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SYNTHESIS OF 6-DEOXY-6-SULFO-α-D-GLUCOPYRANOSYL PHOSPHATE*

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ABSTRACT

6-Deoxy-6-sulfo-α-D-glucopyranosyl phosphate was obtained as the crystalline tris-cyclohexylammonium salt from 6-*O*-acetyl-2,3,4-tri-*O*-benzyl-D-glucopyranose by the following sequence of reactions: conversion into the glycosyl trichloroacetimidate, reaction with dibenzyl hydrogen phosphate, *O*-deacetylation, trifluoromethanesulfonylation, reaction with tetrabutylammonium hydrogensulfide, oxidation with *m*-chloroperbenzoic acid, and hydrogenolysis of the benzyl groups.

INTRODUCTION

"Sulfolipid", synthesized by plants, is a 1,2-di-*O*-acyl-3-*O*-(6-deoxy-6-sulfo-α-D-glucopyranosyl)-*sn*-glycerol. Its structure was elucidated by Benson and coworkers[2] and confirmed by synthesis[3]. Several aspects of the biosynthesis of this compound, particularly the introduction of sulfur and the combination of the sulfosugar with the diacylglycerol moiety, are not clear. It has been suggested[4] that, by analogy with the biosynthesis of many other glycosides, a sulfo-sugar nucleotide (**1**) could be involved in the biosynthesis although other possibilities have been discussed[5] and investigated[6]. The availability of such sulfo-sugar nucleotides would allow the above proposal to be investigated. Therefore, the synthesis was undertaken of a set of nucleotides containing 6-deoxy-6-sulfo-D-glucose (sulfoquinovose). Since the existing methods for the chemical synthesis of sugar nucleotides[7] require the corresponding sugar 1-phosphates, an important intermediate for this approach is 6-deoxy-6-sulfo-α-D-glucopyranosyl phosphate (**2**). A crucial step in the synthesis of **2** is the preparation of the anomerically pure α-phosphate ester, which was accomplished by using the trichloroacetimidate method[8,9]. We now describe the synthesis of the title compound. The preparation of nucleoside di-

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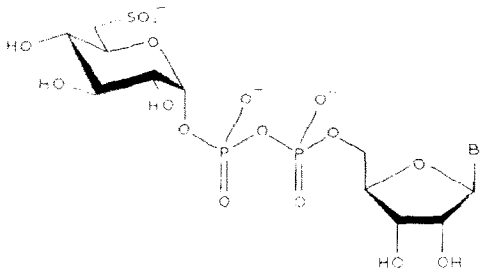
phospho sulfoquinovoses and their use in biological investigations will be described elsewhere[10].



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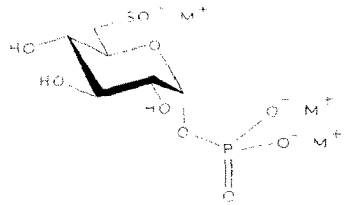

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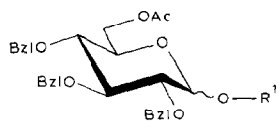
RESULTS AND DISCUSSION

6-Deoxy-6-sulfo-D-glucose and some derivatives have been synthesized[3,11]. The sulfonic acid residue was introduced by nucleophilic displacement of iodo, tosyl, or triflyl derivatives, or by the addition of sulfite to 6-deoxyhex-5-enopyranosides. The synthesis of sulfoquinovosyl phosphate has not been reported hitherto, and the following sequence was used.

6-*O*-Acetyl-2,3,4-tri-*O*-benzyl-D-glucopyranose (**3**), obtained from 1,6-di-*O*-acetyl-2,3,4-tri-*O*-benzyl-D-glucopyranose[12] by reaction with hydrazine acetate[13], was treated with trichloroacetonitrile to give the trichloroacetimidate **4** as a ~1:3 $\alpha\beta$-mixture. Displacement of the trichloroacetimidate group by dibenzyl hydrogen phosphate was fast and nearly quantitative, but yielded a substantial proportion of the β anomer $\alpha\beta$-ratio, (~3:1) which would interfere in the subsequent synthesis. Therefore, *p*-toluenesulfonic acid was incorporated in the reaction to promote anomerisation[8], and 98% of the more stable α-anomeric product **5** was obtained. The n.m.r. data confirmed the structure of **5**. In t.l.c., compounds **5**–**9**, each of which contains a protected phosphate ester group, are conveniently detected by a colour reagent specific for phosphate esters[14] (see Experimental).

