Syntheses of New Model Compounds Related to an Antigenic Epitope from *Bupleurum falcatum* L. and Their Distributions in Various Ganglioside-Phospholipid Monolayers

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6-*N*-[2-(Tetradecyl)hexadecanamido]hexyl β-D-glucopyranosyluronic acid-(1→6)-β-D-galactopyranosyl-(1→6)-β-D-galactopyranoside (1) and its clustering compound (2) carrying a tetravalent sugar unit, which are new model compounds related to a major antigenic epitope from antiulcer pectic polysaccharide of *Bupleurum falcatum* L., were synthesized and the distributions of 1 and 2 in mixed ganglioside (GM1, GD1a or GT1b)/phospholipid (DPPC) monolayers were observed using atomic force microscopy (AFM). AFM images showed that 1 was distributed in the GM1, GD1a and GT1b region of the mixed monolayers, in which 1 was miscible with GD1a. Specific distribution of 1 was observed in the mixed GM1/DPPC monolayer. Compound 2 was miscible with GM1, while 2 formed associations with GD1a and GT1b in the mixed monolayers. The distribution mode of 1 and 2 was different among the mixed ganglioside/DPPC monolayers.

Key words *Bupleurum falcatum*; atomic force microscopy; peptidic glycocluster; ganglioside/DPPC monolayer

We synthesized[1] a trivalent analogue, *N*,*N*′,*N*″-tri-{5-[4-*O*-methyl-β-D-glucopyranosyluronic acid-(1→6)-β-D-galactopyranosyloxy]pentylcarbonylaminoethyl}-1,3,5-benzenetriamide (A) of β-D-GlcA4Me-(1→6)-β-D-Gal, which is related to a major antigenic epitope against antibupleurum 2IIc/PG-1-IgG from antiulcer pectic polysaccharide of *Bupleurum falcatum* L. (Japanese name *Saiko*),[2,3] and showed potent mitogenic activity and then previously showed[4] the AFM (atomic force microscopy) image of A in the ganglioside G_{M3} (GM3)/L-α-dipalmitoylphosphatidylcholine (DPPC) monolayer, where selective distribution of A in the GM3 region was found. Furthermore, we developed new peptidic glycoclusters and glycodentron,[5,6] and the fluorescence-labeled cluster derivatives of β-D-GlcA4Me-(1→6)-β-D-Gal-β-D-Gal[7] have been synthesized for biological assay. Recently, we synthesized new glycocluster derivatives carrying double alkyl chains instead of fluorescent dansyl group. The distributions of the new derivatives, monomer type (**1**) and its clustering compound (**2**) in the GM3/DPPC monolayer have been examined using AFM, and the results have been promptly reported (Letters).[8] (The details of synthesis have not been presented in the report[8] because of the limiting space.)

Gangliosides are localized at the surface of mammalian membranes and participate in cellular interaction, differentiation and transformation.[9—12] Ganglioside compositions are different among organs and/or tumors.[13] Ganglioside G_{M1} (GM1) and ganglioside G_{D1a} (GD1a) are present in caveolae membrane, whereas ganglioside G_{T1b} (GT1b) is not present in caveolae.[14] Membrane properties of GM1, GD1a and GT1b have been studied.[15—17] Studying drug distribution in biological membranes is interesting in how it relates to their pharmacological potency and is important to develop a convenient method for drug screening.

In this study, the distributions of **1** and **2** in GM1/DPPC, GD1a/DPPC and GT1b/DPPC monolayers were observed using AFM. We report herein the results along with the syntheses of **1** and **2** (Fig. 1).

Results and Discussion

Syntheses of Model Compounds Preparation of the designed trisaccharide monomer and tetramer derivative **1** and **2** were straightforward (Chart 1). Monomer amine derivative **3** and tetramer-cluster amine **6** were prepared according to the previous paper.[6] 2-(Tetradecyl)hexadecanoic acid (**4**), was chosen as a fatty-alkyl residue. Coupling of amine derivatives **3** and **6** with **4** in the presence of diethyl phosphorocyanidate (DEPC) in dry DMF gave **5** (60%) and **7** (50%), respectively. Subsequent removal of all acyl groups and esters with sodium methoxide afforded the target compounds **1** and **2** in excellent yield.

