Studies on Selectin Blockers. 2. Novel Selectin Blocker as Potential Therapeutics for Inflammatory Disorders

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As a part of our studies of selectin blockers, we prepared 1-(2-tetradecylhexadecyl)-3′-*O*-sulfo Le^X **1** and 1-(2-tetradecylhexadecyl) sLe^X **2** and examined their inhibitory activities against natural ligand (sLe^X) binding to E-, P-, and L-selectins. Compounds **1** and **2** were 2 times more potent than the sLe^X tetrasaccharide toward E-selectin binding and up to 4 times more potent than sLe^X toward P- and L-selectin binding. Interestingly, compound **1** provided dose-dependent protective effects against an immunoglobulin E-mediated skin reaction in mouse ears. This protective effect was associated with diminished tissue accumulation of neutrophils in the ear (as assessed by myeloperoxidase). These findings indicate that the modification of sLe^X or 3′-*O*-sulfo Le^X with a "branched anchor", a 2-tetradecylhexadecyl group, is useful in the design of a more potent selectin blocker, which has broad inhibitory activities toward all selectins.

Recent studies have indicated the involvement of selectin-oligosaccharide interactions in various inflammatory diseases.[1] It is known that E-selectin (ELAM-1), P-selectin (GMP-140), and L-selectin (LECAM-1) play important roles in the migration of inflammatory cells from the blood stream to inflammatory sites. Selectins are expressed on a variety of cell surfaces. For example, E-selectin is an adhesion molecule that is expressed on vascular endothelial cells during inflammation.[2] P-selectin is an adhesion molecule that is expressed on platelets and vascular endothelial cells,[3] and L-selectin is an adhesion molecule expressed on leukocytes.[4] These selectins are believed to be involved in the progression of the clinical manifestations of complex diseases, such as chronic inflammation.[5] Attempts have been made, therefore, to find a selectin blocker that effectively inhibits cell-adhesion activities at an early stage of inflammation.[6] To this end, it would be desirable for the blocker to exert its cell adhesion-inhibitory effects on all members of the selectin family.

Phillips et al. reported that the native ligand for E-selectin is the tetrasaccharide sialyl Lewis X (sLe^X).[7] Feizi et al. found that a 3′-sulfated analog of sLe^X was a better ligand for the P- and L-selectins than sLe^X.[8] Since then, numerous sLe^X derivatives have been reported.[9] We have also reported that 1-deoxy-3′-*O*-sulfo Le^X **3** was more potent than sLe^X against P-selectin in a competitive binding assay.[10] As a part of our studies of selectin blockers, we prepared 1-(2-tetradecylhexadecyl)-3′-*O*-sulfo Le^X **1** and 1-(2-tetradecylhexadecyl) sLe^X **2** with a branched anchor attached, similar to the natural ceramide, and examined their inhibitory activities against natural ligand (sLe^X) binding to E-, P-, and L-selectins. In our current studies, we investigated the in vivo activity of compound **1** and demonstrated a significant protective effect against an immunoglobulin E (IgE)-mediated skin reaction in the mouse ear.

Results and Discussion

Chemistry. Compounds **1**−**3** were synthesized according to a published procedure.[11]

For the synthesis of compound **1**, the glycosylation of 2-tetradecylhexadecan-1-ol (**5**) with the key intermediate **4**, in dichloromethane in the presence of boron trifluoride etherate, gave exclusively the β-glycoside **6**, in an 85% yield. Compound **6** was treated with hydrazine monoacetate in ethanol−tetrahydrofuran at room temperature to give the 3-hydroxy derivative **7** in a quantitative yield. Compound **7** was treated with a sulfur trioxide pyridine complex in DMF for 1 h at room temperature to afford the sulfated Le^X **8**, and this was transformed quantitatively, by the removal of the protecting groups, into the desired compound **1**.