Treatment of **5** with K_2CO_3 in methanol selectively removed the 6-*O*-acetyl group and demonstrated the expected stability of the phosphate triester group under these acyl transfer conditions. The product **6** is unstable under acid conditions and the presence of triethylamine was required during chromatography on silica gel.

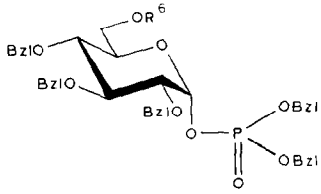
Chromatography of **6** was not necessary since it was sufficiently pure to be used in the subsequent reaction. A similar practice could be followed with **7**, which was generated rapidly and quantitatively from **6** at low temperature on the addition of triflic anhydride. Displacement of the triflyl group by tetrabutylammonium hydrogensulfide, which is soluble in organic solvents, gave **8**. The disulfide structure was deduced from the mass spectrum which contained an appropriate molecular ion. Apparently, the thiol formed initially is oxidized rapidly by molecular oxygen. The same phenomenon was observed after liberation of the thiol group from the 6-thioacetate during the synthesis of the sulfolipid[3].



3 R^1 = H

4 R^1 = C(=NH)—CCl_3

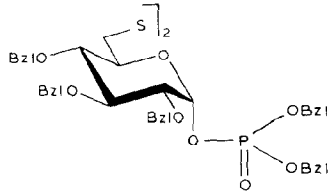
R = Bzl



5 R^6 = Ac

6 R^6 = H

7 R^6 = CF_3SO_2



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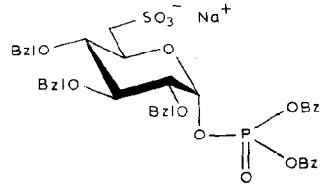

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m-Chloroperbenzoic acid was used to oxidize the disulfide **8** to the sulfonic acid **9**. The downfield location of the signals for H-6,6′ in the ^{1}H-n.m.r. spectrum reflected the presence of the sulfonic acid residue at this position. Hydrogenolysis of **9** in aqueous 1,4-dioxane removed the benzyl groups and gave a strongly acidic product. Despite repeated adjustment of the pH during the reaction, which was extended to 4 days, the product contained some free phosphate. Compound **9** was converted into the H^+ form, and titrated to pH 7.0 with cyclohexylamine. It is important that this pH, despite its proximity to the pKa_2 (see Table I), is not exceeded, otherwise crystallization will yield **2** heavily contaminated by inorganic phosphate.

TABLE I

PHYSICOCHEMICAL PROPERTIES OF SUGAR PHOSPHATES[a]

Compound	*pKa_2′*	*pKa_2*	*k × 10^{-4} (min^{-1})*	*$t_{1/2}$ (min)*	*$[\alpha]^{20}_{589}$ (degrees)*	*$[M]^{20}_{589}$ (degrees)*
6-Deoxy-6-sulfo-α-D-glucopyranosyl phosphate (**2**)	6.59 (3)	6.77	72 ±3 (5)	96	+37[b]	+230
α-D-Glucopyranuronic acid 1-phosphate	6.61 (3)	6.74	3.6 ±0.1 (4)	1930	+51[c]	+245
α-D-Glucopyranosyl phosphate	6.24 (4)	6.42	250 ±3 (5)	28	+78[d]	+290

[a]The pKa_2 values were calculated from the measured pKa_2′ values (see Experimental). The number of independent experiments is given in brackets and the mean variation was ±0.01: *k* is the first-order rate constant for hydrolysis of the phosphate ester bond in M HCl at 40°, and $t_{1/2}$ is the corresponding half life. Optical rotations were measured on aqueous solutions: [M] = [α] × mol. wt. × 0.01. [b]As the tris-cyclohexylammonium salt. [c]As the tri-potassium salt. [d]As the di-potassium salt dihydrate.

In t.l.c. with detection by a modified Hanes–Isherwood reagent, **2** gives a blue colour due to the inorganic phosphate which is liberated by the increasing concentration of HNO_3 during drying. The response of **2** to the reagent was significantly slower than that of glucosyl phosphate, but faster than that of glucuranosyl phosphate, in accord with their rate constants of acid hydrolysis (Table I), and reflecting the inductive effect of the 6-substituent. Rate constants for acid hydrolysis have been determined[15] and show that glucuronosyl phosphate is more stable than glucosyl phosphate. However, since the conditions differed from those used here, comparison of the data is not possible. The hydrolysis experiments showed that **2** is stable at neutal pH, which is important for future biochemical work.

