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Chemical Synthesis of a Biosynthetic Precursor of Lipid A with a Phosphorylated Tetraacyl Disaccharide Structure

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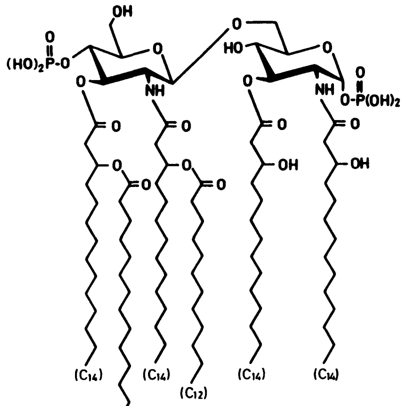
2,2′-*N*:3,3′-*O*-Tetrakis[(*R*)-3-hydroxytetradecanoyl]-*β*(1-6)-D-glucosamine disaccharide 1,4′-bis(phosphate) and its dephospho derivatives were synthesized. The bisphosphate prepared was shown to be identical with a natural biosynthetic precursor of lipid A which corresponds to the lipophilic part of lipopolysaccharide (LPS) in bacterial cell wall. The synthetic bis- and monophosphates exhibited many of typical endotoxic activities of LPS. Consequently, this work established the chemical structure of the biosynthetic precursor of lipid A and elucidated the fundamental structure required for the expression of these activities.

Gram-negative bacteria contain in their cell surface a toxic principle which causes fever and lethal shock in higher animals. This toxin is called endotoxin since it is firmly bound to the cells in living bacteria and released only after the cells are destroyed. Endotoxin exhibits not only undesired toxic activities mentioned above but also beneficial ones such as stimulation of immune responses and induction of a tumor-necrotizing factor.[1] These toxic and beneficial biological activities have been shown to be attributed to the lipopolysaccharide (LPS) moiety which is a complex amphipathic molecule characteristic of the outer membrane of Gram-negative bacterial cells.[1] In 1954, Westphal and Lüderitz[2] isolated the lipophilic part of LPS by mild acidic hydrolysis and termed it "lipid A". They described that lipid A manifested most of the endotoxic activities of LPS. Although there had been some confusions due to the heterogeneous character of natural lipid A preparations,[3] this concept was unequivocally confirmed by our recent total synthesis of *Escherichia coli* lipid A (**1**).[4] In this paper we described in detail a total synthesis of a biosynthetic precursor (**2**) of lipid A and its dephospho derivatives. As reported previously in a preliminary form,[5] this synthetic work was the first successful chemical construction of a compound possessing definite endotoxic activities.[6]



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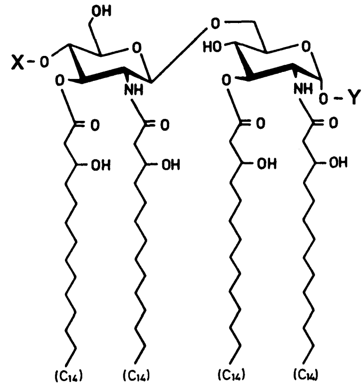


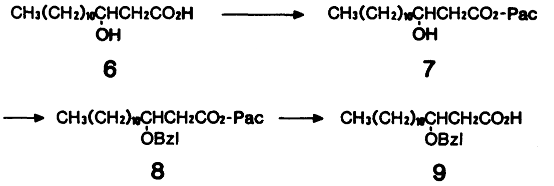
2 X = Y = PO(OH)$_2$
3 X = PO(OH)$_2$, Y = H
4 X = H, Y = PO(OH)$_2$
5 X = Y = H

The chemical structure of lipid A had been studied by the group of Westphal.[1] They demonstrated that lipid A's from many bacterial species share a common basic structure composed of polyacylated D-glucosamine *β*(1→6) disaccharide 1,4′-bis(phosphate). However, because of the heterogeneity of natural lipid A preparations studied before our synthetic work, the exact number and location of acyl groups could not be determined, the positions of acylation being rather tentatively assigned to the 2,2′-amino and 3,4,6′-hydroxyl groups. Since this assignment then proved incorrect by our synthesis of corresponding compounds,[7] we started a structural study on a purified main component of *E. coli* lipid A and elucidated that the bisphosphorylated disaccharide backbone is acylated at 2,2′-amino and 3,3′-hydroxyl groups with four moles of (*R*)-3-hydroxytetradecanoic acid[8] which is a typical constituent acid of LPS.

With this information, we took the structure **2** as the first target of our synthesis in order to confirm the newly proposed acylation pattern. We already knew that a biosynthetic precursor of lipid A which contained only four moles of 3-hydroxytetradecanoic acid possessed most of endotoxic activities.[9,10] The precursor designated "acidic precursor" and now renamed "precursor Ia"[11] was first isolated from a temperature-sensitive mutant of *Salmonella typhimurium*.[12] The location of the fatty acids in precursor Ia had not been determined but the structure **2** could be most reasonably assigned to it by taking our results on *E. coli* lipid A into consideration.[13]

The strategy for the synthesis was decided on the basis of our knowledges accumulated during the previous works[7] on the compounds with the incorrect old-type acylation pattern. Thus the target compound **2** was constructed via the following steps: 1) preparation of a β(1→6) disaccharide without long-chain acyl groups, 2) introduction of 3-benzyloxytetradecanoic acids to the two amino and two hydroxyl groups of the disaccharide, 3) phosphorylation of 4′-position, 4) phosphorylation of the glycosidic position, 5) hydrogenolytic deprotection. Phosphorylation of the glycosidic position had to be carried out at the last stage of the synthesis, since glycosyl phosphates are unstable. Thus, the 1-hydroxyl function was protected with allyl group which could be removed without affecting other functional and protecting groups. For persistent protection of other hydroxyl and 4′-phosphate groups were used benzyl and phenyl groups respectively.

