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Studies of alkaline mediated phosphate migration in synthetic phosphoethanolamine L-*glycero*-D-*manno*-heptoside derivatives

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Abstract

Synthetic 2-, 3-, 4- and 6-monophosphate derivatives of methyl α-D-mannopyranosides, the 4-, 6- and 7-monophosphate derivatives of methyl L-*glycero*-α-D-*manno*-heptopyranosides and the corresponding phosphoethanolamine derivatives and a 6,7-cyclic phosphate analogue of methyl L-*glycero*-α-D-*manno*-heptopyranoside were used to study phosphate migration and hydrolysis when subjected to strong alkaline conditions (4 M KOH, 120 °C, 18 h). The resulting products were analyzed by ^{1}H NMR spectroscopy and electrospray mass spectrometry. It was found that phosphate substituents were stable under these conditions and neither migration nor hydrolysis was observed except for the heptose 7-phosphate, which gave a substantial amount of phosphate hydrolysis. In phosphoethanolamine-substituted compounds migration to adjacent positions with concomitant loss of ethanolamine was found together with hydrolysis.

Keywords: Sugar phosphates; Phosphate migration; Structural analysis; Lipopolysaccharides

1. Introduction

The inner-core region in lipopolysaccharides (LPS) of Gram-negative bacteria most often contains L-*glycero*-D-*manno*-heptose residues, which are commonly substituted by phosphate (*P*) and phosphoethanolamine (*P*Etn) groups [1]. Recently, phosphocholine (*P*Chol) was also identified as a substituent in *Haemophilus influenzae* LPS [2]. Phosphate groups are known to be important in maintaining the integrity of outer membranes in Gram-negative bacteria [3], but their role in biological and immunological functions is not clear. However, recent studies have implicated the presence of phosphate substituents as potential triggers that control the biosynthesis of important oligosaccharide epitopical structures [4]. Structural analysis of LPS requires complete identification of the number and sites of these substituents in order to determine their role in biological function. It has been observed that ethanolamine was lost from *P*Etn-substituted heptose with concomitant migration of the phosphate group when LPS was subjected to strong alkaline treatment [2,5,6]. Moreover, choline was observed to be lost from *P*Chol when LPS of *H. influ-*

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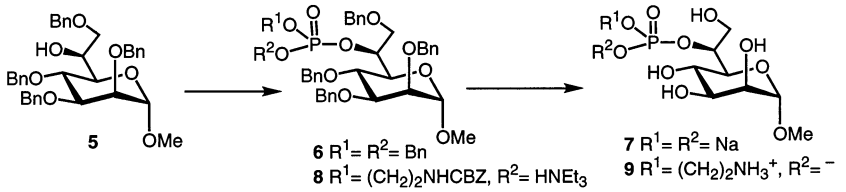


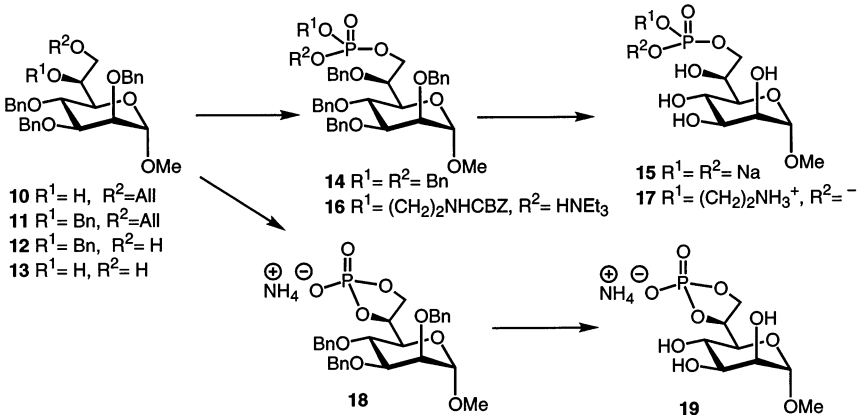
Scheme 1.

enzae was treated under strong alkaline conditions [2]. Migration of appended substituents due to experimental conditions is a well-known problem. The migration of acetate esters has been studied, and it was found that they migrate under basic conditions to the thermodynamically more favored positions in a sugar residue, but are more stable under acidic conditions [7,8]. Strong alkaline treatment of LPS is a standard procedure in structural analyses to remove N- and O-linked fatty acids. It was thus of interest to obtain more insight into the stability of *P* and *P*Etn groups under those conditions and to find experimental conditions where no degradation or migration occurred. For this reason monophosphorylated D-mannose derivatives were subjected to strong alkaline treatment. Monophosphorylated L,D-heptose derivatives with and without ethanolamine and a 6,7-cyclic phosphate analog were synthesized and treated in the same manner. The resulting products were analyzed by NMR spectroscopy and electrospray mass spectrometry (ESMS).

2. Results and discussion

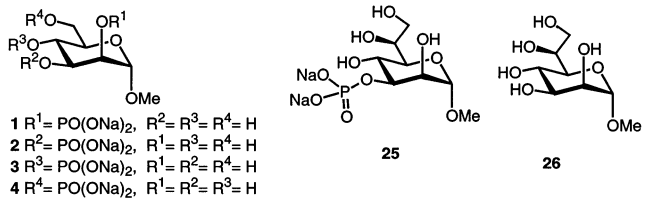
The synthesis of methyl α-D-mannopyranoside 2-phosphate (**1**), methyl α-D-mannopyranoside 3-phosphate (**2**), methyl α-D-mannopyranoside 4-phosphate (**3**), and methyl α-D-mannopyranoside 6-phosphate (**4**) has been described earlier [9].

