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New glycolipids (chitooligosaccharide derivatives) possessing immunostimulating and antitumor activities

Vladimir I. Gorbach [a,*], Inna N. Krasikova [a], Pavel A. Luk'yanov [a], Yury N. Loenko [a], Tamara F. Solov'eva [a], Yury S. Ovodov [a], Vadim V. Deev [b], Alexey A. Pimenov [b]

[a] *Pacific Institute of Bioorganic Chemistry, Far East Division, The Russian Academy of Sciences, Vladivostok-22, Russian Federation*

[b] *Oncological Center, Academy of Medical Sciences of Russia, Moscow, Russian Federation*

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Abstract

New glycolipids, derived from chitooligosaccharides of dp 2–4 and containing both free and acylated amino groups, were synthesized. The structure of the key compounds (di-, tri-, and tetra-saccharides acylated with different fatty acids) were elucidated by ^{13}C NMR spectroscopy. Only the amino group of the reducing end of the chitooligosaccharides was found to be acylated when equimolecular amounts of reagents were used. The compounds obtained were shown to possess a low toxicity and certain immunostimulatory and antitumor activities. An induction of interleukin-1 and tumor necrosis factor by the immunocompetent cells and an augmentation by 140–180% of the mean life of mice with the Erlich carcinoma were observed.

1. Introduction

Amino sugars and their derivatives are known to represent an important group of biologically active compounds influencing the immune system of mammals and plants. Muramyldipeptides have been reported to exhibit immunostimulating properties [1]; oligosaccharides of dp 4–6 were found to possess a hemoattractive effect on macrophages [2]; chitohexaose and its *N*-peracetylated derivative were shown to inhibit the growth of murine cancer cells [3] and to protect from *Candida albicans* infection [4]. Chitosan of low molecular weight and chitooligosaccharide mixtures were shown to inhibit the growth of phytopathogens in plants [5].

Among the amino sugar derivatives, a special place belongs to lipid A — a component of Gram-negative bacteria lipopolysaccharides. It is composed of a

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1,4-bisphosphorylated β-(1 → 6)-linked glucosamine disaccharide with amide- and ester-linked 3-hydroxy- or 3-acyloxy-acyl fatty acids [6]. Lipid A and its synthetic analogues possess a wide variety of bioactivities, as follows: adjuvanticity, mitogenicity, antitumor and radioprotective properties, and the ability to induce synthesis of interferon, interleukin-1, and tumor necrosis factor by cells of the immune system [7]. It has, however, not found widespread clinical use because of its inherent endotoxicity. The present paper describes our attempts to create novel glucosamine-containing derivatives as glycolipids possessing a good balance between toxicity and beneficial bioactivities.

2. Results and discussion

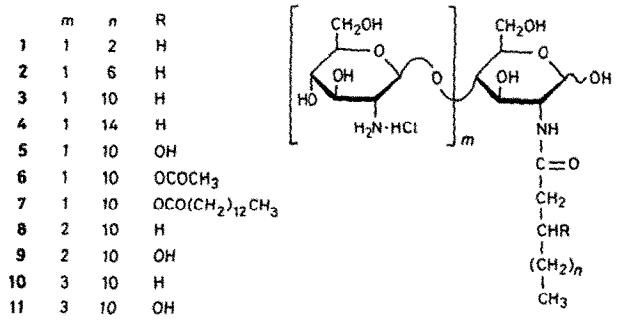
We have synthesized earlier [8] lipid A analogues, using *O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-amino-2-deoxy-D-glucose (chitobiose) as the parent compound differing from lipid A in the type of glycosidic linkage in the backbone. Upon *N*-acylation of the initial disaccharide, a new compound was obtained in addition to the *N*,*N*-disubstituted derivative. It was shown to be chitobiose having only one amino group acylated by the 3-hydroxytetradecanoic acid residue. The material obtained was found to dissolve readily at the acidic pH of the medium and to display immunostimulating activity.

As mentioned above, chitooligosaccharides are known to be biologically active compounds [2–4]. We speculated that fatty acid residues may intensify or modify the biological functions of these compounds. This idea impelled us to extend the range of similar compounds by varying the dp of the chitooligosaccharides and the nature of the fatty acids.