For the synthesis of compound **2**, the glycosylation of **5** with the key intermediate **9** gave exclusively the β-glycoside **10**, in a 66% yield. Deacylation of **10** with sodium methoxide in methanol and hydrolysis of the methyl group quantitatively yielded the desired sLe^X analog **2**.

Biological Activities: Inhibition Assay of E-, P-, and L-Selectin−sLe^X Binding. The method using selectin-IgG chimeras reported by Foxall et al. was followed.[12] As shown in Table 1, compounds **1** and **2** were more potent (IC_{50}, 0.28 mM for **1**, 0.33 mM for **2**) than sLe^X (IC_{50}, 0.6 mM) and 1-deoxy-3′-*O*-sulfo Le^X (IC_{50}, >1.0 mM) in the ligand−E-selectin competitive binding assay. In addition, compounds **1** and **2** were 25 times more potent (IC_{50}, 0.03 mM for **1**, 0.04 mM for **2**) than sLe^X and **3** (IC_{50}s, >1.0 mM for both) against L-selectin in the competitive binding assay, which demonstrated that the attachment of a branched alkane,

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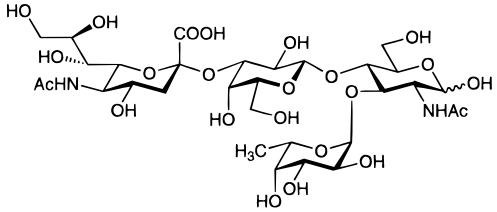
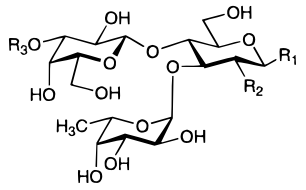
Chart 1

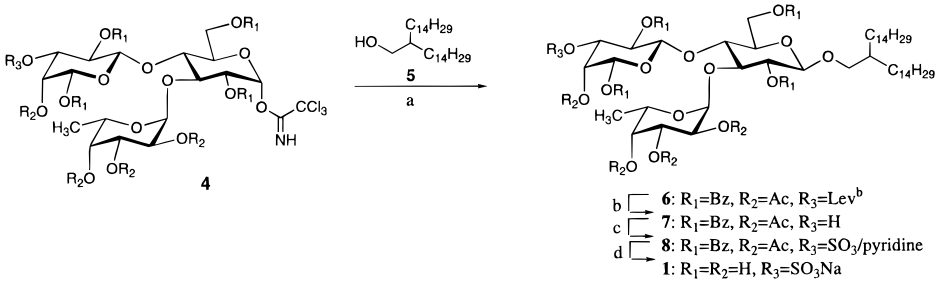


sLe^x



1: R_1= $OCH_2CH(C_{14}H_{29})_2$, R_2=OH, R_3=SO_3Na
2: R_1=$OCH_2CH(C_{14}H_{29})_2$, R_2=NHAc, R_3=sialic acid
3: R_1=H, R_2=OH, R_3=SO_3Na

Scheme 1[a]



4

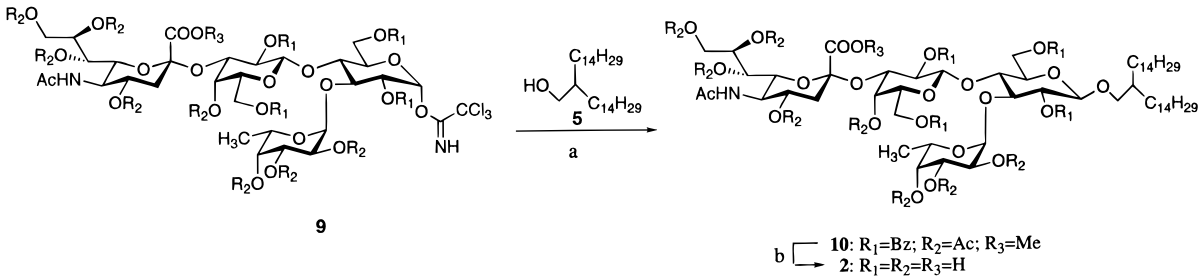
5

a

b → **6**: R_1=Bz, R_2=Ac, R_3=Lev[b]
c → **7**: R_1=Bz, R_2=Ac, R_3=H
d → **8**: R_1=Bz, R_2=Ac, R_3=SO_3/pyridine
1: R_1=R_2=H, R_3=SO_3Na