The syntheses of the phosphate and phosphoethanolamine substituted methyl L,D-heptosides and the 6,7-cyclic phosphate derivative are summarized in Schemes 1–3. The one-carbon elongations to obtain the variously protected heptose precursors were accomplished by a Grignard reaction using benzyloxy- or allyloxymethyl chloride and methyl 2,3,4-tri-*O*-benzyl-1,6-dialdo-α-D-mannopyranoside. In contrast to earlier similar syntheses by Grzeszczyk and Zamojski [10], the reactions were performed using Barbier reaction conditions, i.e., the chloride and the aldehyde were added simultaneously to the magnesium turnings [11]. The yields and stereoselectivities were comparable to the previous ones, but in our hands the Barbier variant made the elongation less sensitive to reaction conditions, and, furthermore, less of an excess of the toxic chloride could be used, which also simplified purification. Thus, heptose derivatives **5** and **10** were prepared in good yields and with high stereoselectivity from the known mannose precursor. To allow the synthesis of 7- and cyclic 6,7-phosphorylated derivatives, compound **10** was benzylated and deallylated or directly deallylated to give intermediates **12** and **13**, respectively. In order to synthesize the 4-phosphorylated derivatives, compound **5** was debenzylated and treated with dimethoxypropane to give **20**. To obtain the 6-, 7- and 4-monophosphates, compounds **5**, **12** and **20** were treated with PCl_3, imidazole and Et_3N followed by benzyl alcohol to give the dibenzyl phosphites. These compounds were in situ oxidized by *m*-chloroperoxybenzoic acid to yield the dibenzylphosphates **6**, **14** and **21**, respectively, which were catalytically hydrogenolyzed, and **21** also further treated with aqueous acid to complete deisopropylidenation, to give the corresponding deprotected monophosphates **7**, **15** and **22**. Treatment of the diol **13** with commercial methyl dichlorophosphate followed by basic work-up afforded **18**, which was deprotected to give the cyclic 6,7-phosphate **19**. Initially, an approach using bis(*N*,*N*-diisopropylamino)benzyloxyphosphine [12] and 1H-tetrazole was employed for the synthesis of the ethanolaminephosphate compounds. This procedure, however, gave only moderate yields of the secondary 4-



Scheme 2.

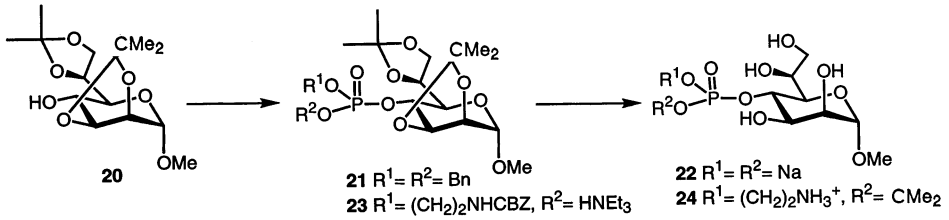
and 6-phosphates **24** (40%) and **9** (48%) and proved inefficient for the preparation of the primary 7-phosphoethanolamine derivative **17**. Hence, the H-phosphonate method was tried instead. Pivaloyl chloride-promoted coupling of *N*-benzyloxycarbonyl-protected ethanolamine phosphonate [13] to **5**, **12** and **20**, respectively, followed by oxidation by iodine in aqueous pyridine gave intermediates **8** (84%), **16** (75%) and **23** (89%), respectively. The latter compounds were deprotected to give the target compounds **9** (94%), **17** (82%) and **24** (79%), respectively.



The monophosphates **1**–**4**, **7**, **15** and **22**, the cyclic phosphate **19** and the ethanolaminephosphates **9**, **17** and **24** were used as model substances for studying the stability of these substituents under alkaline conditions. All samples were subjected to the strong alkaline treatment that is used in *N*/*O*-deacylation of LPS, i.e., 4 M KOH, 18 h, 120 °C. The reaction mixtures were neutralized, subjected to gel-permeation chromatography and the obtained products were analyzed by NMR and ESMS.

It was evident that the monophosphorylated compounds **1**–**4**, **7**, and **22** were not affected by the alkaline treatment. The resulting products were identical with the starting materials. The 7-monophosphate **15,** however, showed a substantial amount of the phosphate hydrolysis product **26** (36%), indicating a mechanism involving a 6,7-anhydro intermediate [14] (Scheme 4), since the other primary monophosphate **4** was inert.

The heptose 4-, 6- and 7-phosphoethanolamine derivatives **24**, **9** and **17** gave mixtures of components after the alkaline treatment as revealed by NMR spectroscopy. ESMS, run in the negative mode, of the products showed pseudomolecular ions at m/z 303 corresponding to monophosphorylated methyl heptosides ($C_8H_{17}O_{10}P$), which clearly indicated the loss of ethanolamine from the starting materials. The products of **24** were identified to be the 4-phosphate **22** and the 3-phosphate **25** as characterized by NMR. The products of **9** were identified to be the 6-phosphate **7** and the 7-phosphate **15**. The products of **17** were identified also to be the 6-phosphate **7** and 7-phosphate **15**. In addition dephosphorylation of **24**, **9** and **17** was evident since methyl L,D-heptoside **26** could be identified in the ^{1}H NMR spectra and inorganic phosphate in the ^{31}P NMR spectra. The relative proportions of the resulting products are given in Table 1. Thus, in all the phosphoethanolamine derivatives, the phosphate groups partly migrated to the adjacent positions with concomitant loss of ethanolamine. Migration was found to occur also when compound **9** was treated with KOH at lower concentration (i.e., 2 M) and under even milder reaction conditions (2 h, 60–100 °C).



Scheme 3.