Partial *N*-acylation of the chitooligosaccharides was carried out by reaction of equimolecular amounts of the sugar and acylating agent in a mixture of water and organic solvent. Three acylating agents were used: (*A*) the fatty acids together with dicyclohexylcarbodiimide, (*B*) the acid chlorides, or (*C*) the *N*-hydroxysuccinimide esters of the acids.

Isolation of the partially acylated compounds from the reaction mixtures was achieved by taking advantage of their solubility in acidic aqueous solutions from which the hydrochlorides could be extracted into 1-butanol. Final purification was effected by precipitation of the compounds obtained by the addition of acetone to a methanol solution. In some cases (compounds **5** and **9**, procedure *A*), an additional treatment was necessary to remove the impurities of carbodiimide and urea derivatives. Liquid chromatography on a hydrophobic adsorbent was used for this purpose.

The various methods of acylation led to substantial differences in the yields of glycolipids. Procedure *A*, using carbodiimide, gave low yields (up to 20%) of mono-*N*-acylated chitooligosaccharides (compounds **5** and **9**). The results of synthesis by procedure *B* depended on the reactivity of the fatty acid chloride used; the yields of compounds **1**, **2**, **3**, **4**, **8**, and **10** were in a range 11 to 38%. Procedure *C* with the preformed active fatty acid ester proved to be the best. It gave reliable



Scheme 1.

30–50% yields of compounds **2**, **3**, **5**, **6**, and **11** with the exception of the synthesis using the ester of 3-tetradecanoyloxytetradecanoic acid which was poorly soluble in the reaction mixture (compound **7**, yield 15.5%). All the compounds synthesized were chromatographically homogeneous. Their structures, listed in Scheme 1, were confirmed by the analytical data and physico-chemical methods of investigation.

The IR-spectra of the glycolipids (**1–11**) showed absorption bands at 1528, 1644, 2856, and 2922 cm^{-1}, characteristic of the fatty acid amides. In addition, the spectra of compounds **6** and **7** containing acyloxy fatty acid residues showed an absorption band at 1716 cm^{-1} characteristic of the ester linkage.

Each of the compounds synthesized was proved by analytical data to contain only one fatty acid residue. ^{13}C NMR spectroscopy was used to estimate the position of the acyl group in the glycolipid molecules. Satisfactory ^{13}C NMR spectra were obtained; as an example, the spectra of compound **3** and the parent chitobiose are given in Fig. 1.

The spectral data for some key compounds **3**, **5**, **8**, and **11** are listed in Table 1. The assignment of signals in the spectra was made by comparison with ^{13}C NMR data of the initial chitooligosaccharides. Interpretation of ^{13}C NMR data was substantially facilitated by the preference for the α configuration of the reducing end of the glycolipids in contrast to the β configuration of the other residues. As is known [9,10], acetylation of the amino group at C-2 of an amino sugar has little effect on the chemical shift of C-2. However, β-effects of such substitutions on the adjacent carbon atoms amount to 2–3 ppm. Experimentally, the signals of C-1, C-3, and C-4 of the reducing end of compounds **3**, **5**, **8**, and **11** were shifted downfield by 2.0, 1.0, and 0.9 ppm in comparison with the signals of these atoms in spectra of chitooligosaccharides.

On the other hand, *N*-acetylation of chitobiose at the nonreducing end leads to a downfield shift of C-1′ and C-4′ signals by ~4 ppm [11]. Signals with such values of chemical shifts were absent in the spectra of compounds **3**, **5**, **8** and **11**, thus indicating that the nonreducing sugar residues possessed free amino groups. Therefore, only the amino groups of the reducing sugar residues of the glycolipids synthesized were acylated with the fatty acids.