In order to investigate the role of phosphate moieties for the biological activities, the corresponding monophosphates (**3** and **4**) and the dephospho derivative **5** were also prepared by slight modification of the synthetic route.



$$\underset{\mathbf{6}}{CH_3(CH_2)_{10}\underset{OH}{CH}CH_2CO_2H} \longrightarrow \underset{\mathbf{7}}{CH_3(CH_2)_{10}\underset{OH}{CH}CH_2CO_2\text{-}Pac}$$

$$\longrightarrow \underset{\mathbf{8}}{CH_3(CH_2)_{10}\underset{OBzl}{CH}CH_2CO_2\text{-}Pac} \longrightarrow \underset{\mathbf{9}}{CH_3(CH_2)_{10}\underset{OBzl}{CH}CH_2CO_2H}$$

Pac : $C_6H_5COCH_2$ Bzl : $C_6H_5CH_2$

(*R*)-3-Benzyloxytetradecanoic acid (**9**) was prepared from optically pure (*R*)-3-hydroxytetradecanoic acid (**6**)[14] via the corresponding phenacyl ester **7**. For the next *O*-benzylation of **7**, strongly basic conditions should not be used since the 3-benzyloxy derivative formed was susceptible to base-induced β-elimination. Acid catalyzed *O*-benzylation by means of benzyl trichloroacetimidate-trifluoromethanesulfonic acid[15] proved to be preferable to the benzyl bromide-silver(I) oxide method employed in our previous work.[7] With the former method, a comparable yield of the benzylated product **8** could be obtained in a much shorter reaction time. The phenacyl group of **8** was removed with zinc dust in acetic acid to give the 3-benzyloxy acid **9**.

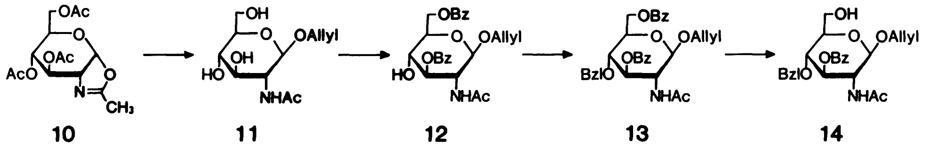
As the glycosyl acceptor was designed the compound **14**, whose 4-hydroxyl function was protected with persistent benzyl group and glycosidic position with allyl group for the reasons mentioned above. The starting material, β-allyl glycoside of *N*-acetyl-D-glucosamine (**11**), was prepared from D-glucosamine hydrochloride via peracetylated oxazoline **10** obtained by the new method, which is suitable for a large-scale preparation of the oxazoline **10**. Compound **11** thus obtained was selectively benzoylated with benzoyl chloride-pyridine at low temperature (−65 °C) to afford the 3,6-dibenzoate **12**.[16] Acid-catalyzed benzylation[15] of **12** gave 4-*O*-benzylated derivative **13**. Under these conditions, the *N*-acetyl group was partially converted into benzyl imidate, which could be reconverted to *N*-acetyl group via mild acidic hydrolysis and reacetylation. Compound **13** was purified by column chromatography on DIAION HP-20 (high-porous polystyrene gel). The recovered starting material **12** and trichloroacetamide formed from the reagent were readily removed by this chromatography. The benzoyl group on the primary hydroxyl function of **13** was selectively split off by transesterification to afford the glycosyl acceptor **14**.

Coupling of **14** with the oxazoline **10** yielded a disaccharide **15**. The β-configuration of the newly formed glycosidic linkage was confirmed by the coupling constant value (8 Hz) of the signal due to the anomeric proton in the ^{1}H NMR spectrum of **15**.

The next step in the synthesis was introduction of 3-benzyloxytetradecanoic acid (**9**) into the disaccharide backbone. Both *N*-acetyl groups of the disaccharide **15** were cleaved by an action of Meerwein's reagent, triethyloxonium tetrafluoroborate, followed by acid hydrolysis of the resultant imidates. The free amino functions were acylated with 3-benzyloxy acid **9** and dicyclohexylcarbodiimide (DCC) to give *N,N′*-diacylated disaccharide **16**. Thereafter all the ester-type protecting groups of **16** were removed and the 4′- and 6′-hydroxyl groups were protected by isopropylidenation. The remaining two hydroxyl functions on 3- and 3′-position of **18** were acylated with 3-benzyloxy acid **9**, DCC, and 4-dimethylaminopyridine (DMAP). Hydrolysis of the isopropylidene group afforded the product **19**, which possessed four acyl groups at the desired positions.

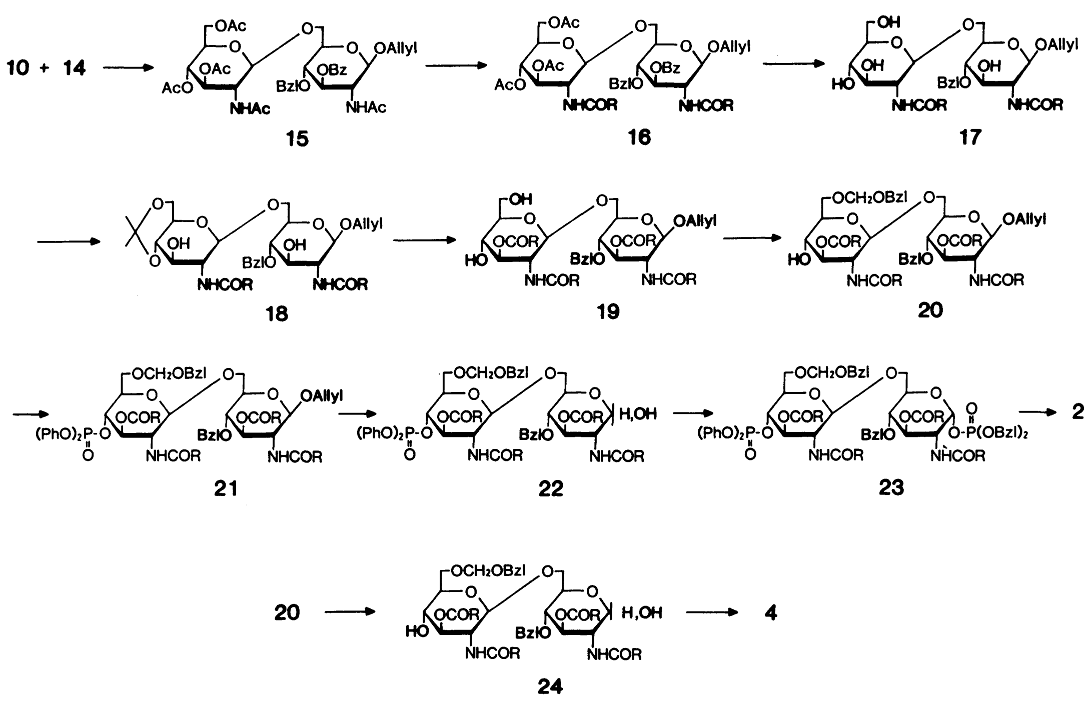
After protection of the primary hydroxyl function of **19** by benzyloxymethylation, the hydroxyl function on 4′-position of the product **20** was phosphorylated with diphenyl phosphorochloridate-pyridine-DMAP according to the procedure by Szabo et al.[17] to give **21**.