[a] Reagents and conditions: (a) BF_3-Et_2O, CH_2Cl_2 (69%); (b) NH_2NH_2, EtOH (100%); (c) pyridine-SO_3, DMF (87%); (d) NaOMe, MeOH (92%). [b] Lev, levulinyl group.

Scheme 2[a]



9

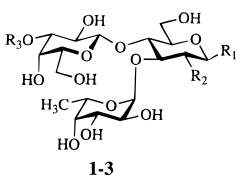
5

a

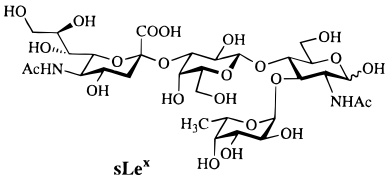
b → **10**: R_1=Bz; R_2=Ac; R_3=Me
2: R_1=R_2=R_3=H

[a] Reagents and conditions: (a) BF_3-Et_2O, CH_2Cl_2 (66%); (b) NaOMe, KOH (100%).

Table 1. Blocking Activity of Compounds **1**−**3** and sLe^X



1-3



sLe^x

				IC$_{50}$, mM		
compd	R1	R2	R3	E-selectin	P-selectin	L-selectin
1	B-30[a]	OH	SO_3Na	0.28	0.10	0.03
2	B-30	NHAc	NeuAc[b]	0.33	0.25	0.04
3	H	OH	SO_3Na	>1.0	0.30	>1.0
sLe^X				0.60	>1.0	>1.0

[a] B-30, $OCH_2CH(C_{14}H_{29})_2$. [b] NeuAc, sialic acid.

a 2-tetradecylhexadecyl group, to sLe^X and 3′-sulfo Le^X was important for binding to the E- and L-selectins.

On the other hand, the competitive inhibitory activities of **1** and **2** against P-selectin binding (IC$_{50}$, 0.10 mM for **1**, 0.25 mM for **2**) were similar to that of compound **3** (IC$_{50}$, 0.30 mM), which indicates that a branched alkane at the 1-position of either compound **1** or **2** is not essential for binding to P-selectin. In contrast, the inhibitory effect of sLe^X (IC$_{50}$, >1.0 mM) for P-selectin was less potent than those of compounds **1** and **2**. These results indicate that the modification of sLe^X and 3′-*O*-sulfo Le^X with an "artificial anchor", the 2- tetradecylhexadecyl group, is a useful tool to exert cell adhesion-inhibitory effects on all members of the selectin family.

In Vivo Activity: Effects on IgE-Mediated Skin Reaction in Mouse Ears. Compound **1** was administered intravenously at 2, 5, and 8 h after ovalbumin (OA) challenge. Measurements of ear swelling were made at 24 h together with neutrophil infiltration, as assessed by myeloperoxidase (MPO).

As indicated in Figure 1, ear swelling and neutrophil infiltration into tissue occurred at 24 h after the OA challenge. The IgE-mediated skin reactions in the ears of the mice were significantly inhibited by dexamethasone at 3 mg/kg, po. Notably, selectin blocker **1** (3, 10, and 30 mg/kg, iv) provided dose-dependent protective effects against IgE-mediated skin reactions in the mouse ear, and the content of MPO in the ear was significantly reduced. In this model, migration and activation of neutrophils is crucial, and compound **1** caused a significant reduction of the IgE-mediated skin reactions and a corresponding reduction in neutrophil accumulation in the ear tissue. Although the requisite role of selectins in IgE-mediated skin reactions is not clear, there is experimental evidence suggesting that in animals, selectins may be one of the participants in inflammatory responses featuring neutrophil migration.[13]