Similar migrations are known to proceed via a cyclic phosphate intermediate, where a five-membered ring intermediate is preferred over a six-membered one [14,15]. Our results were in accordance with this, as only $4 \rightarrow 3$ and $6 \leftrightarrow 7$, and no $4 \leftrightarrow 6$ migrations were observed, together with the similarity in the product distribution of the hydrolysis of **9** and **17**. To further verify this, the 6,7-cyclic phosphate **19** was synthesized and subjected to alkaline treatment. The products obtained were the 6-phosphate **7**, the 7-phosphate **15** and the non-phosphorylated **26** in the molar ratio 50:33:17 (Table 1), the latter product probably formed through phosphate hydrolysis of **15** as found above. Thus, the 6,7-cyclic phosphate opens up to give about a 1:1 mixture of the 6 and the 7-phosphate of which the latter then is partly hydrolyzed to give **26**. The product ratios obtained from the alkaline treatment of **9** and **17** are very similar (Table 1). Hence, the basic hydrolysis of **9** and **17** seems to proceed almost exclusively through the cyclic intermediate **19** as depicted in Scheme 4.

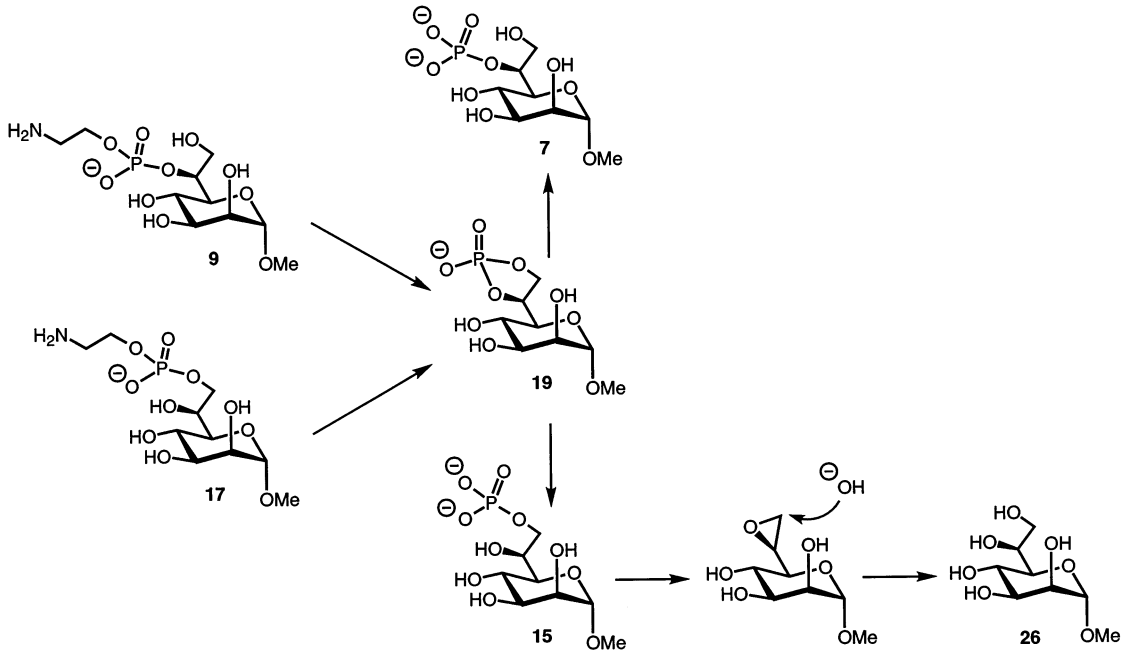
A primary objective of this study was to find experimental conditions of alkaline treatment of LPS in which no degradation of *P*Etn and migration of *P* occurs. For this reason LPS from a strain of *Neisseria meningitidis* [5] was subjected to different *N*/*O*-deacylation conditions (4 M KOH, 2–18 h, 120 °C or 2 M KOH, 2 h, 60 °C). However, reducing the KOH concentration, reaction time or temperature did not change the fact that ethanolamine is lost from the heptose bearing the *P*Etn substituent as revealed by ESMS. It can be concluded that the strong alkaline conditions used for *N*/*O*-deacylation of LPS do not degrade the material if it is substituted with *P* but in case of substitution with *P*Etn and/or *P*Chol, information will be lost during that experiment. Thus, alternative ways of *N*/*O*-deacylation should be taken into consideration.

3. Experimental

General methods.—These were as described earlier [16]. Benzyloxymethyl chloride was synthesized according to Connor et al. [17], dried over $CaCl_2$ and stored without drying agent in a sealed container at −18 °C. The same procedure was also applied in the synthesis of allyloxymethyl chloride.

General procedure for the synthesis of the protected heptose dibenzyl phosphates.—A solution of phosphorus trichloride (350 μL, 4.01 mmol) in CH_2Cl_2 (5 mL) was added dropwise to a solution of imidazole (820 mg, 12.0 mmol) in CH_2Cl_2 (25 mL) at 0 °C, followed by Et_3N (1.76 mL, 12.6 mmol). The mixture was stirred for 10 min and the suitably protected monosaccharide (1.00 mmol) in CH_2Cl_2 (10 mL) was then added dropwise. After stirring for 1 h, benzyl alcohol (1.30 mL, 12.6 mmol) in CH_2Cl_2 (5 mL) was added and stirring was continued at 0 °C for 40 min. Recrystallized *m*-chloroperoxybenzoic acid (1.00 g, 5.79 mmol) in CH_2Cl_2 (10 mL) was added, and the mixture was stirred for 1.5 h at room temperature, then washed with aq 5% $Na_2S_2O_3$ (20 mL) and water (20 mL), dried (Na_2SO_4), and concentrated. The crude products were purified on silica gel columns.