The final two steps of the synthesis were the introduction of the 1-α-phosphate moiety and deprotection.



10 **11** **12** **13** **14**

Allyl : $CH_2{=}CH\text{-}CH_2$ Bz : C_6H_5CO Bzl : $C_6H_5CH_2$



Allyl : $CH_2{=}CH{-}CH_2$ Bz : C_6H_5CO Bzl : $C_6H_5CH_2$ Ph : C_6H_5 RCO : $CH_3(CH_2)_{10}\underset{\displaystyle OBzl}{\underset{|}{C}}HCH_2CO$

Thus, the glycosidic allyl group of **21** was removed by two-step conversion. It was first isomerized into 1-propenyl group with 1,5-cyclooctadienebis(methyldiphenylphosphine)iridium hexafluorophosphate[18] and then cleaved off by an action of iodine in aqueous THF[19] to afford the compound **22** with a free glycosidic position. 1-α-Phosphorylation of **22** was accomplished by using butyllithium-dibenzyl phosphorochloridate.[20] The protected 1-α,4′-bis(phosphate) **23** thus obtained was immediately hydrogenolyzed in order to prevent a decomposition of the labile dibenzyl ester of the glycosyl phosphate. After removal of all the benzyl-type groups with palladium catalyst, the phenyl groups on the 4′-phosphate were hydrogenolyzed with platinum.

The final product **2** was isolated by silica-gel column chromatography. Although **2** showed a behavior identical with that of natural precursor Ia in TLC analysis, the solubility of the former in water was lower than that of the natural compound. This seemed to be due to the formation of salts at the phosphate residues with inorganic cations from silica gel. It is known that the solubility as well as the biological activities of lipid A's are much influenced by counter cations of phosphate moieties.[21] Therefore the synthetic product was once converted into the free acid form and then to the triethylammonium salt, which is the most soluble form in water, by the following procedures. An aqueous suspension of synthetic **2**, the pH of which was adjusted to 9 with triethylamine, was electrodialyzed[21] under the conditions described in the experimental part. By this procedure, the solubility of the material increased considerably, but it was found that the phosphate groups still formed a monobasic salt as judged from TLC analysis after treatment with diazomethane. Removal of cations was completed by acidic precipitation method.[22] By addition of cold diluted hydrochloric acid to an aqueous solution of the electrodialyzed material under ice-cooling, pure **2** of free acid form was precipitated. The material thus obtained gave a satisfactory result in elemental analysis. The sample was supplied for biological tests as bis(triethylammonium) salt, which was prepared by lyophilization from aqueous solution of **2** after addition of a calculated amount of triethylamine.

The 4′-phosphate **3** was prepared by direct deprotection of the above intermediate **22**. Thus, the benzyl and phenyl groups of **22** were cleaved by hydrogenolysis with palladium and platinum respectively. In order to remove inorganic impurities resulted from catalyst, the product was subjected to the acidic precipitation method to afford the pure 4′-monophosphate **3**. This compound gave the same R_f value on silica-gel TLC with the corresponding 4′-monophosphate which was derived from natural precursor Ia by mild acidic hydrolysis of the unstable glycosyl phosphate. For more strict identification, synthetic and natural 4′-monophosphates were converted into the dimethyl esters by treatment with ethereal diazomethane. Both dimethyl esters of 4′-phosphate were indistinguishable on TLC as well as 360 MHz ^1H NMR spectrometry.

1-Monophosphate **4** was derived from the intermediate **20** by removal of the glycosidic allyl group followed by 1-*O*-phosphorylation. Since the reactivity of the hindered 4′-hydroxyl group is much lower than that of the glycosidic hydroxyl group, selective phosphorylation of the latter position of **24** was accomplished with a limited amount of butyllithium and dibenzyl phosphorochloridate.[7] Hydrogenolytic deprotection followed by column chromatography on silica gel afforded the desired 1-monophosphate **4**, which was purified by means of electrodialysis and acidic precipitation as described for **2**. Dephospho derivative **5** was prepared from the nonphosphorylated intermediate **19** by direct deprotection.

As had already been reported elsewhere,[6] the synthetic bisphosphate **2** showed fully identical biological activities with those of natural biosynthetic precursor obtained from *Salmonella*. The activities include lethal toxicity, B-cell mitogenicity, induction of interferon and so on.[23] These results together with the NMR spectroscopic and TLC identification of the synthetic and natural compounds described above led us to the following unequivocal conclusions. First, biosynthetic precursor Ia of lipid A has the structure formulated as **2**. Secondly, our new proposal of the location of the acyl groups on the disaccharide backbone[8] was correct. Finally, at least the most of the typical biological activities[23] of bacterial endotoxin can be attributed to this type of phosphorylated acyl disaccharide, which is now synthetically accessible.

Furthermore, this work demonstrated for the first time that a precise study became possible on the relationship between the chemical structures and biological activities of lipid A analogs by synthetic approaches. For example, the importance of the phosphate moieties was elucidated by a comparative study on the biological activities of the above synthetic derivatives: The two monophosphates **3** and **4** exhibited somewhat weaker than **2** but definite endotoxic activities whereas the dephospho derivative **5** was completely inactive.[6] Further synthetic studies in this line is described in the accompanying paper.

Experimental

All melting points are uncorrected. ^{1}H NMR spectra were measured on a Varian XL-100-15 spectrometer (100 MHz) or a JEOL PMX-60 spectrometer (60 MHz) for chloroform-*d* solutions unless noted otherwise. The chemical shifts are given in δ values with TMS as the internal standard. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. High resolution EI-MS was obtained on a JEOL O1-SG mass spectrometer. Conventional and medium-pressure (2—4 kg cm^{-2}) column chromatography were carried out with Kieselgel 60 (E. Merck), 0.063—0.2 mm and 0.040—0.063 mm, respectively. Precoated Kieselgel F_{254} plates (0.5 mm thickness; E. Merck) were used for preparative TLC. For electrodialysis and acidic precipitation procedures was used a pyrogen-free water (Otsuka). Organic solutions were dried over magnesium sulfate and evaporated in vacuo.