This study supports the proposition that the interaction between the ligand and each selectin becomes

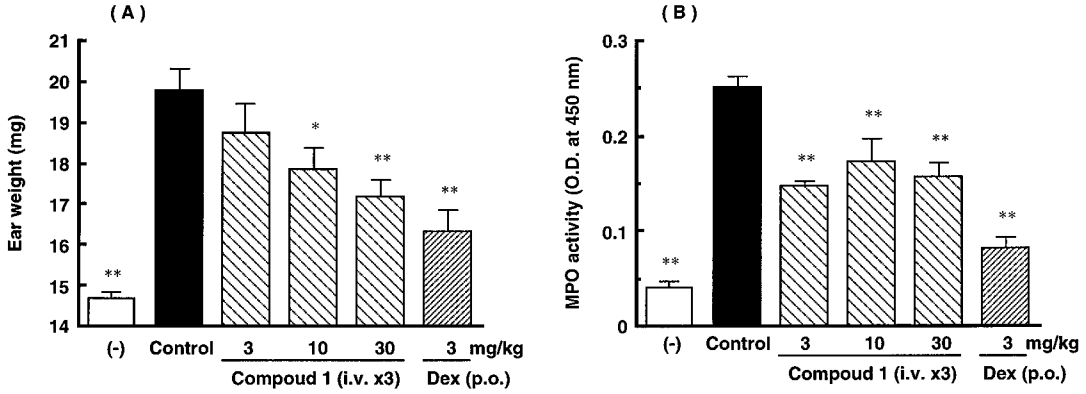


Figure 1. Effects of compound **1** and dexamethasone (Dex) on IgE-mediated skin reaction in mouse ears. Ovalbumin (OA; 3 μg/animal) was given intraperitoneally with 4 mg/animal of alum 2 weeks before the challenge of 10 μg/ear of OA. Compound **1** was given intravenously 2, 5, and 8 h after the OA challenge. Dexamethasone was given orally 2 h before the OA challenge: (A) effects on the increase of ear swelling and (B) effects on neutrophil infiltration. Each column represents the mean of four to six animals. Vertical bars indicate SE. *P < 0.05 and **P < 0.01, significantly different from control; (−) nonsensitized mice.

favorable by the attachment of a "branched anchor", a 2-tetradecylhexadecyl group, at the 1-position of sLe^X and 3′-*O*-sulfo Le^X. Our data indicate that the modification of the 1-position of either sLe^X or 3′-*O*-sulfo Le^X could be useful for the design of more potent selectin blockers, which have broad inhibitory activities toward all selectins. In addition, compound **1** has the desirable effect against neutrophil infiltration into tissue, suggesting that compound **1** could be an effective antiinflammatory candidate.

Experimental Section

Inhibition Assay of E-, P-, and L-Selectin−sLe^X Binding. The construction of the selectin−immunoglobulin was carried out according to a previous paper.[12c] A solution of sLe^X-pentaceramide, in a 1:1 mixture of methanol and distilled water, was pipetted into microtiter plate wells (96 wells; Falcon PRO-BIND) at 100 pmol/50 μL/well and adsorbed by evaporating the solvent. The wells were washed twice with distilled water, blocked with 5% BSA (bovine serum albumin)-PBS (phosphate-buffered saline) for 1 h at room temperature, and washed three times with PBS.