General procedure for the synthesis of the protected heptose N-*(benzyloxycarbonyl)ethanolamine phosphates.*—Suitably protected heptose derivatives (1.00 mmol) and *N*-(benzyloxycarbonyl)ethanolamine triethylammonium H-phosphonate [12] (432 mg, 1.20 mmol) were concentrated from dry pyridine and dried on a vacuum pump for 1 h. The



Scheme 4. Suggested pathways for the alkaline treatment of **9**, **17** and **19**.

residue was dissolved in MeCN (6 mL) and pyridine (2 mL) under Ar, and cooled to 0 °C. Freshly distilled pivaloyl chloride (493 μL, 4.00 mmol) was added and the solution was stirred for 10 min at 0 °C and then for 50 min at room temperature, when TLC showed a complete conversion into a more hydrophilic product. The solution was diluted with toluene (50 mL) and washed with water (25 mL). After drying ($MgSO_4$) and concentration, the residue was dissolved in pyridine (5 mL) and cooled (0 °C). Water (100 μL) and iodine (279 mg, 1.10 mmol) were added and the solution was stirred for 1 h at 0 °C, diluted with EtOAc (60 mL) and washed with aq satd $Na_2S_2O_3$ (30 mL) and aq 1 M triethylammonium bicarbonate, pH 8 (50 mL). The combined aqueous phases were back-extracted with CH_2Cl_2 (2 × 25 mL for compounds **8** and **16**, and 5 × 25 mL for compound **23**). The organic phases were concentrated without drying, and purified by column chromatography using $CHCl_3$–MeOH containing 1% Et_3N.

Table 1
Products obtained for compounds **9**, **17**, **19** and **24** after alkaline treatment with 4 M KOH (120 °C, 18 h)

Compound	Products[a]		
9	**7** (57)	**15** (36)	**26** (7)
17	**7** (54)	**15** (26)	**26** (20)
19	**7** (50)	**15** (33)	**26** (17)
24	**22** (44)	**25** (49)	**26** (7)

[a] The ratios measured from signal areas of appropriate NMR resonances are given in parentheses.

Methyl 2,3,4,7-tetra-O-*benzyl*-L-glycero-α-D-manno-*heptopyranoside* (**5**).—To a flame-dried flask equipped with an internal thermometer, an efficient stirrer and two dropping funnels were added freshly activated magnesium turnings (1.11 g, 45.7 mmol) and sublimed $HgBr_2$ (132 mg, 0.37 mmol) under Ar. Benzyloxymethyl chloride (4.2 mL, 30.3 mmol) was dissolved in THF (30 mL) and a portion of this solution (approx. 5 mL) was added to the magnesium at room temperature via one of the dropping funnels. Once the exothermic reaction had started (as monitored by the temperature) the flask was partially immersed into an ice bath (0 °C) and crude methyl 2,3,4-tri-*O*-benzyl-1,6-dialdo-α-D-mannopyranoside [10] (7.52 g, 15.3 mmol) in THF (30 mL) and the alkyl halide solution were added simultaneously through the dropping funnels at such a rate that the internal temperature was kept between 20 and 24 °C

(approx. rate: 2 mL/min of each solution). After stirring overnight the mixture was diluted with Et_2O (200 mL) and freshly prepared aq satd NH_4Cl (250 mL) was added. The mixture was stirred for 2 h, when all the metal turnings had dissolved. The organic phase was separated, dried ($MgSO_4$), filtered, and concentrated. To a solution of the residue in $CHCl_3$ was added silica gel (ca. 15 g), and the suspension was carefully concentrated and dried under reduced pressure for at least 1 h. The silica gel was poured on top of a packed column, and elution with 6:1 toluene–EtOAc then gave **5** (6.65 g, 57%); $[\alpha]_D$ +13° (*c* 1.0, $CHCl_3$) lit. $[\alpha]_D$ +10.5° [18]; ^{13}C NMR ($CDCl_3$): δ 54.7 (MeO), 68.0, 70.8, 71.5, 72.2, 72.8, 73.3, 74.3, 74.5, 75.2, and 80.3 (C-2–7, Ph*C*H_2), 99.4 (C-1), 127.6–138.6 (aromatic).

Methyl L-glycero-α-D-manno-*heptopyranoside 6-(disodium phosphate)* (**7**).—Phosphorylation of 1.03 mg (1.75 mmol) of **5** according to the general procedure gave methyl 2,3,4,7-tetra-*O*-benzyl-6-*O*-dibenzyloxyphosphoryl-L-*glycero*-α-D-*manno*-heptopyranoside (**6**; 1.30 mg, 88%) after silica gel chromatography (two columns: 3:2 light petroleum ether [bp 40–65 °C]–EtOAc and 4:1 toluene–EtOAc). NMR: ^{13}C ($CDCl_3$), δ 54.7 (MeO), 69.1, 69.4 (d), 70.1 (d), 71.9, 72.7, 73.2, 73.8, 74.30 (d), 74.34, 74.8, and 80.7 (C-2–7, Ph*C*H_2), 99.1 (C-1), 127.4–138.7 (aromatic); ^{31}P (EtOAc), δ −0.5 (m). Compound **6** (641 mg, 0.759 mmol) dissolved in a mixture of absolute EtOH (25 mL) and aq 60% HOAc (4 mL) was hydrogenolyzed over Pd–C at 9 atm. After 3 days, the mixture was filtered and H_2O was added. The pH was adjusted to 9 by the addition of aq NaOH, and the aqueous phase was washed once with Et_2O. After a slight concentration under reduced pressure, the crude product was desalted on a Bio-Gel P-2 column eluted with H_2O containing 1% *n*-BuOH. Freeze-drying gave **7** (256 mg, 97%); $[\alpha]_D$ +41° (*c* 1.0, H_2O); NMR: ^{13}C (D_2O), δ 55.5 (MeO), 62.5, 66.5, 70.7, 70.8, 72.2 (d), and 72.4 (d) (C-2–7), 102.0 (C-1); ^{31}P (H_2O), δ 4.8 (s). HRMS Calcd for $C_8H_{17}O_{10}P$ [M + Na]: 327.0457. Found: 327.0467.