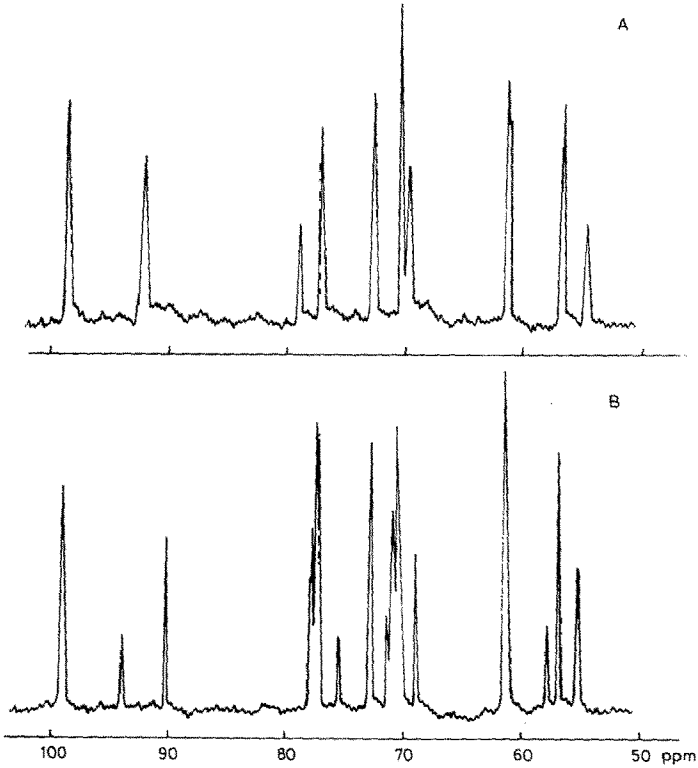


Fig. 1. ^{13}C NMR spectra of compound **3** (A) and chitobiose (B). Only the signals for carbon atoms of the carbohydrate moiety of **3** in the field 50–100 ppm are given in this figure. Also, the signals of carbon atoms of the tetradecanoic acid residue at 14.4, 23.2, 26.3, 30.0, 30.4, 32.6, and 36.7 ppm were recorded in spectrum A.

As may be seen from Table 1, only one signal is observed in the region of the anomeric carbon atoms of compounds **3**, **5**, **8**, and **11**, thus pointing to a high isomeric purity of the new glycolipids obtained.

An absence of isomers resulting from acylation of the nonreducing amino sugar residues is surprising. Analysis of the reaction mixture by TLC points to the presence in small quantities of other products with similar mobility, but the homogeneous compounds were obtained in the process of purification. The well-resolved NMR spectra of the purified substances suggest that they are pure single isomers.

It should be noted that the compounds examined by ^{13}C NMR spectroscopy were synthesized by different methods (compounds **3**, **5**, **8**, and **11** by methods *C*, *A*, *B*, and *C*, respectively). Evidently, a preferred substitution of the amino group of the reducing end occurs on reaction of equimolecular amounts of reagents, independent of the method. It suggests that, in all of the other compounds synthesized, the amino group of the reducing end is also acylated. The reasons for such a regiospecific acylation are not obvious. There are several conceivable

Table 1
The ^{13}C NMR data [a] for chitooligosaccharides and some mono-*N*-acylated derivatives

Compound	Reducing end						Central residues						Nonreducing end					
	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-5	C-6
Chitobiose	89.4	54.8	68.5	77.3	70.5	60.9							98.2	56.6	72.4	70.3	77.0	60.9
3	91.5	54.4	69.7	78.3	70.4	61.2 [b]							98.3	56.6	72.5	70.4	77.2	61.4 [b]
5	91.3	54.6	69.5	77.9	70.3	61.2							98.2	56.5	72.5	70.3	77.1	61.2
Chitotriose	89.4	54.9	68.5	77.3	70.5	61.0 [b]	98.0 [b]	56.5	70.7	77.3	75.3	61.0 [b]	98.2 [b]	56.5	72.3	70.3	77.0	60.8 [b]
8	91.5	54.5	69.6	78.0	70.3	61.1 [b]	97.9 [b]	56.6	70.7	77.3	75.5	61.1 [b]	98.2 [b]	56.6	72.4	70.3	77.1	61.4 [b]
Chitotetraose	89.5	54.9	68.5	77.3	70.4	61.0 [b]	98.0 [b]	56.6	70.7	77.4	75.4	61.1 [b]	98.3 [b]	56.6	72.3	70.4	77.0	61.0 [b]
							97.8 [b]											
11	91.3	54.6	69.4	78.2	70.5	61.1 [b]	98.1 [b]	56.6	70.7	77.6	75.4	61.0 [b]	98.3 [b]	56.6	72.4	70.4	77.1	61.4 [b]
							97.8 [b]											

[a] Carbon atom signals for the α anomers (prevalent in solution) of chitooligosaccharides and their derivatives. The signals of carbon atoms of fatty acid residues in compounds **3**, **5**, **8**, and **11** are not given in this table. [b] Precise assignment is uncertain.

