[\alpha]_D$ −20° (*c* 1 in $CHCl_3$) (lit.,[19] m.p. 152—153 °C; $[\alpha]_D$ −21°) (Found: C, 67.95; H, 7.15; N, 3.2 Calc. for $C_{25}H_{31}NO_6$: C, 68.0; H, 7.1; N, 3.2%).

*Benzyl* 2-*Acetamido*-3-O-*benzyl*-2-*deoxy*-β-D-*glucopyranoside* (**5**).—A solution of the preceding compound (5.1 g, 11.56 mmol) in a mixture of methanol (200 ml), water (41.4 ml), and TFA (9.6 ml) was stirred overnight. T.l.c. [(8:4:1) ethyl acetate–hexane–ethanol] then showed hydrolysis to be complete. The solution was passed through a column of Amberlite IR 45 ($HO^-$) resin (300 ml) in methanol–water (8:2), and the column was eluted with the same solvents. Fractions containing compound (**5**) were pooled and neutralised with solid sodium hydrogen carbonate. Solids were filtered off, the solvent was removed, and toluene was evaporated several times from the residue which was then crystallised from methanol–ether–pentane to give compound (**5**) (3.74 g, 80.65%), m.p. 180—181 °C; $[\alpha]_D$ −20° (*c* 1 in MeOH) (lit.,[17] m.p. 183—184 °C; $[\alpha]_D$ −19°; lit.,[18] m.p. 187—189 °C; $[\alpha]_D$ −18.8°; lit.,[21] m.p. 169—170 °C; $[\alpha]_{578}$ −20°) (Found: C, 65.7; H, 6.9; N, 3.4. Calc. for $C_{22}H_{27}NO_6$: C, 65.8; H, 6.8; N, 3.5%).

*Benzyl* 2-*Acetamido*-3,6-*di*-O-*benzyl*-2-*deoxy*-β-D-*glucopyranoside* (**6**).—A mixture of the preceding compound (4.21 g, 10.5 mmol) and bis(tributylstannyl)oxide (2.63 g, 10.5 mmol) in toluene (310 ml) was refluxed for 16 h with continuous removal of water. The resulting solution was concentrated (*ca.* 250 ml), tetrabutylammonium iodide (3.87 g, 10.5 mmol) and benzyl bromide (2.625 ml, 22 mmol, 2.1 mol equiv.) were added, and the mixture was refluxed for 4 h, at which time t.l.c. [(95:5) chloroform–methanol] showed the reaction to be complete. The solution was cooled and concentrated to dryness. The residue was dissolved in chloroform and the solution was washed with aqueous sodium arsenite–sodium hydrogen carbonate until the iodine colour disappeared, and then with water and was dried ($Na_2SO_4$). The solvent was removed and the residue was chromatographed on a column (50 × 4.5 cm) of silica gel (solvents as for t.l.c.). The title compound (5 g, 78%), obtained by removal of the solvents from the appropriate pooled fractions and recrystallisation of the residue from ethyl acetate–hexane, had m.p. 179—180 °C; $[\alpha]_D$ −35° (*c* 1.1 in $CHCl_3$) (lit.,[17] m.p. 181 °C; $[\alpha]_D$ −37°; lit.,[18] m.p. 182—184 °C; $[\alpha]_D$ −35°) (Found: C, 70.7; H, 6.75; N, 2.8. Calc. for $C_{29}H_{33}NO_6$: C, 70.9; H, 6.8; N, 2.85%).