The pKa_2 values for the three sugar phosphate (Table I) differed far less than the rate constants for acid hydrolysis because the inductive effect of the 6-substituent is less pronounced due to the greater distance to the P–OH bonds. α-D-Glucopyranuronic acid and **2** have similar pKa_2 values, which are 0.3 pH unit higher than that of α-D-glucopyranosyl phosphate. The value (6.42) found for the latter compound is slightly lower than those [6.46 (ref. 16) and 6.51 (ref. 17)] determined previously. β-D-Glucopyranosyl phosphate[16] (and, most likely, the β forms of the two other sugar phosphates as well) is slightly more acidic than the α anomer (pKa_2 6.24).

EXPERIMENTAL

General. — Solvents were dried by refluxing and distilling from appropriate desiccants and stored over 4Å molecular sieves. Light petroleum refers to fraction b.p. 40–60°. Solvents were removed in a rotatory evaporator under reduced pressure at 30–40° (bath). Melting points are uncorrected. N.m.r. spectra (internal Me_4Si for ^1H and ^{13}C, external H_3PO_4 for ^{31}P) were recorded with Bruker WM 250 Cryospec (^1H) and Jeol JNM-GX 400 instruments (^{13}C and ^{31}P). Mass spectra were obtained with a Varian MAT CH-7 instrument. Optical rotations were measured with a Perkin–Elmer 241 MC polarimeter.

Flash chromatography was carried out with Silica Gel 7024/1 (Baker), using solvents purified by distillation. T.l.c. was carried out on Silica Gel 60 F_{254} (Merck) with detection by u.v. light or by charring with sulfuric acid. Protected phosphate esters gave a blue colour with the phospholipid reagent[14]. Unprotected sugar phosphates gave a blue colour with modified Hanes–Isherwood solution[18]. This solution is stable for only a few hours, hence two separate and stable solutions were prepared and equal volumes were mixed just before use. The solutions were (*a*) ammonium heptamolybdate (2 g) in aqueous 70% HNO_3 (21 mL), water (2 mL), and methanol (80 mL); and (*b*) ascorbic acid (2 g) and anthranilic acid (2 g) in methanol (100 mL). These solutions are stable for many weeks when kept in the dark. Inorganic phosphate in the presence of labile organic phosphate esters was quantitated colorimetrically at 720 nm by the Fiske–Subbarow method[19].

The presence of sugar or sugar phosphates in the eluates of ion-exchange

columns was detected with anthrone[20]: the eluate (0.1 mL) was mixed rapidly with 2 vol. of anthrone solution (30 mg in 10 mL of conc. sulfuric acid). The green colour developed from sulfoquinovosyl phosphate had λ_{max} 588 nm (*cf.* 617 nm for glucose[18]). α-D-Glucopyranuronic acid 1-phosphate gave a violet colour with the reagent.

6-O-Acetyl-2,3,4-tri-O-benzyl-D-glucopyranose (**3**). — To a solution of 1,6-di-*O*-acetyl-2,3,4-tri-*O*-benzyl-D-glucopyranose[12] (5.29 g, 9.90 mmol) in dry *N,N*-dimethylformamide (200 mL) was added hydrazinium acetate[13] (1.09 g, 10.19 mmol) at room temperature. After 4 h, the mixture was poured into water and extracted with ether (2 × 100 mL), and the combined extracts were washed with water, dried ($MgSO_4$), and concentrated. Flash chromatography (2:1 light petroleum–ethyl acetate) gave αβ-**3** (4.0 g, 82%), isolated as a colourless oil, R_F 0.21. ^{1}H-N.m.r. data (250 MHz, $CDCl_3$): δ 7.34–7.25 (m, 15 H, 3 Ph), 5.20–3.35 (m, 13 H), 3.29 (d, 0.5 H, HO-1), 2.95 (s, 0.5 H, HO-1), 2.04, 2.02 (2 s, Ac).

This compound was directly used for the formation of **4**.