Distributions of 1 and 2 in the GM1/DPPC Monolayer First, the AFM image of the GM1/DPPC (4 : 6) monolayer at 37 °C and 35 mN/m without **1** and **2** is shown in Fig. 2a. In Fig. 2a, the ratio of dark area to bright area is not 0.4, although the molar ratio of GM1 to DPPC is 0.4. This result

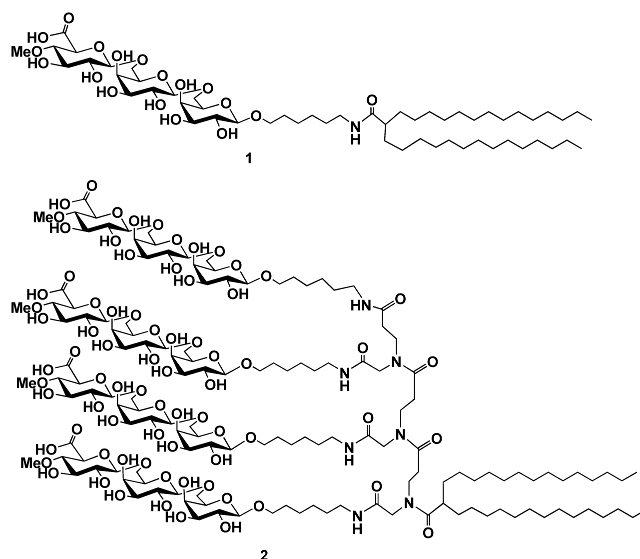

Fig. 1. Structures of Synthetic Compounds

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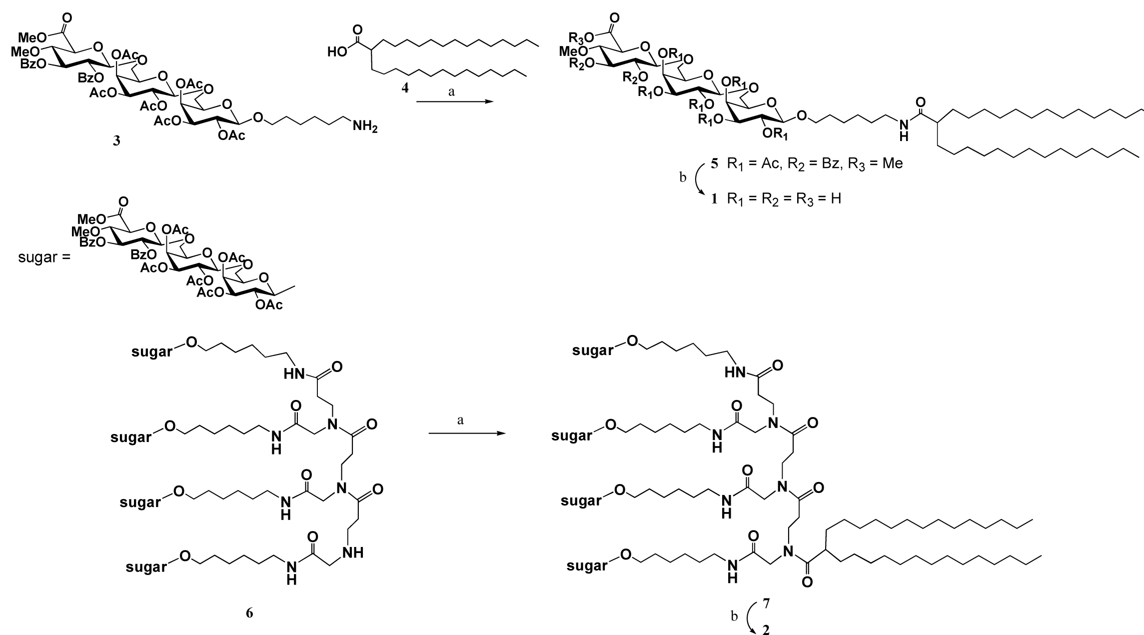

Reagents: (a) 2-(tetradecyl)hexadecanoic acid (**4**), DEPC, Et_3N, DMF. (b) NaOMe, MeOH-1,4-dioxane-H_2O.