Phenacyl (*R*)-3-Hydroxytetradecanoate (7). To a suspension of (*R*)-3-hydroxytetradecanoic acid (**6**) (21.8 g, 89.2 mmol) in ethyl acetate (400 ml) were added phenacyl bromide (19.1 g, 96.0 mmol) and triethylamine (10.6 g, 105 mmol) at 0 °C. The mixture was stirred at 0 °C for 10 min then at room temperature overnight. The precipitate was filtered off and the filtrate was washed with water, dried, and evaporated. The residue was recrystallized from hexane; yield 30.0 g (94%); mp 76—77 °C; $[\alpha]_D^{11}$ −5.1° (*c* 2.17, chloroform). Found: C, 72.88; H, 9.42%. Calcd for $C_{22}H_{34}O_4$: C, 72.89; H, 9.45%. ^{1}H NMR (60 MHz) δ=0.9 (3H, br t, CH_3), 1.1—1.7 (20H, br s, CH_2), 2.6 (2H, m, $-COCH_2-$), 3.1 (1H, s, -OH), 4.1 (1H, m, $-C\underline{H}(OH)-$), 5.4 (2H, s, $-C\underline{H}_2COC_6H_5$), and 7.3—8.0 (5H, m, aromatic H).

Phenacyl (*R*)-3-Benzyloxytetradecanoate (8). To a solution of **7** (36.2 g, 100 mmol) and benzyl trichloroacetimidate (30.0 g, 119 mmol) in anhydrous dichloromethane (500 ml) was added dropwise trifluoromethanesulfonic acid (1.8 ml, 20 mmol) at 0 °C under argon atmosphere. After stirring at 0 °C for 2 h, the mixture was washed with a saturated aqueous solution of sodium hydrogencarbonate and water, dried, and concentrated. The residue was subjected to silica-gel column chromatography (1 kg). Elution with benzene gave a syrup; yield 33.3 g (74%). ^{1}H NMR (60 MHz) δ=0.9 (3H, br t, CH_3), 1.0—1.8 (20H, br s, CH_2), 2.8 (2H, AB-type, $-COCH_2-$), 3.9 (1H, m, $-C\underline{H}(OCH_2C_6H_5)-$), 4.5 (2H, s, $-C\underline{H}_2C_6H_5$), 5.3 (2H, s, $-COOCH_2-$), 7.1—7.9 (5H, m, $-COC_6H_5$), and 7.3 (5H, s, $-CH_2C_6\underline{H}_5$).

Further elution with benzene-ethyl acetate (9 : 1) gave the starting material **7**, which was recrystallized from hexane; 8.5 g (19 %).

(*R*)-3-Benzyloxytetradecanoic Acid (9). Zinc dust (30 g) was added to a solution of **8** (33.3 g, 73.6 mmol) in acetic acid (650 ml) and the mixture was stirred at 50 °C. After 2 h zinc dust (15 g) was added and the mixture was stirred for additional 1 h. Insoluble materials were filtered and washed with ethyl acetate. After evaporation of the solvent, the residue was dissolved in ethyl acetate, washed with water, dried, and concentrated. The residue was subjected to silica-gel column chromatography (500 g). The product eluted with chloroform–methanol (3 : 1) was dissolved in ethyl acetate, washed with 1 M[†] hydrochloric acid and water, and dried. Evaporation of the solvent gave a colorless syrup, which was used for the following reactions without further purification; yield 19.8 g (80%); $[\alpha]_D^{17}$ −6.6° (*c* 3.65, chloroform). EI-MS: *m/z* 334 (M^+). Found: C, 75.64; H, 10.10%. Calcd for $C_{21}H_{34}O_3$: C, 75.40; H, 10.34%. ^{1}H NMR (60 MHz) δ=0.9 (3H, br t, CH_3), 1.0—1.7 (20H, br s, CH_2), 2.6 (2H, d, J=7 Hz, $-COCH_2-$), 3.9 (1H, m, $-C\underline{H}(OCH_2C_6H_5)-$), 4.6 (2H, s, $-C\underline{H}_2C_6H_5$), and 7.3 (5H, s, aromatic H).

3,4,6-Tri-*O*-acetyl-2-amino-2-deoxy-1-*α*-*O* : 2-*N*,*N*-ethylidyne-D-glucopyranose (10). 2-Acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-D-glucopyranose (100 g, 0.257 mol) was dissolved in anhydrous chloroform (1 l). The solution was saturated with dry hydrogen bromide at 0 °C and left overnight at room temperature in a stoppered flask. Pyridine (100 ml) was added dropwise to the mixture over 10 min at 0 °C with stirring. After stirring for additional 20 min at room temperature, the mixture was washed with water, dried, and evaporated. The residual pyridine was removed by coevaporation with toluene three times. The pale yellow syrup thus obtained was used for the following step without further purification; yield 79.4 g (94%). High resolution EI-MS Found: *m/z* 329.1092. Calcd for $C_{14}H_{19}NO_8$: M, 329.1111. ^{1}H NMR (60 MHz) δ=2.1 (12H, s, CH_3), 3.6 (1H, dt, $J_{5,4}$=8 Hz, $J_{5,6}$=4 Hz, H-5), 4.1 (1H, m, H-2), 4.2 (2H, d, $J_{6,5}$=4 Hz,

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[†] 1 M=1 mol dm^{-3}.

H-6), 4.9 (1H, dd, $J_{4,3}$=2 Hz, $J_{4,5}$=4 Hz, H-4), 5.2 (1H, t, $J_{3,2}=J_{3,4}$=2 Hz, H-3), and 5.9 (1H, d, $J_{1,2}$=8 Hz, H-1).

Allyl 2-Acetamido-3,6-di-*O*-benzoyl-2-deoxy-*β*-D-glucopyranoside (12). Allyl 2-acetamido-2-deoxy-*β*-D-glucopyranoside (**11**) was prepared by heating a solution of oxazoline **10** in chloroform with allyl alcohol and *p*-toluenesulfonic acid followed by removal of *O*-acetyl groups with 0.01 M sodium methoxide; mp 173—175 °C.

To a solution of **11** (10.0 g, 76.0 mmol) in pyridine (200 ml) and THF (90 ml) was added benzoyl chloride (30.6 ml, 264 mmol) dropwise at −65 °C. The mixture was stirred at −60 °C for 1 h. After addition of water (150 ml), the mixture was concentrated. The residue was dissolved in ethyl acetate and worked up as usual, and the product was recrystallized from ethyl acetate; yield 24.7 g (69%); mp 194—195 °C; $[\alpha]_D^{19}$ +2.1° (*c* 1.02, chloroform). Found: C, 62.77; H, 5.80; N, 2.99%. Calcd for $C_{25}H_{27}NO_8\cdot 0.5H_2O$: C, 62.75; H, 5.90; N, 2.93%. ^{1}H NMR (100 MHz) δ=1.82 (3H, s, NAc), 4.74 (1H, d, *J*=9 Hz, H-1), 5.04—5.35 (2H, m, $-OCH_2CH=C\underline{H}_2$), 5.66—6.04 (1H, m, $-OCH_2C\underline{H}=CH_2$), 6.12 (1H, d, *J*=9 Hz, NH), and 7.2—8.1 (10H, m, aromatic H).