6-O-Acetyl-2,3,4-tri-O-benzyl-D-glucopyranosyl trichloroacetimidate (αβ-**4**). — To a solution of αβ-**3** (12.7 g, 25.7 mmol) in dry dichloromethane (100 mL) was added trichloroacetonitrile (26.2 mL, 258 mmol) and K_2CO_3 (14.8 g, 25.8 mmol) at room temperature. After 6 h, the solvent was evaporated and flash chromatography (2:1 light petroleum–ethyl acetate) of the residue gave αβ-**4** [12.1 g, 74%; αβ-ratio, 1:3 (n.m.r. data)]; R_F (3:1 toluene–ethyl acetate) 0.61 (α-**4**) and 0.55 (β-**4**). ^{1}H-N.m.r. data (250 MHz, $CDCl_3$): δ 8.70 (s, 0.75 H, NH-β), 8.61 (s, 0.25 H, NH-α), 7.32–7.24 (m, 15 H, 3 Ph), 6.46 (d, 0.25 H, $J_{1,2}$ 3.7 Hz, H-1α), 5.81 (d, 0.75 H, $J_{1,2}$ 7.3 Hz, H-1β), 4.97–3.64 (m, 12 H), 2.02 (s, 0.75 H, Ac-α), 2.00 (s, 2.25 H, Ac-β).

Anal. Calc. for $C_{31}H_{32}Cl_3NO_7$: C, 58.46; H, 5.06; N, 2.2. Found: C, 58.02; H, 5.07; N, 2.0.

6-O-Acetyl-2,3,4-tri-O-benzyl-α-D-glucopyranosyl dibenzyl phosphate (**5**). — To a solution of αβ-**4** (12.1 g, 19.0 mmol) in dry dichloromethane was added dibenzyl hydrogen phosphate (5.3 g, 19.1 mmol) and freshly distilled, dry *p*-toluenesulfonic acid (0.5 g) at room temperature. After 6 h, the solvent was evaporated and flash chromatography (3:1 toluene–ethyl acetate) of the residue gave **5** (14.2 g, 98%), isolated as a colourless oil, $[\alpha]_{589}^{20}$ +66.5° (*c* 1, chloroform); R_F (3:1 toluene–ethyl acetate) 0.30. N.m.r. data ($CDCl_3$): ^{1}H (250 MHz), δ 7.33–7.24 (m, 25 H, 5 Ph), 5.93 (dd, 1 H, $J_{1,2}$ 3.4, $J_{1,P}$ 7.0 Hz, H-1), 5.09–4.52 (m, 10 H, 5 PhCH_2), 4.18–3.90 (m, 4 H), 3.52–3.36 (m, 2 H), 1.92 (s, 3 H, Ac); ^{31}P (161.7 MHz), δ −1.59.

Anal. Calc. for $C_{43}H_{45}O_{10}P$: C, 68.61; H, 6.03. Found: C, 68.63; H, 6.05.

2,3,4-Tri-O-benzyl-α-D-glucopyranosyl dibenzyl phosphate (**6**). — To a solution of **5** (33.0 g, 43.8 mmol) in dry dichloromethane (250 mL) and dry methanol (40 mL) was added freshly dried K_2CO_3 (10 g) at room temperature. After 1 h, more methanol (40 mL) and K_2CO_3 (10 g) were added. After 2 h, t.l.c. indicated complete conversion into **6**. The mixture was filtered through Kieselguhr and evaporated to dryness to yield **6** (30 g, 96%) which was sufficiently pure for the

next step. An analytical sample, purified by flash chromatography (1:2 light petroleum–ethyl acetate containing 1% of triethylamine), was isolated as a colourless, unstable oil, R_F 0.51. N.m.r. data ($CDCl_3$): ^1H (250 MHz), δ 7.32–7.25 (m, 25 H, 5 Ph), 5.90 (dd, 1 H, $J_{1,2}$ 3.4, $J_{1,P}$ 6.7 Hz, H-1), 5.92–4.61 (m, 10 H, 5 PhCH_2), 3.97–3.55 (m, 6 H), 2.06 (bs, 1 H, OH); ^{31}P (161.7 MHz), δ +1.28.

2,3,4-Tri-O-benzyl-6-O-trifluoromethanesulfonyl-α-D-glucopyranosyl dibenzyl phosphate (**7**). — To a solution of **6** (30.0 g, 42.2 mmol) in dry dichloromethane (250 mL) and triethylamine (10.0 mL, 72 mmol) was added a solution of trifluoromethanesulfonic anhydride (8.5 mL, 52 mmol) in dry dichloromethane (40 mL) at −15°. After removal of the solvents and concentration for 2 h under high vacuum, **7** (36.0 g, 97%), R_F 0.2 (3:1 toluene–ethyl acetate), was obtained which was sufficiently pure to be used immediately in the next step.