Chart 1

suggests that the bright area is composed of DPPC and small amount of GM1, taking the area per molecule of 95 and 71 Å^2 for GM1[18] and DPPC,[19] respectively, into account. GM1 and DPPC would be partly miscible and formed DPPC-rich domains and GM1-rich matrix.

Next, the distributions of **1** and **2** in the mixed GM1/DPPC monolayer were examined, and the results are shown in Figs. 2b and c. The mole fraction of **1** or **2** to the lipid was 0.3 and 0.1, respectively. Compound **2** has a tetravalent sugar unit, so we used a smaller amount of **2** than **1** for AFM observation. Compound **1** is detected as bright spots in Fig. 2b, while large islands are DPPC domains. The darkest region in the matrix is GM1 alone, which formed a net-like pattern. Compound **1** was localized at the GM1 region. We have previously found[8] that **1** and **2** are not distributed in the DPPC alone monolayer. The distribution characteristic of **1** in the GM1/DPPC monolayer was similar to that[2] of mitogenic active compound A in the net-like region of the GM3/DPPC monolayer.

In Fig. 2c, gathered dark area and widely uniform region are observed, whose AFM image is different from Fig. 2a. This phenomenon is considered as follows: GM1 molecules in the DPPC-rich phase were attracted by **2**, thereby the DPPC-rich phase shown in Fig. 2a became flat and uniform. An attractive interaction between **2** and GM1 is considered to be related to a sugar chain–sugar chain interaction as similar to cell recognition.

Distributions of 1 and 2 in the GD1a/DPPC Monolayer The AFM images of the GD1a/DPPC (4 : 6) monolayer without and with **1** and **2** are shown in Fig. 3. The GD1a/DPPC binary system (Fig. 3a) exhibited DPPC domains (bright area) and GD1a-rich matrix. Compound **1** was distributed in the GD1a matrix (Fig. 3b), in which **1** was miscible with GD1a. Compound **2** interacted with GD1a and formed associations (Fig, 3c).

Distributions of 1 and 2 in the GT1b/DPPC Monolayer The AFM images of the GT1b/DPPC (4 : 6) monolayer without and with **1** and **2** are shown in Fig. 4.

Dark area in Fig. 4a is GT1b. Compound **1** was distributed in the GT1b matrix and is observed as bright spots in Fig. 4b, while larger islands are DPPC domains. Compound **2** strongly interacted with GT1b and formed large and swollen associations. GT1b has one more sialic acid residue than GD1a. Interaction of sugar chains between GT1b and **2** is stronger than that between GD1a and **2**, thereby larger associations are formed between GT1b and **2**. On the other hand, there is an electrostatic repulsion between uronic acid residue of **2** and tri-sialic acid residue of GT1b, which brings about bulky structure of association.

The reason why **2** formed large associations with GT1b, comparing with an interaction between **1** and GT1b, is the clustering structure of **2**: namely, the dissociation degree of uronic acid residue of cluster type is suppressed compared with that of monomer type, as similar as the dissociation degree of monomer (100%) and micelles (20—30%) of ionic surfactants, thus electrostatic repulsion between **2** and GT1b carrying sialic acid residue is less than that between **1** and GT1b, thereby large associations are formed between **2** and GT1b.

In conclusion, compound **1** was distributed in the GM1, GD1a, GT1b region of the mixed monolayers with DPPC. Specific distribution of **1** was observed in the mixed GM1/DPPC monolayer, whose distribution characteristics was similar to that of mitogenic active compound A[2] previously studied. Compound **2** interacted with GM1, GD1a and GT1b in the mixed monolayers. Compound **2** was gently miscible with GM1, while **2** formed associations with GT1b and GD1a. The terminal second and third sialic acid residues of GD1a and GT1b are likely to strongly interact with **2**. The distribution mode of **1** and **2** was different among the mixed ganglioside/DPPC monolayers.