*Methyl 6-*O*-(2-aminoethyl)phosphoryl-*L*-glycero-α-*D*-manno-heptopyranoside* (**9**).—Following the general procedure, compound **5** (331 mg, 0.566 mmol) gave methyl 2,3,4,7-tetra-*O*-benzyl-6-*O*-(2-benzyloxycarbonylamino)ethyl-phosphoryl-L-*glycero*-α-D-*manno*-heptopyranoside, triethylammonium salt (**8**; 446 mg, 84%) after purification on silica gel (19:1 $CHCl_3$–MeOH + 1% Et_3N). NMR: ^{13}C ($CDCl_3$), δ 8.4 (Et_3N), 42.2 (d, CH_2CH_2N), 45.3 (Et_3N), 54.5 (MeO), 64.8 (d), 66.3, 68.6, 69.7 (d), 71.1, 72.0, 73.0, 73.8, 74.4, 75.3, and 80.8 (C-2–7, Ph*C*H_2, O*C*H_2CH_2), 98.9 (C-1), 127.2–139.2 (aromatic), 156.6 (CON); ^{31}P (9:1 EtOAc–$CHCl_3$), δ 0.8 (m). To a solution of **8** (295 mg, 0.313 mmol) in 4:3 THF–H_2O (14 mL) and HOAc (1 mL) was added a catalytic amount of $Pd(OH)_2$ on carbon. The mixture was hydrogenolyzed at 9 atm for 2 days, filtered (Celite), concentrated, and dissolved in H_2O (10 mL). The solution was set to neutral pH and washed once with Et_2O. Size-exclusion chromatography on a Bio-Gel P-2 column eluted with aq 0.1 mM pyridinium acetate buffer, pH 5.4 gave **9** (102 mg, 94%) after freeze-drying; $[\alpha]_D$ +26° (*c* 1.0, H_2O); NMR: ^{13}C (D_2O), δ 41.0 (d, CH_2CH_2N), 55.5 (MeO), 61.8, 63.3, 66.7, 70.6, 71.1, 71.3 (d), and 74.4 (d) (C-2–7, O*C*H_2CH_2), 101.9 (C-1); ^{31}P (H_2O), δ 0.5 (m). HRMS Calcd for $C_{10}H_{23}O_{10}NP$ [M + H]: 348.1060. Found: 348.1104.

*Methyl 7-*O*-allyl-2,3,4-tri-*O*-benzyl-*L*-glycero-α-D-manno-heptopyranoside* (**10**).—Methyl 2,3,4-tri-*O*-benzyl-1,6-dialdo-α-D-mannopyranoside (5.42 g, 11.7 mmol) was processed as described for the formation of **5** above, except that allyloxymethyl chloride (3.2 mL, 30.9 mmol) was used instead of benzyloxymethyl chloride, to yield, after silica gel chromatography (4:1 toluene–EtOAc), **10** (2.82 g, 45%); $[\alpha]_D$ +13° (*c* 1.0, $CHCl_3$); ^{13}C NMR ($CDCl_3$): δ 54.7 (MeO), 68.0, 70.9, 71.5, 72.3, 73.0, 74.4, 74.7, 75.3, and 80.4 (C-2–7, Ph*C*H_2, allyl CH_2), 99.5 (C-1), 117.2 (allyl), 127.7–138.7 (aromatic, allyl). Anal. Calcd for $C_{32}H_{38}O_7$: C, 71.89; H, 7.16. Found: C, 71.53; H, 7.23.

*Methyl 7-*O*-allyl-2,3,4,6-tetra-*O*-benzyl-*L*-glycero-α-D-manno-heptopyranoside* (**11**).—Compound **10** (5.44 g, 10.2 mmol) in DMF

(30 mL) was added dropwise to a suspension of hexane-washed NaH (1.85 g, 55–60% in mineral oil) and DMF (20 mL). The mixture was stirred for 1 h at room temperature, whereafter benzyl bromide (3.6 mL, 30.3 mmol) was added and stirring was continued for 90 min. MeOH (5 mL) was added carefully during 20 min. The mixture was diluted with toluene (200 mL) and washed with water (3x100 mL). The organic phase was filtered through a sintered glass funnel containing dry $MgSO_4$ and concentrated. Silica gel chromatography (9:1 toluene–EtOAc) gave **11** (5.96 g, 94%); $[\alpha]_D$ +25° (*c* 1.0, $CHCl_3$); ^{13}C NMR ($CDCl_3$): δ 54.7 (MeO), 70.0, 71.3, 72.0, 72.3, 72.5, 72.8, 74.3, 74.4, 74.6, 75.1, and 80.8 (C-2–7, Ph*C*H_2, allyl CH_2), 99.1 (C-1), 117.0 (allyl), 127.5–138.9 (aromatic, allyl). Anal. Calcd for $C_{39}H_{44}O_7$: C, 74.98; H, 7.10; Found: C, 74.60; H, 6.85.

Methyl 2,3,4,6-tetra-O-*benzyl*-L-glycero-α-D-manno-*heptopyranoside* (**12**).—A sample of $PdCl_2$ (60%; 159 mg, 0.54 mmol) was added to a solution of **11** (2.90 g, 4.64 mmol) in 3:2 EtOH–MeOH (50 mL). The mixture was stirred overnight, filtered through Celite, concentrated, and purified on a silica gel column (2:1 toluene–EtOAc) to yield **12** (2.47 g, 91%); $[\alpha]_D$ +32° (*c* 1.0, $CHCl_3$), lit. $[\alpha]_D$ +37.8° [19]; ^{13}C NMR ($CDCl_3$): δ 54.9 (MeO), 62.5 (C-7), 71.9, 72.1, 72.5, 73.1, 74.2, 74.3, 74.6, 75.9, and 80.4 (C-2–6, Ph*C*H_2), 99.1 (C-1), 127.6–138.6 (aromatic).