Separately, a 1:1 volumetric mixture of a 1:500 dilution in 1% BSA−PBS of biotin−anti-human IgG (Fc) (BioSource International Inc., lot 1201)/streptavidin−alkaline phosphatase (Zymed Lab Inc., lot 50424702) and an E-selectin−immunoglobulin fusion protein (E-selectin-Ig) was incubated at room temperature for 30 min to form a complex. The test compounds were dissolved in distilled water at 10 mM and finally diluted to final concentrations at 100, 25, 6.25, and 1.56 μM, respectively. Reactant solutions were prepared by incubating 30 μL of this solution at each concentration with 30 μL of the above complex solution for 30 min at room temperature. This reactant solution was then added to the above microtiter wells at 50 μL/well and incubated at 37 °C for 45 min. The wells were washed three times with PBS and distilled water, respectively, followed by addition of *p*-nitrophenyl phosphate (1 mg/mL) and 0.01% of $MgCl_2$ in 1 M diethanolamine (pH 9.8) at 50 mL/well. The reactant mixture was developed for 120 min at 23 °C, and absorbance at 405 nm was measured. Percent binding was calculated by the following equation:

$$\% \text{ binding} = (X - C/A - C) \times 100$$

wherein X is the absorbance of wells containing the test compounds at each concentration, C is the absorbance of wells not containing the selectin-Ig and test compounds , and A is the absorbance of control wells not containing the test compounds. Inhibition of P- or L-selectin−sLex binding was repeated except that P-selectin-Ig or L-selectin-Ig was replaced for E-selectin-Ig. The results of inhibitory activities are presented in Table 1 as IC_{50} values. The number of replicates is two.

Protective Effects on IgE-Mediated Skin Reaction in Mice Ears. Eight-week-old female BALB/c mice were sensitized by intraperitoneal injection with 3 μg of OA and 4 mg of alum. Reactions to OA were elicited by intracutaneous injection of 10 μg of OA to each ear of mice 2 weeks after the sensitization. After 24 h, the mice were killed, and 6-mm diameter biopsy of the ears were taken and weighed as an index of tissue swelling. As an index of neutrophil infiltration into tissue, myeloperoxidase activity in biopsy homogenates (in 50 mM potassium phosphate, pH 6.0, with 0.5% hexadecyltrimethylammonium bromide) was measured as the degradation of H_2O_2 in the presence of *o*-dianisidine.[14] Compound **1** dissolved in Ca^{2+}- and Mg^{2+}- free phosphate-buffered saline (PBS(−)) was given intravenously 2, 5, and 8 h after the OA challenge. Dexamethasone suspended in 0.5% carboxymethyl cellulose was given orally 2 h before the OA challenge. Results are expressed as mean ± SE. A one-way analysis of variance with Dunnett's test was used to determine statistical significance.[15]

2-Tetradecylhexadecyl *O*-(4-*O*-Acetyl-2,6-di-*O*-benzyl-3-*O*-levulinyl-β-D-galactopyranosyl)-(1→4)-*O*-[(2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl)-(1→3)]-2,6-di-*O*-benzyl-β-D-glucopyranoside (6). To a solution of **4**[11] (700 mg, 0.51 mM) and 2-tetradecylhexadecanol (**5**) (363 mg, 0.81 mM) in CH_2Cl_2 (8.8 mL) was added 4 Å molecular sieves (1.7 g), and the mixture was stirred for 6 h at room temperature and then cooled to 0 °C. Boron trifluoride etherate (0.13 mL, 1.03 mM) was added to the mixture, and this was stirred for 5 h at room temperature. The precipitate was filtered off and washed with CH_2Cl_2. The filtrate and washing were combined, and the solution was successively washed with 1 M Na_2CO_3 and water, dried (Na_2SO_4), and concentrated. Column chromatography (1:4 EtOAc−hexane) of the residue on silica gel (60 g) gave **6** (585 mg, 69%) as an amorphous mass: $[\alpha]_D$ −67.7° (*c* 1.0, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 0.88−1.43 (m, 58H, 2*Me*, 26CH_2), 1.82−2.15 (5s, 15H, 4AcO, $CH_3COCH_2CH_2$), 2.34, 3.67 (2dd, 2H, J = 9.3 Hz, H-1 and H-1′ of alkyl residue), 4.37 (d, 1H, J = 8.1 Hz, H-1a), 4.79 (d, 1H, J = 8.2 Hz, H-1c), 5.22 (dd, 1H, J = 10.4, 3.8 Hz, H-3c), 5.47 (d, 1H, J = 2.8 Hz, H-1b), 5.53 (dd, 1H, H-2c), 5.72 (dd, 1H, H-4c), 7.35−8.13 (m, 20H, 4Ph). Anal. Calcd for ($C_{89}H_{122}O_{25}$) C, H.