Allyl 2-Acetamido-3,6-di-*O*-benzoyl-4-*O*-benzyl-2-deoxy-*β*-D-glucopyranoside (13). To a solution of **12** (30.0 g, 63.9 mmol) and benzyl trichloroacetimidate (56.5 g, 244 mmol) in anhydrous dichloromethane (1.5 l) was added trifluoromethanesulfonic acid (8.5 ml, 96 mmol) at −45 °C under argon atmosphere. The mixture was stirred overnight at 4 °C. After addition of saturated aqueous sodium hydrogencarbonate, the organic layer was washed with water, dried, and evaporated. The oily residue was dissolved in THF (300 ml) and treated with 1 M hydrochloric acid (130 ml) at room temperature for 1 h. To the solution were added triethylamine (30 ml) and acetic anhydride (13.5 ml). The mixture was stirred overnight at room temperature. After removal of the insoluble materials, the filtrate was concentrated. The residue was subjected to column chromatography on DIAION HP-20 (2 l). Elution by 70% aqueous methanol afforded first trichloroacetamide and then the starting material **12** which was recrystallized from ethyl acetate: 4.6 g (15%). The product was eluted by methanol and recrystallized from ethyl acetate; yield 17.2 g (48%); mp 160—162 °C; $[\alpha]_D^{15}$ +2.5° (*c* 1.21, chloroform). Found: C, 68.39; H, 5.96; N, 2.51%. Calcd for $C_{32}H_{33}NO_8$: C, 68.68; H, 5.94; N, 2.50%. ^{1}H NMR (100 MHz) δ=1.82 (3H, s, NAc), 5.02—5.34 (2H, m, $-OCH_2CH=C\underline{H}_2$), 5.58 (1H, dd, *J*=8 and 10 Hz, H-3), 5.68—6.07 (1H, m, $-OCH_2C\underline{H}=CH_2$), 7.06 (5H, s, $-CH_2C_6\underline{H}_5$), and 7.2—8.2 (10H, m, $-COC_6H_5$).

Allyl 2-Acetamido-3-*O*-benzoyl-4-*O*-benzyl-2-deoxy-*β*-D-glucopyranoside (14). Compound **13** (16.7 g, 30.0 mmol) was treated with 0.02 M sodium methoxide (600 ml) at 0 °C for 6 h. After neutralization with Dowex 50 (H^+ form) and silica-gel column chromatography (300 g, chloroform–methanol 9 : 1) the product was recrystallized from ethyl acetate-hexane; yield 10.8 g (79%); mp 165—167 °C (with sintering at 137 °C); $[\alpha]_D^{19}$ −54.1° (*c* 2.06, chloroform). Found: C, 66.01; H, 6.44; N, 3.06%. Calcd for $C_{25}H_{29}NO_7$: C, 65.92; H, 6.42; N, 3.07%. ^{1}H NMR δ=1.83 (3H, s, NAc), 4.60 (1H, d, *J*=8 Hz, H-1), 4.61 (2H, s, $-C\underline{H}_2C_6H_5$), 5.02—5.36 (2H, m, $-OCH_2-CH=C\underline{H}_2$), 5.51 (1H, dd, *J*=9 and 10 Hz, H-3), 5.68—6.08 (1H, m, $-OCH_2-C\underline{H}=CH_2$), 6.20 (1H, d, *J*=9 Hz, NH), 7.14 (5H, s, $-CH_2C_6\underline{H}_5$), and 7.3—8.1 (5H, m, $-COC_6H_5$).

Allyl 2-Acetamido-6-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*β*-D-glucopyranosyl)-3-*O*-benozyl-4-*O*-benzyl-2-deoxy-*β*-D-glucopyranoside (15). A solution of **10** (13.0 g, 39.6 mmol), **14** (9.0 g, 19.8 mmol), and *p*-toluenesulfonic acid (1.5 g, 7.0 mmol) in anhydrous chloroform (450 ml) was heated under reflux for 7 h. After cooling, the mixture was washed with aqueous sodium hydrogencarbonate and water, dried, and concentrated. The residue was subjected to column chromatography on silica gel (500 g, chloroform–acetone 3 : 1) and recrystallized from methanol; yield 10.0 g (64% based on **10**); mp 222—224 °C (decomp); $[\alpha]_D^{17}$ −38.1° (*c* 0.43, chloroform). Found: C, 59.38; H, 6.16; N, 3.57%. Calcd for $C_{39}H_{48}N_2O_{15}$: C, 59.68; H, 6.16; N, 3.57%. ^{1}H NMR (100 MHz, $CDCl_3-CD_3OD$) δ=1.82 and 1.87 (each 3H, s, NAc), 2.02 (9H, s, OAc), 4.52 (1H, d, *J*=8 Hz, anomeric H), 4.52 (2H, s, $-C\underline{H}_2C_6H_5$), 4.82 (1H, d, *J*=8 Hz, anomeric H), 5.67—6.08 (1H, m, $-OCH_2C\underline{H}=CH_2$), 7.14 (5H, s, $-CH_2C_6\underline{H}_5$), and 7.3—8.1 (5H, m, $-COC_6H_5$).