Table 2
Induction of interleukin-1 (IL-1) and tumor necrosis factor (TNF) with the new glycolipids in vitro and in vivo and their antitumor activity

Compound	IL-a [a]	TNF [b]	TNF [c]	Antitumor activity (%) [d]
3	2.03	54.9		188
5	2.36	45.1	76.5	138
7	4.24	34.9		138
9	1.37	35.9		144
11	2.98	32.1		140
Lipid A from *Y. pseudotuberculosis*	3.45	13.3	70.0	–

[a] Stimulation index for dose of 10 μg/mL. [b] Cytolytic index for dose of 10 μg/mL. [c] Cytolytic index of the compounds injected in mice (dose, 2.5 mg/kg); blood serum was tested in titre 1/4. [d] Increase (%) of mean life of mice with Erlich carcinoma.

explanations of this phenomenon. The presence of the neighbouring free lactol hydroxyl group may increase the reactivity of the amino group of the reducing end of the molecule. Alternatively, steric effects by the reducing end (or central residues of amino sugars) may inhibit attack at the amino group of adjacent residues. The preferred acylation of the amino group of the reducing residue may also result from its existence in the acyclic form.

The compounds obtained are not close analogues of lipid A, but contain a number of its structural elements. For this reason, the next step of our investigation was to examine their toxicity and other biological properties. A study of the acute toxicity of the novel compounds revealed that all of them were nontoxic for the CBA mice in doses of 60 mg/kg (peritoneal injection) and 50 mg/kg (intravenous). They may therefore be considered to possess low toxicity.

Some beneficial activities of the glycolipids synthesized were examined in a bioassay routinely used for the study of lipid A and its analogues. Interleukin-1 and tumor necrosis factor induction by immune system cells in vitro was estimated [12,13]. The influence of the preparations on the production of tumor necrosis factor in vivo was determined by its presence in the blood serum of mice after intravenous injection of the substances tested [13]. The data are given in Table 2.

As may be seen from Table 2, all the preparations studied were inducers of limphokines, and some compounds were more active than lipid A from *Yersinia pseudotuberculosis* LPS. It suggests the possibility that they might be antitumor agents. In fact, an injection of compounds **3**, **5**, **7**, **9**, and **11** in the CBA mice with Erlich carcinoma increased their mean life in comparison with a control group of animals by 188, 138, 138, 144, and 140%, respectively (Table 2). It is noteworthy that the most active compound (**3**) in this test was also the most active inducer of tumor necrosis factor in vitro.

Thus, we have obtained new glycolipids based on chitooligosaccharides containing both free amino groups and amino groups acylated by fatty acids. Owing to their amphiphilicity, these compounds readily dissolved in water and interacted

actively with membranes of the immune system cells. The partial dissociation of harmful and beneficial properties should encourage the use of novel glycolipids in medicine and biotechnology.

3. Experimental

General methods.—^{13}C NMR spectra were recorded with a Bruker-Physik WM-250 spectrometer at 62.9 MHz in D_2O solution with CD_3OD (49.6 ppm) as the internal standard. IR spectra were obtained with a Specord spectrophotometer for KBr pellets. Specific rotations were determined with a Perkin–Elmer 141 polarimeter. Preparative column chromatography was performed on Octadecyl Si60 Polyol (Serva, 0.01 mm). Silica Gel Woelm TLC was used for TLC, with 40:40:15:5 1-butanol–EtOH–water –25% NH_3 as solvent.

Reagents.—(*R,S*)-3-Hydroxytetradecanoic acid was synthesized as described earlier [14]; (*R,S*)-3-acetoxy- and (*R,S*)-3-tetradecanoyloxytetradecanoic acids were synthesized as reported [15]; *N*-hydroxysuccinimide esters of fatty acids were obtained as described by Demary et al. [16].

Chitooligosaccharide hydrochlorides were obtained using acid hydrolysis of chitosan followed by fractionation of the mixture on a column of Amberlite CG-120 (H^+) resin [17]. Lipid A was isolated from *Yersinia pseudotuberculosis* lipopolysaccharide (1B serovar, strain 598) as described previously [18].