2-Tetradecylhexadecyl *O*-(4-*O*-Acetyl-2,6-di-*O*-benzyl-β-D-galactopyranosyl)-(1→4)-*O*-[(2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl)-(1→3)]-2,6-di-*O*-benzyl-β-D-glucopyranoside (7). To a solution of **6** (583 mg, 0.36 mM) in EtOH (15 mL)−THF (3 mL) was added hydrazine monoacetate (168 mg, 0.18 mM), and the mixture was stirred for 1 h at room temperature and then concentrated. Column chromatography (1:3 EtOAc−hexane) of the residue gave **7** (545 mg, 100%) as an amorphous mass: $[\alpha]_D$ −25.0° (*c* 1.0, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 0.85−1.33 (m, 58H, 2*Me*, 26CH_2), 1.59−2.11 (4s, 12H, 4AcO), 3.09, 3.79 (2dd, 2H, J = 9.3 Hz, H-1, H-1′ of alkyl residue), 4.38 (d, 1H, J = 7.7 Hz, H-1a), 4.63 (bs, 1H, OH),

4.67 (d, 1H, J = 8.6 Hz, H-1c), 5.60 (d, 1H, J = 2.9 Hz, H-1b), 7.33−8.15 (m, 20H, 4Ph). Anal. Calcd for ($C_{84}H_{116}O_{23}$) C, H.

2-Tetradecylhexadecyl *O*-(3-*O*-Sulfo-*β*-D-galactopyranosyl)-(1→4)-*O*-[(α-L-fucopyranosyl)-(1→3)]-*β*-D-glucopyranoside Sodium Salt (1). To a solution of **7** (78 mg, 0.05 mM) in DMF (0.1 mL) was added sulfur trioxide pyridine complex (41 mg, 0.26 mM), and the mixture was stirred for 1 h at room temperature. The course of the reaction was monitored by TLC. Methanol was added, and the mixture was concentrated at 25 °C to afford **8** as an amorphous mass: $[\alpha]_D$ −2.2° (*c* 1.0, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 0.85−1.31 (m, 58H, 2*Me*, 26CH_2), 1.38 (d, 3H, J = 6.2 Hz, H-6b), 1.74−2.35 (4s, 12H, 4AcO), 4.37 (d, 1H, J = 7.7 Hz, H-1a), 7.09−8.05 (m, 25H, 4Ph, pyridine).

To a solution of **8** (75 mg, 0.04 mM) in MeOH (2 mL) and THF (1 mL) was added sodium methoxide (5 mg), and the mixture was stirred for 24 h at room temperature and then concentrated at 25 °C. Column chromatography (5:4:0.7 $CHCl_3$−MeOH−H_2O) of the residue on Sephadex LH-20 gave **1** (42 mg, 92%) as an amorphous mass: $[\alpha]_D$ −30.0° (*c* 0.8, 1:1 MeOH−$CHCl_3$); ^{1}H-NMR (C_5D_5N) δ 0.85−1.31 (m, 58H, 2*Me*, 26CH_2), 1.54 (d, 3H, J = 6.4 Hz, H-6b), 5.06 (d, 1H, J = 2.7 Hz, H-1b), 5.21 (dd, J = 10.1, 3.8 Hz, H-3c), 5.41 (d, 1H, J = 7.3 Hz, H-1a), 5.48 (d, 1H, J = 6.7 Hz, H-1c). The mass spectrum of **1** showed the base peak at *m*/*z* 988.3 $(M - H)^-$.