*Benzyl* 2-*Acetamido*-3,6-*di*-O-*benzyl*-2-*deoxy*-β-D-*glucopyranoside* 4-(*Dibenzyl Phosphate*) (**7**).—A solution of LDA in tetrahydrofuran (THF) [prepared by adding butyl-lithium (1.6M solution in hexane; 3 ml, 4.8 mmol) to THF (4 ml) containing di-isopropylamine (0.8 ml) at −20 °C] (1.95 ml, 1.2 mmol) was added to a solution of the preceding compound (491 mg, 1 mmol) in THF (15 ml) at −78 °C, followed 15 min later by a solution of tetrabenzyl pyrophosphate (0.67 g, 1.25 mmol) in THF (10 ml). The temperature of the cooling bath was raised to 0 °C and the reaction mixture was kept for 1 h at this temperature. It was then diluted with ether (100 ml). The organic phase was washed successively with cold saturated aqueous sodium hydrogen carbonate and ice–water, and dried. After removal of the solvents, the residue was chromatographed on a column (26 × 2.2 cm) of silica gel with (9:2) chloroform–acetone as eluant. The *dibenzyl phosphate* (**7**) (412 mg, 55%) had m.p. 95—97 °C, $[\alpha]_D$ −9.7° (*c* 1.025 in $CHCl_3$) (Found: C, 68.5; H, 6.45; N, 1.9. $C_{43}H_{46}NO_5P$ requires C, 68.7; H, 6.2; N, 1.9%).

2-*Acetamido*-2-*deoxy*-D-*glucopyranose* 4-(*Ammonium Hydrogen Phosphate*) (**8**).—A solution of the preceding compound (1.65 g, 2.2 mmol) in ethanol (160 ml) was hydrogenated in the presence of 10% palladium–charcoal catalyst. When t.l.c. [(5:3:1:1) propan-1-ol–ammonia–water–acetic acid, $R_F$ 0.44] showed the reaction to be complete, the catalyst was filtered off, aqueous ammonia was added to bring the pH to *ca.* 6 (pH paper), and solvents were removed. Treatment of a hot solution of the residue in water (4 ml) with hot ethanol (10 ml) furnished the crystalline title compound (**8**) (521 mg, 81.4%), m.p. 165 °C (decomp.); $[\alpha]_D$ +41° (equilib.) (*c* 1.03 in water) {lit.,[13] m.p. 165 °C (decomp.); $[\alpha]_D$ +42° (equilib.); lit.,[14] m.p. 141.5—142 °C; $[\alpha]_D$ +46.7° (equilib.)} (Found: C, 28.4; H, 6.5; N, 8.3. Calc. for $C_8H_{19}N_2O_9P{\cdot}H_2O$: C, 28.6; H, 6.3; N, 8.3%).

*Benzyl* 2-*Acetamido*-2-*deoxy*-β-D-*glucopyranoside* 6-(*Diphenyl Phosphate*) (**9**).—A solution of diphenyl chlorophosphate (6.2 g, 23.15 mmol) in anhydrous pyridine (20 ml) was added dropwise to a stirred solution, cooled to −35 °C, of benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**2**) (6 g, 19.29 mmol) in pyridine (500 ml). The reaction mixture was allowed to come to room temperature and was left overnight. Water (1 ml) was added dropwise to the stirred solution and, after 1 h, solvents were removed, the last traces of pyridine being removed by repeated distillations with toluene. Upon trituration with ice–water, the residue solidified. The precipitate (8.72 g, 83%), practically pure on t.l.c. [(8:3) ethyl acetate–ethanol], was filtered off and dried. A sample of the *title compound* (**9**), recrystallised from aqueous ethanol, had m.p. 78—79 °C; $[\alpha]_D$ −22° (*c* 1 in EtOH) (Found: C, 59.5; H, 5.6; N, 2.4. $C_{27}H_{30}NO_9P$ requires C, 59.7; H, 5.6; N, 2.6%).