6,6-Dithiobis(2,3,4-tri-O-benzyl-6-deoxy-α-D-glucopyranosyl dibenzyl phosphate) (**8**). — A solution of **7** (5.0 g, 5.9 mmol) in dry dichloromethane (30 mL) was stirred with tetrabutylammonium hydrogensulfide (Fluka AG; 2.8 g, 10 mmol) at room temperature. After 2 h, the mixture was diluted with toluene (200 mL), washed with water (3 × 100 mL), dried (Na_2SO_4), and concentrated. Flash chromatography (4:1 light petroleum–ethyl acetate) of the residue gave **8** (3.0 g, 70%) isolated as an oil, R_F 0.60 (3:1 toluene–ethyl acetate), $[\alpha]_{589}^{20}$ +7.2° (*c* 0.5, chloroform). ^1H-N.m.r. data ($CDCl_3$): ^1H (250 MHz), δ 7.36–7.25 (m, 50 H, 10 Ph), 5.93 (dd, 2 H, $J_{1,2}$ 3.4, $J_{1,P}$ 7.0 Hz, 2 H-1), 5.11–4.60 (m, 20 H, 10 PhCH_2), 3.97–3.87 (m, 4 H), 3.63–3.56 (m, 4 H), 2.8–2.5 (m, 4 H, 2 H-6,6′); ^{31}P (161.7 MHz), δ −1.59. Mass spectrum (70 eV): *m/z* 1451 M^+).

Anal. Calc. for $C_{82}H_{84}O_{16}P_2S_2$: C, 67.85; H, 5.83. Found: C, 67.52; H, 5.99.

2,3,4-Tri-O-benzyl-6-deoxy-6-sulfo-α-D-glucopyranosyl dibenzyl phosphate sodium salt (**9**). — To a solution of **8** (16.0 g, 22 mmol) in dry dichloromethane (200 mL) was added sodium acetate (6.0 g, 73 mmol), and the stirred mixture was cooled to 0°. A solution of *m*-chloroperbenzoic acid (11.5 g, 66 mmol) in dry dichloromethane (170 mL) was added dropwise with vigorous stirring and cooling during 1 h. The mixture was stirred for 1 h at room temperature, then $CHCl_3$ (130 mL) and methanol (250 mL) were added, and the mixture was washed with aqueous 5% $NaHCO_3$ (170 mL), dried (Na_2SO_4), and concentrated. Flash chromatography (20:1 chloroform–methanol) of the residue gave **9** (13.0 g, 74%), R_F 0.3 (10:1 chloroform–methanol), which was sufficiently pure for the next step. N.m.r. data (Me_2SO): ^1H (250 MHz), δ 7.57–7.26 (m, 25 H, 5 Ph), 5.95 (dd, 1 H, $J_{1,2}$ 3.1, $J_{1,P}$ 7.8 Hz, H-1), 5.30–4.62 (m, 10 H, 5 PhCH_2), 4.30 (dd, 1 H, $J_{4,5} = J_{5,6}$ = 9 Hz, H-5), 3.80 (dd, 1 H, $J_{2,3} = J_{3,4}$ = 9 Hz, H-3), 3.69–3.65 (m, 1 H, H-2), 3.40–3.34 (dd, 1 H, H-4), 2.97 (d, 1 H, $J_{6,6'}$ 13.7 Hz, H-6′), 2.73 (dd, 1 H, $J_{5,6}$ 9, $J_{6,6'}$ 13.7 Hz, H-6); ^{31}P (161.7 MHz), δ −1.86.

6-Deoxy-6-sulfo-α-D-glucopyranosyl phosphate tris-cyclohexylammonium salt (**2**). — A solution of **9** (11.0 g, 13.8 mmol) in 1,4-dioxane (80 mL) was diluted with water (80 mL), and stirred under hydrogen in the presence of 10% Pd/C (10 g). The pH of the mixture was adjusted to 5.5 at daily intervals by the addition of

aqueous KOH. After 4 days, the mixture was diluted with water and centrifuged (15 min, 1000 r.p.m.), and the supernatant solution was filtered and passed through a column of Amberlite IR-120 (H^+) resin (20–50 mesh, 2 mequiv/mL, 200-mL bed volume). Elution with water was continued until the pH of the eluate was neutral. The combined eluates were titrated to pH 7.00 with a solution of cyclohexylamine in 1,4-dioxane, and the neutralized solution (800 mL) was concentrated to a small volume, diluted with acetone until crystallization started, and kept overnight at ~4°. The crystals were collected and recrystallized from water–acetone to give **2** (2.0 g). The hydrogenolysis was monitored by t.l.c. in either chloroform–methanol (10:1), to follow the disappearance of **9**, or in ethanol–H_2O–acetic acid (30:10:1), to show the appearance of **2** (R_F 0.6).