2-Tetradecylhexadecyl *O*-(Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2→3)-*O*-(4-*O*-acetyl-2,6-di-*O*-benzyl-*β*-D-galactopyranosyl)-(1→4)-*O*-[(2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl)-(1→3)]-*O*-2,6-di-*O*-benzyl-*β*-D-glucopyranoside (10). To a solution of **9**[11] (65.3 mg, 0.04 mM) and 2-tetradecylhexadecanol (**5**) (34 mg, 0.077 mM) in CH_2Cl_2 (4 mL) was added 4 Å molecular sieves (1 g), and the mixture was stirred for 6 h at room temperature and then cooled to 0 °C. Boron trifluoride etherate (0.013 mL, 0.10 mM) was added to the mixture, and this was stirred for 5 h at room temperature. The precipitate was filtered off and washed with CH_2Cl_2. The filtrate and washing were combined, and the solution was successively washed with 1 M Na_2CO_3 and water, dried (Na_2SO_4), and concentrated. Column chromatography (1:4 EtOAc−hexane) of the residue on silica gel (60 g) gave **10** (51 mg, 66%) as an amorphous mass: $[\alpha]_D$ −0.6° (*c* 1.0, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 0.88−1.26 (m, 58H, 2Me, 26CH_2), 1.33 (d, 3H, J = 6.4 Hz, H-6d), 1.43−2.20 (9s, 27H, 8AcO, AcN), 2.52 (dd, 1H, J = 12.5 Hz, H-3eq, J = 4.6 Hz, H-3c-eq), 3.07, 3.52 (2dd, 2H, J = 9.4 Hz, H-1, H-1′ of fatty alkyl), 3.77 (s, 3H, MeO), 4.33 (d, 1H, J = 8.1 Hz, H-1a), 5.28 (d, 1H, J = 2.8, 10.5 Hz, H-7c), 5.46 (d, 1H, J = 2.8 Hz, H-1d), 5.63 (m, 1H, H-8c), 7.27−8.22 (m, 20H, 4Ph). Anal. Calcd for ($C_{104}H_{143}NO_{35}$) C, H, N.

2-Tetradecylhexadecyl *O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-(*β*-D-galactopyranosyl)-(1→4)-*O*-[(α-L-fucopyranosyl)-(1→3)]-*β*-D-glucopyranoside (2). To a solution of **10** (105 mg, 0.048 mM) in MeOH was added NaOMe (10 mg), and the mixture was stirred for 24 h at 40 °C. Potassium hydroxide (0.2 M, 5 mL) was added, and the mixture was stirred for an additional 6 h at room temperature and then neutralized with Amberlite IR-120 (H^+) resin. The resin was filtered off and washed with 1:1 $CHCl_3$−MeOH. The filtrate and washings were combined and concentrated. Column chromatography (5:4:0.7 $CHCl_3$−MeOH−H_2O) of the residue on Sephadex LH-20 (30 g) gave **2** (63.3 mg, 100%) as an amorphous mass: $[\alpha]_D$ −21.5° (*c* 1.1, 1:1 $CHCl_3$−MeOH); ^{1}H-NMR (DMSO-d_6) δ 0.86 (t, 6H, 2MeCH_2), 0.91 (d, 3H, J = 6.4 Hz, H-6d), 1.25 (s, 52H, 26CH_2), 1.89 (s, 3H, AcN), 2.85 (dd, 1H, J = 12.5, 4.9 Hz, H-3c-eq), 4.16 (d, 1H, J = 7.9 Hz, H-1a), 4.39 (d, 1H, J = 7.9 Hz, H-1b), 5.16 (d, 1H, J = 3.9 Hz, H-1d). Anal. Calcd for ($C_{59}H_{109}NO_{23}$) C, H, N.

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