2-*Acetamido*-2-*deoxy*-D-*glucose* 6-(*Dihydrogen Phosphate*) (**10**) *and* 6-(*Ammonium Hydrogen Phosphate*) (**11**).—The preceding crude diphenyl phosphate (**9**) (8.72 g, 16 mmol) in methanol (200 ml) was hydrogenated in the presence of 10% palladium–charcoal catalyst until t.l.c. [(8:3) ethyl acetate–ethanol] showed the removal of the benzyl group to be complete. (It was sometimes necessary, if the hydrogenation was sluggish, to filter off the catalyst and add fresh catalyst). The catalyst was filtered off and the filtrate was hydrogenated in the presence of Adams platinum catalyst. When t.l.c. [(7:1:2) propan-1-ol–ammonia–water] showed removal of the phenyl groups to be complete (*ca.* 24 h), the catalyst was filtered off and the solvent was removed. The residue (5 g, 95%), practically pure on t.l.c. [5:3:1:1) propan-1-ol–ammonia–water–acetic acid, $R_F$ 0.39], was crystallised from methanol–ethanol–ether. The *pure sesquihydrate product* (**10**) (4.37 g, 83%) had m.p. 144—146 °C (decomp.), $[\alpha]_D$ +30° (*c* 1.04 in water) {lit.,[28] anhydrous compound $[\alpha]_D$ +29.5 (*c* 8 in 0.5M-sodium acetate)} (Found: C, 29.3; H, 5.7; N, 4.25. $C_8H_{16}NO_9P{\cdot}1.5H_2O$ requires C, 29.3; H, 5.8; N, 4.3%). If the methanolic solution was neutralised with dil. aqueous ammonia before concentration, upon concentration the monoammonium salt (**11**) crystallised out. It had m.p. 143—145 °C (decomp)., $[\alpha]_D$ +31° (*c* 1 in water) [lit.,[27] 146.5—147.5 °C (decomp.)] (Found: C, 29.6; H, 6.2; N, 8.3. Calc. for $C_8H_{19}N_2O_9P{\cdot}0.5H_2O$: C, 29.4; H, 6.2; N, 8.6%).

2-*Amino*-2-*deoxy*-D-*glucose* 6-(*Dihydrogen Phosphate*) (**12**).—A solution of the dihydrogen phosphate (**10**) (2.57 g, 7.8 mmol) in 0.5M-sulphuric acid (102 ml) was heated for 3 h at 100 °C. Half-saturated aqueous barium hydroxide was added to the cooled solution to bring the pH to 2 (pH meter). The precipitated barium sulphate was centrifuged off and the supernatant solution was concentrated (50 ml). Ethanol was added to slight turbidity and the mixture was kept for several days at 4 °C. The crystalline monohydrate of the phosphate (0.93 g, 42%), pure on t.l.c. [(5:3:1:1) propan-1-ol–aqueous ammonia–water–acetic acid, $R_F$ 0.26], had m.p. 165—170 °C (decomp.), $[\alpha]_D$ +55° (*c* 1 in water) {lit.,[12] m.p. 170—180 °C (decomp.); $[\alpha]_D$ +54°; lit.,[28] for the anhydrous compound m.p. 160—167 °C; $[\alpha]_D$ +58.8°} (Found: C, 25.9; H, 5.8; N, 5.1. Calc. for $C_6H_{14}NO_8P{\cdot}H_2O$: C, 26.0; H, 5.8; N, 5.05%).

Concentration of the mother liquors followed by hydrolysis [0.5M-sulphuric acid (50 ml)] and work up as described above gave a further crop (0.43 g) of chromatographically pure crystalline phosphate (total yield 62%).

*Alternative Preparation of* 2-*Amino*-2-*deoxy*-D-*glucose* 6-(*Dihydrogen Phosphate*) (**12**).—A solution of compound (**10**) (300 mg, 0.92 mmol) in 1M-hydrochloric acid (30 ml) was heated for 1.5 h at 100 °C. The quantity of dry Amberlite IR 45($HO^-$) resin previously determined to be necessary to bring the pH of the same amount of M-hydrochloric acid to 2 was added to the cooled, stirred solution. The resin was filtered off and washed with water and the combined filtrate and washings were concentrated (bath temperature 30 °C) to small volume (*ca.* 1 ml) and ethanol was added to turbidity. The crystalline phosphate (156 mg, 61.6%) was identical with that described above.