The mother liquors, which contained **2** contaminated by inorganic phosphate and a sugar of higher mobility (probably 6-deoxy-6-sulfo-D-glucopyranose), were concentrated to a small volume and diluted with water to 100 mL, and the pH was adjusted to 4.0 with dilute HCl. Part (50 mL) of this solution was applied to a column (1.3 × 13 cm) of Dowex 1-X2 (Cl^-) resin (200–400 mesh) equilibrated in 10^{-4}M HCl and washed with 130mM LiCl (150 mL) in 10^{-4}M HCl. Elution with 220mM LiCl (150 mL) then gave **2**. The second 50 mL of the mother liquors were treated in the same way. The combined final solutions were adjusted to pH 7.0 with dilute LiOH and concentrated under reduced pressure, and a solution of the residue in water (8 mL) was treated[21] (2-mL portions) with 4:1 acetone–ethanol (35 mL) and then centrifuged (15 min, 1000 r.p.m.). Each sediment was dissolved in water (2 mL) and reprecipitated as described above. After the fifth precipitation, Cl^- could not be detected in the supernatant solution or the sediments. Part (75%) of the combined solutions of the tri-lithium salt of **2** (1.3 mmol as determined by the anthrone reaction) was passed through a column of Amberlite IR-120 (H^+) resin to give the tris-cyclohexylammonium salt of **2**. Crystallization from aqueous acetone, as described above, gave more crystalline **2** (2.4 g, 3.8 mmol). The total yield of **2** was 8.3 mmol (60%), $[\alpha]_{589}^{20}$ +37° (*c* 1, water). N.m.r. data (D_2O): ^1H (250 MHz), δ 5.21 (dd, 1 H, $J_{1,2}$ 3.1, $J_{1,P}$ 7.6 Hz, H-1), 4.00 (m, 1 H), 3.55 (dd, 1 H, $J_{2,3} = J_{3,4}$ = 9.7 Hz, H-3), 3.22 (dd, 1 H, $J_{1,2}$ 3.1, $J_{2,3}$ 9.7 Hz, H-2), 3.21–2.88 (m, 6 H, 3 C*H*ND_3, 3 sugar H), 1.73–0.9 (m, 30 H, 15 CH_2); ^{31}P (161.7 MHz), δ 3.22; ^{13}C (100.54 MHz), δ 94.3 (d, 3.3 Hz, C-1), 74.2, 74.1, 73.5, 73.4, 73.3, 69.8, 53.1, 51.3, 51.2, 49.4, 49.2, 49.0, 48.8, 48.6, 31.3, 31.2, 31.1, 25.2, 25.1, 24.7, 24.5.

Anal. Calc. for $C_{24}H_{52}N_3O_{11}PS$: C, 46.37; H, 8.43; N, 6.76; O, 28.31; P, 4.98; S, 5.16. Found: C, 46.16; H, 8.40; N, 6.66; P, 4.94; S, 5.31.

Determination of pKa_2 values. — Separate solutions of α-D-glucopyranuronic acid 1-phosphate (crystalline tripotassium salt pentahydrate from Sigma; 22–35 μmol), α-D-glucopyranosyl phosphate (crystalline dipotassium salt monohydrate from Sigma; 80–190 μmol), and tris-cyclohexylammonium salt **2** (30–70 μmol) in water (8–13 mL) were each mixed with 0.5 equiv. of HCl (1.1–9.5 mL of a 10mM solution). The pH values (6.21–6.62, corresponding to pKa_2') were measured with a glass electrode at 25° and used for calculation of pKa_2 by application of published

correction terms[16,17,22]. The same results were obtained when each of the sugar phosphates was first converted into the acid forms by passage through a small column of Amberlite IR-120 (H^+) resin and reading the pH values after the addition of 1.5 (glucose 1-phosphate) or 2.5 equiv. (the other 1-phosphates) of 10mM NaOH.

Determination of rate constants for acid hydrolysis. — A solution of each sugar phosphate (0.65mM, 3.5 mL total volume) in M HCl was kept at 40°. After an appropriate time (5–30 min for glucose 1-phosphate, 10–120 min for **2**, and 3–49 h for glucuronic acid 1-phosphate), aliquots (0.4 mL) were withdrawn for colorimetric determination of released inorganic phosphate by using a standard curve covering the range of 30–150 nmol. Heating at neutral pH did not result in release of free phosphate.

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