Methyl 2,3,4-tri-O-*benzyl*-L-glycero-α-D-manno-*heptopyranoside* (**13**).—Compound **10** (2.10 g, 3.93 mmol) was deallylated as described for **11** to give, after silica gel chromatography (3:1 → 1:2 toluene–EtOAc), **13** (1.28 g, 66%); $[\alpha]_D$ +21° (*c* 1.0, $CHCl_3$) lit. $[\alpha]_D$ +30.5° [20]; ^{13}C NMR ($CDCl_3$): δ 54.8 (MeO), 65.0, 69.4, 72.2, 72.4, 73.0, 74.3, 74.5, 75.3, and 80.1 (C-2–7, Ph*C*H_2), 99.5 (C-1), 127.6–138.4 (aromatic).

Methyl L-glycero-α-D-manno-*heptopyranoside 7-(disodium phosphate)* (**15**).—Compound **12** (789 mg, 1.35 mmol) was subjected to phosphorylation following the general procedure above. Before silica gel chromatography the crude product was acetylated (2:1 pyridine–Ac_2O) in order to acetylate excess benzyl alcohol, and concentrated. The mixture was purified on a silica gel column (19:1 $CHCl_3$–MeOH) followed by a silanized SiO_2 column (acetone containing a 30 → 20% H_2O gradient). After concentration, the residue was dissolved in toluene and washed once with water to remove polar impurities. The organic phase was dried ($MgSO_4$), filtered, and concentrated to give methyl 2,3,4,6-tetra-*O*-benzyl-7-*O*-dibenzyloxyphosphoryl-L-*glycero*-α-D-*manno*-heptopyranoside (**14**; 856 mg, 75%). NMR: ^{13}C ($CDCl_3$), δ 54.9 (MeO), 66.0 (d), 69.3 (d), 70.6, 71.9, 72.5, 72.8, 73.9, 74.2, 74.4, 74.9 (d), and 80.5 (C-2–7, $PhCH_2$), 99.1 (C-1), 127.4–138.8 (aromatic); ^{31}P (EtOAc), δ 0.3 (m). To a solution of compound **14** (772 mg, 0.914 mmol) in 1:1 EtOAc–EtOH (30 mL) was added Pd–C and H_2O (0.5 mL), and the mixture was stirred overnight under H_2 (9 atm). The mixture was filtered through Celite, diluted with MeOH, and the pH was adjusted to 9 by aq 1 M NaOH. After concentration, the residue was dissolved in H_2O, washed once with Et_2O, and purified by size-exclusion chromatography (Bio-Gel P-2, eluent H_2O containing 1% of *n*-BuOH). Freeze-drying of appropriate fractions gave **15** (283 mg, 89%); $[\alpha]_D$ +33° (*c* 1.0, H_2O); NMR: ^{13}C (D_2O), δ 55.6 (MeO), 66.4, 66.8, 68.8 (d), 70.7, 71.6, and 71.7 (C-2–7), 101.7 (C-1); ^{31}P (H_2O), δ 4.1 (dd). HRMS Calcd for $C_8H_{17}O_{10}P$ [M + Na]: 327.0457. Found: 327.0447.

Methyl 7-O-(2-aminoethyl)phosphoryl-L-glycero-α-D-manno-*heptopyranoside* (**17**).—Following the general phosphoethanolamine-coupling procedure, compound **12** (766 mg, 1.31 mmol) afforded methyl 2,3,4,6-tetra-*O*-benzyl-7-*O*-(2-benzyloxycarbonylamino)ethylphosphoryl-L-*glycero*-α-D-*manno*-heptopyranoside, triethylammonium salt (**16**; 923 mg, 75%) after purification on a silica gel column eluted with $CHCl_3$ containing 10 → 20% MeOH and 1% Et_3N. NMR: ^{13}C ($CDCl_3$), δ 8.4 (Et_3N), 42.4 (d, CH_2CH_2N), 45.4 (Et_3N), 55.0 (MeO), 64.2 (d), 64.4 (d), 66.4, 71.8, 72.4, 74.17, 74.22, 74.5, 75.4 (d), and 80.6 (C-2–7, Ph*C*H_2, O*C*H_2CH_2), 98.9 (C-1), 127.2–139.2 (aromatic), 156.6 (CON); ^{31}P (H_2O), δ 0.7 (m). Compound **16** (780 mg, 0.827 mmol) in 1:1 EtOAc–EtOH (30 mL) and 0.25 mL H_2O was hydrogenolyzed over Pd–C at 9 atm for 5 days. Removal of the catalyst (Celite filtra-

tion) and concentration followed by gel filtration (two Bio-Gel P-2 columns eluted with H_2O containing 1% *n*-BuOH and pyridinium acetate buffer, respectively) gave **17** (235 mg, 82%); $[\alpha]_D$ +30° (*c* 1.0, H_2O); NMR: ^{13}C (D_2O), δ 40.7 (d, CH_2CH_2N), 55.5 (CH_3O), 62.5 (d), 66.6, 67.2 (d), 68.0 (d), 70.6, 71.4, and 71.5 (C-2–7, OCH_2CH_2), 101.7 (C-1); ^{31}P (H_2O), δ 0.1 (m). HRMS Calcd for $C_{10}H_{23}O_{10}NP$ [M + H]: 348.1060. Found: 348.1053.