2-*Benzyloxyethyl* 2-[(3R)-3-*Acetoxytetradecanamido*]-3,6-*di*-O-*acetyl*-2-*deoxy*-β-D-*glucopyranoside* 4-(*Diphenylphosphate*) (**14**).—Dry PTSA (87 mg, 0.5 mmol, 0.33 mol equiv.) and ethylene glycol monobenzyl ether (693 mg, 4.56 mmol, 3 mol equiv.) were added to a solution of 2′-[(2*R*)-2-acetoxytridecyl]-3,6-di-*O*-acetyl-1,2-dideoxy-4-*O*-diphenoxyphosphoryl-1,2-dihydro-α-D-glucopyranoso[2,1-*d*]oxazole (**13**) (1.13 g, 1.52 mmol) in dry 1,2-dichloroethane (25 ml) and the mixture was heated at 50 °C. When t.l.c. [(3:2) ethyl acetate–hexane] showed the reaction to be complete (*ca.* 1 h), the mixture was cooled, diluted with chloroform (200 ml), and washed successively with ice-cold saturated aqueous sodium hydrogen carbonate and ice–water, and dried ($Na_2SO_4$). Solvents were removed, and the residue was purified on a column (50 × 5 cm) of silica gel (eluant as for t.l.c.). The *title compound* (**14**) (709 mg, 52%) had $[\alpha]_D$ −11° (*c* 1.6 in $CHCl_3$) (Found: C, 62.9; H, 7.05; N, 1.6. $C_{47}H_{64}NO_{14}P$ requires C, 62.9; H, 7.2; N, 1.6%).

2-*Hydroxyethyl* 2-*Deoxy*-2-[(3R)-3-*hydroxytetradecanamido*]-β-D-*glucopyranoside* 4-(*Ammonium Hydrogen Phosphate*) (**15**).—A solution of the preceding compound (688 mg, 0.77 mmol) in ethanol (35 ml) was hydrogenated in the presence of 10% palladium–charcoal catalyst until t.l.c. [(3:1) ethyl acetate–hexane] showed complete removal of the benzyl group, and then in the presence of Adams platinum catalyst. When t.l.c. [(8:4:1) ethyl acetate–ethanol–water] showed removal of the phenyl groups to be complete, the catalysts were filtered off, solvents were removed, and the residue was dissolved in anhydrous methanol. The cooled solution was saturated with gaseous ammonia. When t.l.c. [(6.5:3.5:1) dichloromethane–methanol–ammonia] showed deacetylation to be complete, solvents were removed, and the residue was triturated with ethanol and recovered by centrifugation. This procedure was repeated twice. The dried *title compound* (**15**) (359 mg, 84%) had m.p. 173—175 °C (decomp.) (Found: C, 47.6; H, 8.5; N, 5.1. $C_{22}H_{47}N_2PO_{11}\cdot 0.5H_2O$ requires C, 47.6; H, 8.7; N, 5.0%).

Because of its insolubility in all common solvents, the $[\alpha]_D$ of compound (**15**) could not be determined; the free acid, prepared by addition of dry Amberlite IRN 77 ($H^+$) resin to a suspension of the ammonium salt in methanol, had $[\alpha]_D$ −12° [*c* 1 (calculated as the free acid) in MeOH].

*Hydrolyses of* N-*Acetylglucosamine* 4- *and* 6-*Phosphates*.—For qualitative experiments where the hydrolysis products were identified by t.l.c. [(5:3:1:1) propan-1-ol–ammonia–water–acetic acid], solutions containing sugar phosphate (*ca.* 1 mg per ml of M- or 4M-hydrochloric acid) were heated in screw-topped tubes at 100 °C in an oil-bath for appropriate times [$R_F$ *N*-acetylglucosamine 4-phosphate 0.44; *N*-acetylglucosamine 6-phosphate 0.39; glucosamine 4-phosphate (produced by hydrolysis of the *N*-acetyl derivative) 0.32; glucosamine 6-phosphate 0.26; glucosamine 0.6]. For the quantitative determination of total phosphorus content and of free amino groups and inorganic phosphate formed during hydrolysis with M-hydrochloric acid, the ammonium salts of the sugar phosphates (*ca.* 5 mg) in water (*ca.* 0.5 ml) were decationised by passage through small columns of Amberlite IRN 77 ($H^+$) resin. The eluate and washings were made up to 5 ml with water. Samples (0.75 ml) to which was added 4M-hydrochloric acid (0.25 ml) were heated in screw-topped tubes in an oil-bath at 100 °C for the appropriate times. The solutions were cooled and diluted with the amount of water necessary to adjust the concentrations of nitrogen and of phosphorus to *ca.* 9 and 20 μg per ml respectively.