Biological assays.—Acute toxicity was determined on CBA mice (males, 8 weeks old; weight, 18–20 g) by peritoneal and intravenous injections of all the glycolipids. The inducing activities of interleukin-1 and tumor necrosis factor in vitro and tumor necrosis factor in vivo were determined according to literature methods [12,13]. To examine the antitumor activity of synthetic compounds, groups of CBA mice (10 animals; male, 8 weeks old; weight, 18–20 g) were inoculated intraperitoneally with 1×10^6 Erlich carcinoma cells. The test compounds in saline (60 mg/kg) and controls (saline alone) were injected intraperitoneally into mice 5 times on days +1, +2, +3, +4, and +5. Survivors were counted every day, then the mice mean life and its enhancement were calculated.

Synthesis of samples: Procedure A.—*O-(2-Amino-2-deoxy-β-*D*-glucopyranosyl)-(1 → 4)-2-deoxy-2-[(*R,S*)-3-hydroxytetradecanoylamino]-*D*-glucose hydrochloride* (**5**). Chitobiose hydrochloride (0.29 g, 0.7 mmol) was dissolved in water (5 mL) and the aqueous solution was applied on a column of Dowex 1-X8 (HO^-) resin followed by elution with water. The eluate obtained was evaporated to 2 mL. To this mixture were added solutions of (*R,S*)-3-hydroxytetradecanoic acid (0.19 g, 0.77 mmol) in dry pyridine (10 mL) and dicyclohexylcarbodiimide (0.29 g, 1.4 mmol) in pyridine (10 mL). The mixture was kept for 24 h at 20°C, the precipitate was removed by filtration, the filtrate was evaporated, and the residual material was extracted with acetone. The undissolved residue was stirred with water (20 mL), and acidified by HCl to pH 2.0. The mixture was filtered and the filtrate was extracted by 1-butanol (2×20 mL). The butanolic extract was evaporated, the residual material was dissolved in MeOH (5 mL), and the product was precipitated by adding acetone

(50 mL). The precipitate was separated by centrifugation to afford **5** (82.4 mg, 19.5%). For purification, the material was dissolved in water and subjected to chromatography on a column of Octadecyl Si6O Polyol (5 mL), eluting with water and then an increasing gradient of EtOH in water (control by TLC). The yield of compound **5**, after precipitation from MeOH with acetone, was 68.9 mg (16.3%); white powder; $[\alpha]_D^{25}$ + 16.0° (*c* 0.3, H_2O). Anal. Calcd for $C_{26}H_{51}ClN_2O_{11}$ (603.1): C, 51.78; H, 8.52; N, 4.64. Found: C, 51.62; H, 8.45; N, 4.65.

Procedure *A* was also used to obtain **9**.

O-(2-Amino-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-O-(2-amino-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-deoxy-2-[(R,S)-3-hydroxytetradecanoylamino]-D-glucose dihydrochloride (**9**). Yield 11.6%; $[\alpha]_D^{25}$ +6.3° (*c* 0.4, H_2O). Anal. Calcd for $C_{32}H_{63}Cl_2N_3O_{15}$ (800.8): C, 48.0; H, 7.93; N, 5.25. Found: C, 48.05; H, 8.40; N, 5.07.

Procedure B.—*O-(2-Amino-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-O-(2-amino-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-deoxy-2-tetradecanoylamino-D-glucose dihydrochloride* (**8**).—Triethylamine (0.56 mL) and tetradecanoyl chloride (212 mg, 0.86 mmol) were slowly added to a solution of chitotriose hydrochloride (500 mg, 0.82 mmol) in a mixture of water (5 mL) and EtOH (45 mL). The mixture was stirred for 18 h at 20°C, acidified by aq HCl, and evaporated. The residue was extracted with acetone. The undissolved residue was stirred with water (10 mL), the mixture was filtered, and the filtrate was extracted with 1-butanol (3 × 10 mL). The butanol extract was evaporated, the residue was dissolved in MeOH (5 mL), and the product was precipitated with acetone (50 mL). The precipitate was filtered off to afford **8** (240 mg, 37.4%); white powder; $[\alpha]_D^{25}$ +7.3° (*c* 0.3, H_2O). Anal. Calcd for $C_{32}H_{63}Cl_2N_3O_{14}$ (784.8): C, 48.98; H, 8.09; N, 5.35. Found: C, 48.54; H, 8.02; N, 5.14.

Procedure *B* was also used to obtain **1–4** and **10**.