Allyl 6-*O*-[3,4,6-Tri-*O*-acetyl-2-[(*R*)-3-benzyloxytetradecanoylamino]-2-deoxy-*β*-D-glucopyranosyl]-3-*O*-benzoyl-4-*O*-benzyl-2-[(*R*)-3-benzyloxytetradecanoylamino]-2-deoxy-*β*-D-glucopyranoside (16). To a solution of **15** (8.2 g, 10.4 mmol) in anhydrous dichloromethane (300 ml) were added anhydrous potassium carbonate and triethyloxonium tetrafluoroborate (6.0 g, 31.2 mmol). After stirring at room temperature for 13 h, insoluble materials were filtered off, the filtrate washed with aqueous sodium hydrogencarbonate and water, dried, and evaporated. To a solution of the residue in THF (180 ml) was added 1 M hydrochloric acid (20.8 ml). After stirring for 3 h at room temperature, the mixture was concentrated and coevaporated with anhydrous benzene three times. To a solution of the residue in anhydrous THF (250 ml), 3-benzyloxytetradecanoic acid (**9**) (10.5 g, 31.3 mmol) and DCC (6.47 g, 31.2 mmol) were added at 0 °C. The mixture was stirred for 19 h at room temperature, and then a small amount of acetic acid was added. After removal of the precipitate by filtration, ethereal diazomethane was added to the filtrate. The product was purified by silica-gel chromatography (300 g, chloroform–acetone 9 : 1) and recrystallized from ethyl acetate–hexane; yield 5.8 g (41%); mp 165—169 °C; $[\alpha]_D^{18}$ −13.2° (*c* 1.11, chloroform). Found: C, 68.99; H, 8.15; N, 2.01%. Calcd for $C_{77}H_{108}N_2O_{17}\cdot 0.5H_2O$: C, 68.88; H, 8.18; N, 2.09%. ^{1}H NMR (100 MHz) δ=0.90 (6H, t, *J*=6 Hz, CH_3), 1.1—1.5 (40H, br s, CH_2), 1.98 (3H, s, OAc), 2.01 (6H, s, OAc), 2.24—2.42 (4H, m, α-CH_2 of fatty acids), 6.31 (1H, d, *J*=9 Hz, NH), 6.41 (1H, d, *J*=9 Hz, NH), and 7.0—8.1 (20H, m, aromatic H).

Allyl 4-*O*-Benzyl-2-[(*R*)-3-benzyloxytetradecanoylamino]-6-*O*-[2-[(*R*)-3-benzyloxytetradecanoylamino]-2-deoxy-*β*-D-glucopyranosyl]-2-deoxy-*β*-D-glucopyranoside (17). To a solution of **16** (15.0 g, 11.2 mmol) in methanol (225 ml) and THF (225 ml) was added 1 M sodium methoxide (22.4 ml). After stirring at room temperature for 5 h, the mixture was neutralized with Dowex 50 (H^+ form). The solvent was evaporated and the residue recrystallized from methanol; yield 10.4 g (84%); mp 193—197 °C (decomp); $[\alpha]_D^{18}$ −18.3° (*c* 1.04, chloroform–methanol 3 : 1). Found: C, 68.04; H, 8.87; N, 2.42%. Calcd for $C_{64}H_{98}N_2O_{13}\cdot 1.5H_2O$: C, 68.00; H, 9.01; N, 2.48%. ^{1}H NMR (100 MHz, $CDCl_3-CD_3OD$) δ=0.90 (6H, br t, CH_3), 1.2—1.4 (40H, br s, CH_2), 2.34—2.56 (4H, m, α-CH_2 of fatty acids), 5.6—6.0 (1H, m, $-OCH_2C\underline{H}=CH_2$), and 7.25—7.4 (15H, br s, aromatic H).

**Allyl 4-*O*-Benzyl-2-[(*R*)-3-benzyloxytetradecanoylamino]-6-*O*-[2-[(*R*)-3-benzyloxytetradecanoylamino]-2-deoxy-4,6-*O*-isopropylidene-*β*-D-glucopyranosyl]-2-deoxy-*β*-D-glucopyr-

anoside (18). A mixture of **17** (10.0 g, 9.06 mmol), 2,2-dimethoxypropane (1.66 ml, 13.5 mmol), and *p*-toluenesulfonic acid (345 mg, 1.81 mmol) in chloroform (150 ml) and THF (150 ml) was stirred at room temperature for 4 h. After neutralization with triethylamine and evaporation of the solvent, the residue was dissolved in chloroform, washed with water, and dried. The product was recrystallized from chloroform–methanol; yield 8.75 g (84%); mp 105—108 °C (with sintering at 95 °C); $[\alpha]_D^{19}$ −28.3° (*c* 1.12, chloroform). Found: C, 68.92; H, 8.98; N, 2.34%. Calcd for $C_{67}H_{102}N_2O_{13} \cdot 1.5H_2O$: C, 68.75; H, 9.04; N, 2.39%. ^{1}H NMR (100 MHz) δ=0.89 (6H, br t, CH_3), 1.2—1.4 (40H, br s, CH_2), 1.44 and 1.52 (each 3H, s, isopropylidene), 2.31—2.53 (4H, m, α-CH_2 of fatty acids), and 7.2—7.35 (15H, aromatic H).

Allyl 4-*O*-Benzyl-3-*O*-[(*R*)-3-benzyloxytetradecanoyl]-6-*O*-[3-*O*-[(*R*)-3-benzyloxytetradecanoyl]-2-[(*R*)-3-benzyloxytetradecanoylamino]-2-deoxy-*β*-D-glucopyranosyl]-2-[(*R*)-3-benzyloxytetradecanoylamino]-2-deoxy-*β*-D-glucopyranoside (19). To a solution of **18** (2.0 g, 1.8 mmol) in anhydrous dichloromethane (80 ml) were added **9** (1.8 g, 5.3 mmol), DCC (1.1 g, 5.3 mmol), and DMAP (107 mg, 0.88 mmol). The mixture was stirred at room temperature for 4 h. After addition of acetic acid (1 ml) and removal of the precipitate, the filtrate was washed with 1 M hydrochloric acid and water, dried, and evaporated to give a pale yellow solid. It was heated in 90% acetic acid (80 ml) at 90 °C for 2 h. After the evaporation of the solvent, the residue was recrystallized from methanol; yield 2.5 g (82 %); mp 117—119 °C; $[\alpha]_D^{17}$ −14.5° (*c* 0.40, chloroform). Found: C, 72.31; H, 9.32; N, 1.59%. Calcd for $C_{106}H_{162}N_2O_{17} \cdot H_2O$: C, 72.57; H, 9.42; N, 1.60%.