*Hydrolysis of* 2-*Hydroxyethyl* 2-*Deoxy*-2-[(3R)-3-*hydroxytetradecanamido*]-β-D-*glucopyranoside* 4-(*Ammonium Hydrogen Phosphate*) (**15**).—For the quantitative estimation of total phosphorus and of the free amino groups and inorganic phosphate formed by hydrolysis with M-hydrochloric acid, the ammonium salt of the sugar phosphate was dissolved in the minimum amount of methanol with the help of dry Amberlite IRN 77 ($H^+$) resin, and the solution was passed through a small column of the same resin which was then washed with methanol. The effluent and washings (2.5 ml) were diluted with water (2.5 ml). Water (0.25 ml) and 4M-hydrochloric acid (0.25 ml) were added to samples (0.5 ml) of this solution in screw-topped tubes which were heated in an oil-bath at 100 °C for appropriate times. Methanol–water (1:3) was used to dilute the cooled solutions to give concentrations of nitrogen and phosphorus the same as those described above.

*Transformation of Acidic Hydrolysates of* N-*Acetylglucosamine* 4- *and* 6-*Phosphates into Phosphorylated Polyol Fragments and Their Identification by Paper Electrophoresis*.—(*A*) The phosphorylated *N*-acetylglucosamine (0.05 mmol) was hydrolysed at 100 °C with 4M-hydrochloric acid (1.5 ml). The cooled solution was concentrated to dryness, toluene was evaporated several times from the residue which was then dissolved in water (1.5 ml), and a solution of sodium metaperiodate (85 mg) in water (3 ml) was added. The solution was kept in the dark for 18 h, neutralised with aqueous sodium hydroxide, cooled in ice, and added dropwise to a cooled, stirred solution of sodium borohydride (157 mg) in water (1 ml). The reaction mixture was allowed to come to room temperature and was left overnight. It was then acidified with Amberlite IRN 77 ($H^+$) resin. The resin was filtered off, and the filtrate was neutralised with saturated aqueous barium hydroxide and concentrated to small volume. The precipitate (free from phosphorus) was centrifuged off and washed with water, and the combined supernatants were acidified with Amberlite IRN 77 ($H^+$) resin. This was removed by filtration, and the filtrate was extracted with ether to remove free iodine, and concentrated to dryness. The residue was evaporated several times with methanol, dissolved in a little water, and submitted to electrophoresis. $R_{\text{picric acid}}$ ethylene glycol dihydrogen phosphate 1.52; glycerol dihydrogen phosphate 1.37; inorganic phosphate 1.61. The reaction mixture from *N*-acetylglucosamine 4-phosphate contained these three compounds, that from the 6-phosphate only the first.

(*B*) The residue obtained after hydrolysis of the *N*-acetylsugar phosphates as described in (*A*) was dissolved in water (0.2 ml) and made alkaline (pH 9) by addition of aqueous sodium hydroxide. The cooled solution was added dropwise to a stirred, cooled solution of sodium borohydride (10.5 mg) in water (0.3 ml) and the mixture was left to come to room temperature overnight. Acetic anhydride (18 μl) was added followed, after 2 h, by a solution of sodium metaperiodate (44 mg) in water (0.7 ml). After 2 h, the reaction mixture was cooled and added dropwise to a cooled, stirred solution of sodium borohydride (57 mg) in water (1 ml). Next morning, the precipitate (free from phosphorus) which formed was centrifuged off and the supernatant was acidified with Amberlite IRN 77 ($H^+$) resin. The resin was filtered off, and the filtrate was extracted with ether to

remove free iodine and concentrated to dryness. The residue was evaporated several times with methanol before being dissolved in a little water and submitted to paper electrophoresis. Ethylene glycol dihydrogen phosphate was the only phosphorylated product detected in the residue derived from the 6-phosphate. Together with ethylene glycol dihydrogen phosphate and inorganic phosphate, the mixture from the 4-phosphate contained a phosphorylated 5-carbon polyol, $R_{\text{picric acid}}$ 1.2 (*N*-acetylglucosaminitol phosphate 1.15).

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