O-(2-Amino-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-deoxy-2-hexanoylamino-D-glucose hydrochloride (**1**). Yield 11%; $[\alpha]_D^{25}$ +18.7° (*c* 0.25, H_2O). Anal. Calcd for $C_{18}H_{35}ClN_2O_{10}$ (474.9): C, 45.52; H, 7.43; N, 5.90. Found: C, 45.08; H, 7.32; N, 5.67.

O-(2-Amino-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-deoxy-2-decanoylamino-D-glucose hydrochloride (**2**). Yield 22.4%; $[\alpha]_D^{25}$ +15.9° (*c* 0.3, H_2O). Anal. Calcd for $C_{22}H_{43}ClN_2O_{10}$ (531.0): C, 49.75; H, 8.15; N, 5.28. Found: C, 49.66; H, 7.93; N, 5.32.

O-(2-Amino-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-deoxy-2-tetradecanoylamino-D-glucose hydrochloride (**3**). Yield 38.0%; $[\alpha]_D^{25}$ +14.7° (*c* 0.3, H_2O). Anal. Calcd for $C_{26}H_{51}ClN_2O_{10}$ (587.1): C, 53.19; H, 8.76; N, 4.77. Found: C, 52.86; H, 8.64; N, 4.89.

O-(2-Amino-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-deoxy-2-octadecanoylamino-D-glucose hydrochloride (**4**). Yield 14.0%; $[\alpha]_D^{25}$ +14.0° (*c* 0.2, 1:1 H_2O–MeOH). Anal. Calcd for $C_{30}H_{59}ClN_2O_{10}$ (643.3): C, 56.02; H, 9.25; N, 4.36. Found: C, 55.44; H, 9.57; N, 4.64.

O-(2-Amino-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-O-(2-amino-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-O-(2-amino-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-deoxy-2-

tetradecanoylamino-D-glucose trihydrochloride (**10**). Yield 26.5%; $[\alpha]_D^{25}$ +1.5° (*c* 0.3, H_2O). Anal. Calcd for $C_{38}H_{75}Cl_3N_4O_{18}$ (982.4): C, 46.46; H, 7.70; N, 5.70. Found: C, 45.27; H, 8.03; N, 5.24.

Procedure C.—*Compound* **3**. Triethylamine (0.15 mL) and a solution of the *N*-hydroxysuccinimide ester of tetradecanoic acid (167 mg, 0.54 mmol) in EtOH (10 mL) were added to a solution of chitobiose hydrochloride (200 mg, 0.48 mmol) in a mixture of water (1 mL) and *N*,*N*-dimethylformamide (4 mL). The mixture was stirred for 18 h at 20°C followed by treatment as described for procedure *B*, to furnish **3** (123.9 mg, 43.7%).

Procedure *C* was also used to obtain **2**, **5**–**7**, and **11**.

Compound **2**. Yield 29.8%.

Compound **5**. Yield 50.9%.

O-(2-Amino-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-[(R,S)-3-acetoxytetradecanoylamino]-2-deoxy-D-glucose hydrochloride (**6**). Yield 37.3%; $[\alpha]_D^{25}$ +15.4° (*c* 0.2, H_2O). Anal. Calcd for $C_{28}H_{53}ClN_2O_{12}$ (645.2): C, 52.13; H, 8.28; N, 4.34. Found: C, 51.64; H, 7.68; N, 4.11.

O-(2-Amino-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-deoxy-2-(R,S)-3-tetradecanoyloxytetradecanoylamino-D-glucose hydrochloride (**7**). Yield 15.5%; $[\alpha]_D^{25}$ +16.3° (*c* 0.3, H_2O). Anal. Calcd for $C_{40}H_{77}ClN_2O_{12}$ (813.5): C, 59.06; H, 9.54; N, 3.44. Found: C, 57.84; H, 9.38; N, 3.72.

O-(2-Amino-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-O-(2-amino-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-O-(2-amino-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-deoxy-2-[(R,S)-3-hydroxytetradecanoylamino]-D-glucose trihydrochloride (**11**). Yield 37.8%; $[\alpha]_D^{25}$ +2.6° (*c* 0.4, H_2O). Anal. Calcd for $C_{38}H_{75}Cl_3N_4O_{19}$ (998.4): C, 45.72; H, 7.57; N, 5.61. Found: C, 44.91; H, 8.11; N, 5.56.

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