Allyl 4-*O*-Benzyl-6-*O*-[6-*O*-benzyloxymethyl-3-*O*-[(*R*)-3-benzyloxytetradecanoyl]-2-[(*R*)-3-benzyloxytetradecanoylamino]-2-deoxy-*β*-D-glucopyranosyl]-3-*O*-[(*R*)-3-benzyloxytetradecanoyl]-2-[(*R*)-3-benzyloxytetradecanoylamino]-2-deoxy-*β*-D-glucopyranoside (20). To a solution of **19** (7.78 g, 4.48 mmol) in anhydrous dichloromethane (150 ml) were added benzyloxymethyl chloride (1.40 g, 8.94 mmol) and *N,N*-diisopropylethylamine (1.56 ml, 8.95 mmol). The mixture was stirred for 23 h at room temperature with addition of benzyloxymethyl chloride (0.70 and 0.35 g) and *N,N*-diisopropylethylamine (0.78 and 0.39 ml) after 1 and 8 h, respectively. The mixture was worked up as usual and the product was recrystallized from methanol; yield 7.97 g (96%); mp 137—139 °C; $[\alpha]_D^{19}$ −12.3° (*c* 1.12, chloroform). Found: C, 73.12; H, 9.19; N, 1.48%. Calcd for $C_{114}H_{170}N_2O_{18} \cdot H_2O$: C, 73.04; H, 9.25; N, 1.49%.

Allyl 4-*O*-Benzyl-6-*O*-[6-*O*-benzyloxymethyl-3-*O*-[(*R*)-3-benzyloxytetradecanoyl]-2-[(*R*)-3-benzyloxytetradecanoylamino]-2-deoxy-*β*-D-glucopyranosyl]-3-*O*-[(*R*)-3-benzyloxytetradecanoyl]-2-[(*R*)-3-benzyloxytetradecanoylamino]-2-deoxy-*β*-D-glucopyranoside 4′-(Diphenyl Phosphate) (21). To a solution of **20** (1.70 g, 0.92 mmol) in anhydrous dichloromethane (70 ml) were added pyridine (0.24 ml, 3.0 mmol), DMAP (0.33 g, 2.7 mmol) and diphenyl phosphorochloridate (0.57 ml, 2.7 mmol). After stirring at room temperature for 7 h, the mixture was worked up as usual and the product was recrystallized from chloroform–methanol; yield 1.45 g (76%); mp 71—72 °C; $[\alpha]_D^{18}$ −7.3° (*c* 0.51, chloroform). Found: C, 72.50; H, 8.73; N, 1.40%. Calcd for $C_{126}H_{179}N_2O_{21}P$: C, 72.45; H, 8.64; N, 1.34%.

4-*O*-Benzyl-6-*O*-[6-*O*-benzyloxymethyl-3-*O*-[(*R*)-3-benzyloxytetradecanoyl]-2-[(*R*)-3-benzyloxytetradecanoylamino]-2-deoxy-*β*-D-glucopyranosyl]-3-*O*-[(*R*)-3-benzyloxytetradecanoyl]-2-[(*R*)-3-benzyloxytetradecanoylamino]-2-deoxy-D-glucose 4′-(Diphenyl Phosphate) (22). Compound **21** (1.39 g, 0.75 mmol) and 1,5-cyclooctadienebis(methyldiphenylphosphine)iridium hexafluorophosphate (65 mg) were dissolved in degassed THF (50 ml). After activation of the iridium catalyst with hydrogen,[18] the mixture was stirred at 50 °C under nitrogen atmosphere for 80 min and then cooled to room temperature. Water (15 ml) and iodine (0.38 g. 1.50 mmol) were added and the mixture was stirred for 5 min. To the mixture were added 5% aqueous solution of sodium sulfite and chloroform. The organic layer was washed with water, dried and evaporated. The product was isolated by medium-pressure column chromatography on silica gel (83 g, chloroform–acetone 9 : 1) and recrystallized from chloroform–methanol; yield 750 mg (55%); mp 78—83 °C; $[\alpha]_D^{15}$ +10.6° (*c* 1.09, chloroform–methanol 3:1). Found: C, 71.88; H, 8.69; N, 1.39%. Calcd for $C_{123}H_{175}N_2O_{21}P$: C, 72.11; H, 8.61; N, 1.37%. ^{1}H NMR (100 MHz) δ=0.88 (12H, t, J=6 Hz, CH_3), 2.1—2.6 (80H, br s, CH_2), 2.06—2.60 (8H, m, α-CH_2 of fatty acids), and 7.0—7.3 (40H, m, aromatic H).

2-Deoxy-6-*O*-[2-deoxy-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-2-[(*R*)-3-hydroxytetradecanoylamino]-*β*-D-glucopyranosyl]-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-2-[(*R*)-3-hydroxytetracedanoylamino]-*α*-D-glucopyranose 1,4′-Bis(phosphate) (2). Compound **22** (500 mg, 0.24 mmol) was dissolved in anhydrous THF (15 ml) and cooled to −70 °C under argon atmosphere. The solution was treated with 1.6 M butyllithium in hexane (0.18 ml, 0.28 mmol) and then dibenzyl phosphorochloridate (70 µl, 0.28 mmol). The mixture was stirred at −70 °C for 5 min and then palladium black was added to it. The mixture was stirred under 6 kg cm^{-2} of hydrogen at room temperature for 17 h. After removal of palladium catalyst, platinum oxide was added and the mixture was stirred under 7 kg cm^{-2} of hydrogen at room temperature for 2 h. The catalyst was filtered off and the filtrate was neutralized with triethylamine. The product was isolated by medium-pressure column chromatography on silica gel (80 g, chloroform–methanol–water–triethylamine 50 : 50 : 10 : 1) as colorless solid (200 mg).

Electrodialysis and Acidic Precipitation of 2. Electrodialysis was carried out according to the method of Galanos and Lüderitz.[21] The apparatus for electrodialysis consisted of three compartments separated by two sheets of dialysis membrane (prepared by cutting Cellophane Tubings-Seamless, Union Carbide Co.). The two outer chamber were equipped with platinum electrodes and filled with distilled water (each 7 ml).

The crude product **2** (176 mg) obtained by the above silica-gel chromatography was suspended in pyrogen-free water (9 ml). The pH of the suspension was adjusted to 9 with triethylamine, sonicated and put in the middle chamber. Direct electric current was applied to the apparatus, voltage being increased gradually to 150 V, while current was maintained less than 30 mA. After 1 h, the pH value of the middle chamber dropped to 3 and some precipitate appeared, which was dissolved again by adjusting the pH of the solution to 9 with triethylamine. The temperature of the solution was kept below 20 °C by cooling water throughout the operations. A series of these operations was repeated three times. Thereafter the suspension in the middle chamber was lyophilized to afford a white powder (145 mg).