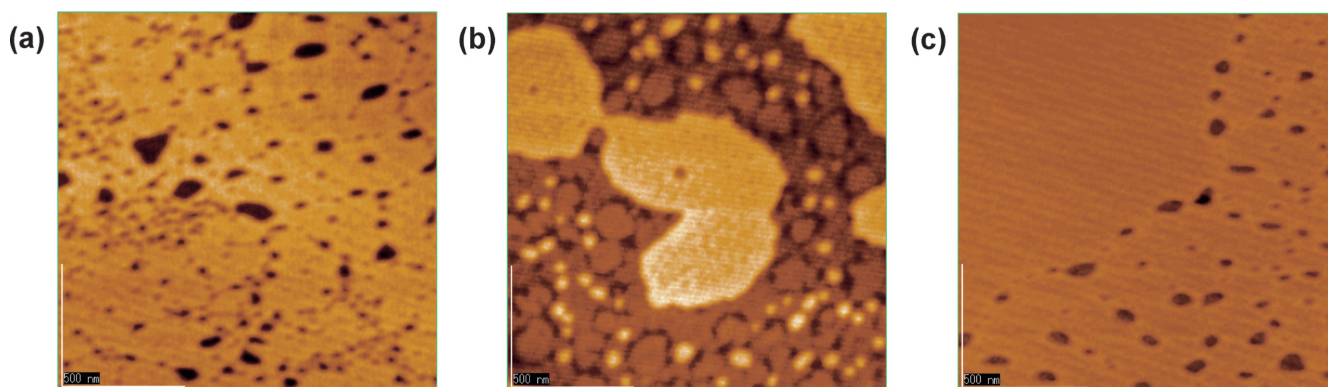

Fig. 2. AFM Images of the GM1/DPPC Monolayers without (a), with **1** (b) and with **2** (c)

Scan area: 1.5×1.5 μm.

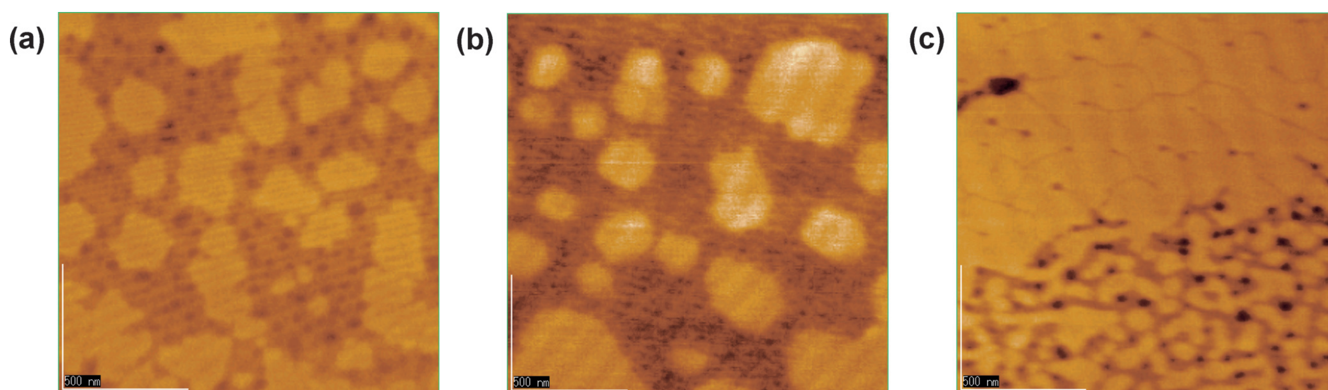

Fig. 3. AFM Images of the GD1a/DPPC Monolayers without (a), with **1** (b) and with **2** (c)