Methyl L-glycero-α-D-manno-*heptopyranoside 6,7-cyclic phosphate, ammonium salt* (**19**).—Methyl dichlorophosphate (238 μL, 2.38 mmol) was dissolved in dry pyridine (4 mL) at 0 °C. The mixture was stirred for 30 min, whereafter a solution of **13** (463 mg, 0.936 mmol) in dry pyridine (3 mL) was added dropwise. After stirring at 0 °C until the conversion into a hydrophilic product was complete (1 h), the reaction was quenched by the addition of aq satd $(NH_4)_2CO_3$ (10 mL). The solution was concentrated, and the residue was dissolved and partitioned between EtOAc and H_2O. The pH was adjusted to 1 prior to separation of the EtOAc phase. The aqueous phase was additionally washed once with EtOAc, and the combined organic phases were concentrated and co-concentrated three times from EtOH. The crude methyl 2,3,4-tri-*O*-benzyl-L-*glycero*-α-D-*manno*-heptopyranoside 6,7-cyclic phosphate, ammonium salt (**18**; 426 mg, 79%) was used in the next step without further purification. NMR: ^{13}C ($CDCl_3$): 55.0 (CH_3O), 64.7, 69.9 (d), 72.3, 73.1, 73.5, 73.8, 75.0, 75.4, and 80.0 (C-2–7, $PhCH_2$), 99.2 (C-1), 127.5–138.2 (aromatic); ^{31}P (EtOAc), δ 18.4 (m). Compound **18** (426 mg, 0.743 mmol) was dissolved in 1:1:1 THF–EtOH–H_2O (24 mL) and hydrogenolyzed in a Parr apparatus (9 atm) using $Pd(OH)_2$ as catalyst. After 3 days the mixture was filtered through Celite, and concentrated. The Celite was suspended in H_2O and centrifuged. The pellet was additionally washed and centrifuged from water. The original residue was dissolved in the combined aqueous phases and washed once with Et_2O. The water phase was concentrated and the residue desalted on a Bio-Gel P-2 column to give, after lyophilization, **19** (195 mg, 87%); $[\alpha]_D$ +41° (*c* 1.0, H_2O); NMR: ^{13}C (D_2O), δ 55.7 (MeO), 66.4, 67.4, 70.6, 71.3, 71.7 (d), and 74.9 (d) (C-2–7), 101.8 (C-1); ^{31}P (H_2O), δ 18.3 (m). HRMS Calcd for $C_8H_{14}O_9P$ [M − H]: 285.0375. Found: 285.0420.

Methyl L-glycero-α-D-manno-*heptopyranoside 4-(disodium phosphate)* (**22**).—Following the general phosphorylation procedure above, **20** [18] (315 mg, 1.04 mmol) gave, after silica gel chromatography (2:1 toluene–EtOAc), methyl 4-*O*-dibenzyloxyphosphoryl-2,3;6,7-di-*O*-isopropylidene-L-*glycero*-α-D-*manno*-heptopyranoside (**21**; 470 mg, 80%); NMR: ^{13}C ($CDCl_3$), δ 25.4, 26.2, 26.3, and 27.7 [$(CH_3)_2C$], 55.2 (CH_3O), 65.7, 68.2 (d), 69.2 (d), 69.6 (d), 74.2, 75.9, 76.4 (d), and 76.8 (C-2–7, $PhCH_2$), 98.3 (C-1), 109.3 and 110.0 [$(CH_3)_2COO$], 127.8–136.1 (aromatic); ^{31}P (EtOAc), δ −0.8 (m). A mixture of **21** (235 mg, 0.416 mmol) and Pd–C in absolute EtOH (10 mL) and aq 80% HOAc (10 mL) was hydrogenolyzed overnight at 9 atm. The mixture was filtered through Celite, evaporated and the residue was stirred with aq 80% HOAc (15 mL) for 1 h at 40 °C in order to complete deisopropylidenation. After concentration, the residue was dissolved in H_2O (10 mL, containing 1% *n*-BuOH) and the pH was adjusted to ~7 using aq NaOH. Freeze-drying, followed by desalting on a Bio-Gel P-2 column (H_2O containing 1% *n*-BuOH) rendered **22** (119 mg, 82%); $[\alpha]_D$ +30° (*c* 1.0, H_2O); NMR: ^{13}C (D_2O), δ 55.5 (MeO), 63.2, 69.2, 70.0 (d), 70.2, 71.3 (d), and 71.7 (C-2–7), 101.5 (C-1); ^{31}P (H_2O): δ 4.7 (d). HRMS Calcd for $C_8H_{17}O_{10}P$ [M + Na]: 327.0457. Found: 327.0478.

Methyl 4-O-(2-aminoethyl)phosphoryl-L-glycero-α-D-manno-*heptopyranoside* (**24**).—The general phosphoethanolamine coupling protocol applied on **20** (384 mg, 1.26 mmol) gave after silica gel chromatography ($CHCl_3$ containing 10→17% MeOH and 1% Et_3N) methyl 4-*O*-(2-benzyloxycarbonylaminoethyl)-phosphoryl-2,3;6,7-di-*O*-isopropylidene-L-*glycero*-α-D-*manno*-heptopyranoside, triethylammonium salt (**23**; 742 mg, 89%). NMR: ^{13}C ($CDCl_3$), δ 8.5 (Et_3N), 25.8, 26.2, 26.3, and 27.7 [$(CH_3)_2C$], 42.1 (d, CH_2CH_2N), 45.3 (Et_3N), 54.9 (CH_3O), 64.7 (d), 66.5, 66.8, 69.9 (d), 74.0 (d), 75.7, and 77.5 (C-2–7, $PhCH_2$,

Table 2
^{1}H NMR data of selected compounds[a] obtained at 38 °C relative to internal acetone (δ_H 2.225)