A suspension of the product in pyrogen-free water (40 ml) was adjusted to pH 9 with triethylamine, sonicated and centrifugated at 17500 rpm for 10 min at 0 °C in order to remove insoluble materials. To the supernatant was added 1 M hydrochloric acid (4.4 ml) at 0 °C and then the resultant precipitate was collected by centrifugation under the same conditions as above. After centrifugal washing with 0.1 M hydrochloric acid and water, the substance was lyophilized to give a white powder; yield 69 mg (20% from **22**); $[\alpha]_{\mathrm{D}}^{19}$ +2.1° (*c* 1.05, chloroform-methanol 3 : 1). Found: C, 56.33; H, 9.37; N, 2.01%. Calcd for $C_{68}H_{130}N_2O_{23}P_2\cdot 2.5H_2O$: C, 56.30; H, 9.38; N, 1.93%.

2-Deoxy-6-*O*-[2-deoxy-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-2-[(*R*)-3-hydroxytetradecanoylamino]-*β*-D-glucopyranosyl]-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-2-[(*R*)-3-hydroxytetradecanoylamino]-D-glucose 4′-Phosphate (3). A solution of **22** (750 mg, 0.37 mmol) in THF (20 ml) and acetic acid (1.5 ml) was hydrogenolyzed for 38 h in the presence of palladium black under 6.5 kg cm^{-2} of hydrogen. After removal of the catalyst and addition of platinum oxide, the mixture was stirred for 15 h under 6.5 kg cm^{-2} of hydrogen. The catalyst was filtered and the filtrate was neutralized with triethylamine and then evaporated. Lyophilization from dioxane gave the crude product as a white powder (360 mg). A part of this material (149 mg) was purified further by means of acidic precipitation as described for compound **2**; yield 104 mg; $[\alpha]_{\mathrm{D}}^{15}$ −13.6° (*c* 0.84, chloroform-methanol 3:1). Found: C, 59.47; H, 9.66; N, 2.17%. Calcd for $C_{68}H_{129}N_2O_{20}\cdot 2.5H_2O$: C, 59.59; H, 9.85; N, 2.04%.

Identification of Synthetic and Natural Dimethyl Ester of 3. Synthetic compound **3** (42.5 mg, 3.2×10^{-5} mol) in chloroform-methanol (9 : 1, 8 ml) was treated with ethereal diazomethane. The product was purified by preparative silica-gel TLC (chloroform-methanol 6 : 1) and lyophilized from dioxane; yield 27.3 mg (64%). This material is identical with the dimethyl ester of 4′-monophosphate prepared from natural precursor Ia as described below.

A suspension of crude natural precursor Ia (25 mg) in 2% aqueous acetic acid (10 ml) was heated at 95 °C for 1 h. The mixture was cooled and lyophilized to give a white powder, which was dissolved in chloroform-methanol (9 : 1, 15 ml). The solution was acidified with 1 M hydrochloric acid (1 ml), sonicated, and concentrated in vacuo. The residue was dissolved again in chloroform-methanol (9 : 1, 10 ml) and treated with ethereal diazomethane. Purification by preparative TLC and lyophilization from dioxane afforded a white powder; yield 3.0 mg.

4-*O*-Benzyl-6-*O*-[6-*O*-benzyloxymethyl-3-*O*-[(*R*)-3-benzyloxytetradecanoyl]-2-[(*R*)-3-benzyloxytetradecanoylamino]-2-deoxy-*β*-D-glucopyranosyl]-3-*O*-[(*R*)-3-benzyloxytetradecanoyl]-2-[(*R*)-3-benzyloxytetradecanoylamino]-2-deoxy-D-glucose (24). Compound **20** (990 mg, 0.53 mmol) was treated with the iridium complex (50 mg) then with iodine (270 mg, 1.06 mmol) as described for compound **22**. The product was isolated by medium-pressure column chromatography on silica gel (75 g, chloroform-acetone 5 : 1) and recrystallized from methanol; yield 660 mg (68%); mp 117—121 °C; $[\alpha]_{\mathrm{D}}^{15}$ +3.1° (*c* 1.11, chloroform-methanol 3 : 1). Found: C, 72.64; H, 9.24; N, 1.47%. Calcd for $C_{111}H_{166}N_2O_{18}$: C, 72.67; H, 9.23; N, 1.53%.

2-Deoxy-6-*O*-[2-deoxy-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-2-[(*R*)-3-hydroxytetradecanoylamino]-*β*-D-glucopyranosyl]-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-2-[(*R*)-3-hydroxytetradecanoylamino]-*α*-D-glucopyranose 1-Phosphate (4). A solution of compound **24** (770 mg, 0.424 mmol) in THF (25 ml) was cooled to −70 °C under argon atmosphere and treated with 1.6 M butyllithium in hexane (0.30 ml, 0.47 mmol) and then with dibenzyl phosphorochloridate (0.12 ml, 0.48 mmol). After 20 min, palladium-black (500 mg) was added and the mixture was hydrogenolyzed under 6 kg cm^{-2} of hydrogen atmosphere at room temperature for 17 h. Isolation by medium-pressure column chromatography on silica gel (80 g, chloroform-methanol-water-triethylamine 250 : 100 : 15 : 1) gave a crude product as white solid (260 mg).

This material was purified by a combination of electrodialysis and acidic precipitation method as described for **2**; yield 183 mg (33%); $[\alpha]_{\mathrm{D}}^{21}$ −5.1° (*c* 1.03, chloroform-methanol 9 : 1); Found: C, 60.21; H, 9.83; N, 2.14%. Calcd for $C_{68}H_{129}N_2O_{20}P\cdot 1.5H_2O$: C, 60.38; H, 9.83; N, 2.07%.

2-Deoxy-6-*O*-[2-deoxy-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-2-[(*R*)-3-hydroxytetradecanoylamino]-*β*-D-glucopyranosyl]-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-2-[(*R*)-3-hydroxytetradecanoylamino]-D-glucose (5). Compound **19** (700 mg, 0.40 mmol) was treated with the iridium complex (35 mg) and then with iodine (137 mg, 0.54 mmol) as described above for **22**. Isolation by medium-pressure column chromatography on silica gel (25 g, chloroform-acetone 2 : 1) gave a solid, which was dissolved in THF (20 ml) and acetic acid (1 ml). The solution was hydrogenolyzed with palladium-black under 7 kg cm^{-2} hydrogen. The product was recrystallized from methanol; yield 141 mg (28%); mp 210—212 °C (decomp). $[\alpha]_{\mathrm{D}}^{18}$ −19.2° (*c* 1.02, chloroform-methanol 9 : 1). Found: C, 64.42; H, 10.40; N, 2.20%. Calcd for $C_{68}H_{128}N_2O_{17}\cdot H_2O$: C, 64.63; H, 10.37; N, 2.22%.

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