Compound	H-1	H-2	H-3	H-4	H-5	H-6	H-7a	H-7b	OMe
7[b]	4.76	3.92	3.80	3.96	3.67	4.42	3.82	3.90	3.41
9	4.78	3.95	3.81	3.95	3.71	4.58	3.81	3.89	3.40
15[b]	4.78	3.91	3.77	3.87	3.62	4.18	3.88	3.98	3.41
17	4.78	3.92	3.78	3.88	3.60	4.20	4.01	4.01	3.41
19	4.81	3.93	3.75	3.82	3.56	4.82	4.36	4.29	3.40
22[b]	4.76	3.93	3.89	4.25	3.63	4.17	3.75	3.75	3.41
24	4.78	3.95	3.94	4.35	3.70	4.08	3.95	3.95	3.40
25	4.77	4.07	4.18	3.95	3.62	4.02	3.74	3.74	3.41
26	4.78	~3.91	~3.74	3.88	3.58	4.05	3.78	3.78	3.41

[a] Compounds **25** and **26** were not synthesized for this study but were obtained after alkaline treatment and characterized by the use of 2D NMR and MS. Signals for ethanolamine protons for compounds **9**, **17** and **24** occurred at δ 4.11, 4.18, 4.12 (O–CH_2), 3.19, 3.30, 3.29 (N–CH_2), respectively.

[b] Assignments were made from COSY experiments and the location of phosphate substituents confirmed by ^{1}H–^{31}P HMQC experiments.

O*C*H_2CH_2), 98.3 (C-1), 108.7 and 109.6 [(CH_3)$_2$*C*OO], 128.0–136.8 (aromatic), 156.7 (CON); ^{31}P (9:1 EtOAc–$CHCl_3$), δ −0.7 (m). A solution of **23** (360 mg, 0.543 mmol) in EtOH (6 mL) and aq 80% HOAc (6 mL) was hydrogenolyzed over Pd–C at 8 atm. After 4 days the mixture was filtered through Celite, and concentrated. The residue was dissolved in 1:1 THF–H_2O (12 mL), new catalyst was added and the hydrogenolysis was continued for 3 days whereby on the last day aq 25% NH_3 (1 mL) was added. The solution was neutralized with HOAc (approx. 1 mL), filtered, and concentrated. The residue was dissolved in H_2O and washed once with Et_2O. Desalting on a Bio-Gel P-2 column (aq 0.1 mM pyridinium acetate buffer, pH 5.4) gave, after freeze-drying of appropriate fractions, **24** (149 mg, 79%); $[\alpha]_D$ +37° (*c* 1.0, H_2O); NMR: ^{13}C (D_2O), δ 9.0 (Et_3N), 40.8 (d, CH_2CH_2N), 47.4 (Et_3N), 55.5 (MeO), 62.9 (d), 63.1, 69.3, 70.8, 70.9 (d), and 72.4 (d), (C-2–7, O*C*H_2CH_2), 101.6 (C-1); ^{31}P (H_2O), δ 0.4 (m). HRMS Calcd for $C_{10}H_{23}O_{10}NP$ [M + H]: 348.1060. Found: 348.1089.

Alkaline treatment.—Phosphate and phosphoethanolamine substituted L,D-heptose and D-mannose methyl glycosides (10 mg) were treated with 4 M KOH (2 mL) for 18 h at 120 °C. In addition, compound **9** was subjected to alkaline treatment with 2 M KOH for 2 h at 60, 80 and 100 °C. LPS from *N. meningitidis* immunotype L3 *lgtB* [5] was treated with 4 M KOH at 120 °C for 18, 8, 4 and 2 h. One experiment was conducted with 2 M KOH for 2 h and 60 °C. Reaction mixtures were cooled, neutralized and fractionated on a Bio-Gel P-2 column (43 × 1.5 cm) with water as eluent. Products were analyzed by ^{1}H NMR and ESMS.

NMR spectroscopy.—^{13}C NMR spectra in D_2O of **7**, **15**, **19** at pD ~ 9, and **9**, **17** and **24** at pD ~ 7 were recorded on a JEOL GSX-270 instrument. ^{1}H NMR data for **7**, **9**, **15**, **17**, **19**, **22**, **24**, **25** and **26** are shown in Table 2 and were obtained with a Bruker AMX 500 spectrometer using standard Bruker software. All ^{1}H measurements were made in D_2O at 38 °C after several lyophilizations with D_2O. Acetone was used as the internal standard (δ_H, 2.225 ppm). 2D COSY experiments were performed by using standard Bruker pulse sequences. ^{31}P spectra were measured at 202 or 109 MHz and aq 85% phosphoric acid was used as the internal standard (δ_P, 0.0 ppm). ^{1}H–^{31}P correlations (HMQC) and ^{1}H–^{31}P HMQC-TOCSY experiments were made in the ^{1}H-detected mode.

Electrospray ionization mass spectrometry (ESMS).—Samples were analyzed on a VG Quattro Mass spectrometer (Micromass, Manchester, UK) fitted with an electrospray

ion source. Samples were dissolved in water, which was then mixed in a 1:1 ratio with aq 50% acetonitrile containing 1% HOAc for mass spectral analysis in the negative mode.

High resolution mass spectra (HRMS).— High resolution mass spectra were obtained by fast atom bombardment in either the positive or negative ion mode on a JEOL AX 505 H double focussing mass spectrometer with a mass resolution of 7000. In the positive ion mode glycerol was used as the matrix and polyethyleneglycol (PEG) 200–400 was used as the internal calibrant. HRMS of methyl L-*glycero*-α-D-*manno*-heptopyranoside 6,7-cyclic phosphate was obtained in the negative mode with triethanolamine as the matrix and PEG 600 diacid as the calibrant.

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