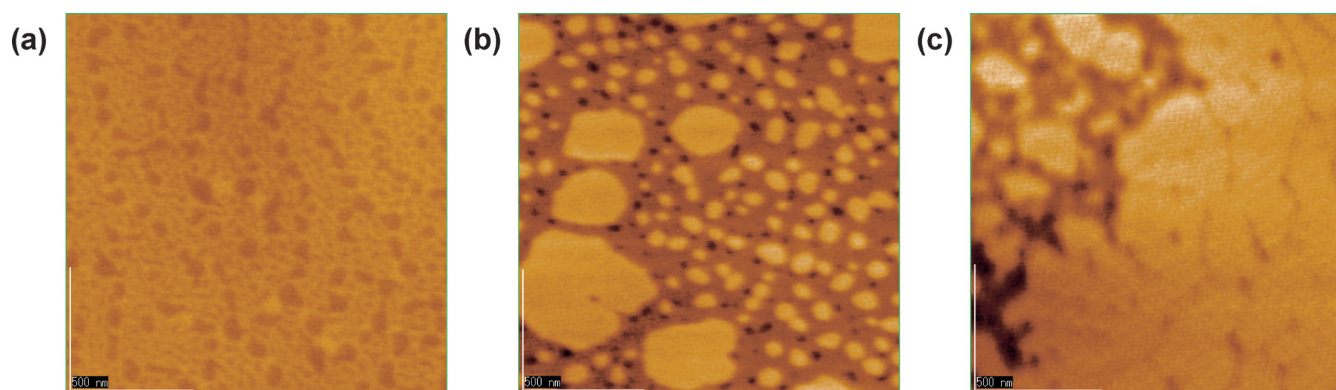

Fig. 4. AFM Images of the GT1b/DPPC Monolayers without (a), with **1** (b) and with **2** (c)

Experimental

Optical rotations were determined with a Jasco digital polarimeter. ^{1}H- and ^{13}C-NMR spectra were recorded with a JNM A 500 FT NMR spectrometer with Me_4Si as the internal standard for solutions in $CDCl_3$ or CD_3OD. MALDI-TOFMS was recorded on a Perceptive Voyager RP mass spectrometer. TLC was performed on silica gel 60-F254 (Merck) with detection by quenching of UV fluorescence and by spraying with 5% ninhydrin and 10% H_2SO_4. Column chromatography was carried out on silica gel 60 (Merck). 2-(Tetradecyl)hexadecanoic acid (**4**) was purchased from Wako Pure Chemical Indusries (Tokyo).

6-*N*-[2-(Tetradecyl)hexadecanamido]hexyl[methyl(2,3-di-*O*-benzoyl-4-*O*-methyl-*β*-D-glucopyranosyl)uronate]-(1→6)-2,3,4-tri-*O*-acetyl-*β*-D-galactopyranosyl)-(1→6)-2,3,4-tri-*O*-acetyl-*β*-D-galactopyranoside (5) To a solution of **3** (21 mg, 19.0 μmol) and 2-(tetradecyl)hexadecanoic acid (**4**) (17.2 mg, 38.0 μmol) in DMF (1 ml) were added triethylamine (4 μl, 28.5 μmol) and DEPC (3.2 μl, 21.1 μmol). After stirring the reaction mixture for 16 h at room temperature, the mixture was extracted with chloroform, washed with water, dried ($MgSO_4$), and concentrated. The product was purified on silica gel column chromatography (chloroform : methanol=40 : 1) to give **5** (17.6 mg, 60.0%); $[\alpha]_D^{23}$ +2.1° (c=0.2, $CHCl_3$). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.92—7.31 (10H, m, Ar-H), 5.53 (1H, t, H-3″), 5.29—5.22 (3H, m, H-2″, H-4, H-4′), 5.12—5.02 (2H, m, H-2, H-2′), 4.94—4.85 (2H, m, H-3, H-3′), 4.70 (1H, d, J=7.9 Hz, H-1″), 4.38—4.35 (2H, m, H-1, H-1′), 4.10 (1H, d, H-5″), 3.89—3.33 (15H, m, H-4″, H-5, H-5′, H-6a, H-6b, H-6a′, H-6b′, $COOCH_3$, OCH_2 of sugar unit, OCH_3), 3.15 (2H, s, NCH_2 of sugar unit), 2.23—1.95 (18H, m, $COOCH_3$×6), 1.52 (4H, s, CH_2×2), 1.35—1.17 (52H, m, (CH_2)×26). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 176.0, 170.2, 170.1, 170.0, 169.5, 169.3, 168.4, 165.5, 165.0, 133.35, 133.26, 129.8, 129.2, 128.4, 101.2, 100.9, 100.7, 78.6, 74.2, 71.9, 71.8, 71.1, 70.9, 69.1, 68.7, 67.5, 67.3, 67.2, 66.8, 60.4, 52.8, 48.2, 39.2, 33.1, 31.9, 29.7,

29.6, 29.5, 29.3, 29.2, 27.7, 26.7, 25.6, 22.7, 20.8, 20.7, 20.64, 20.59, 20.56, 14.1. MALDI-TOF-MS: Calcd for $C_{82}H_{125}NNaO_{26}$: *m/z* 1562.8 [M+Na]$^+$. Found: *m/z* 1563.0 [M+Na]$^+$.

6-*N*-[2-(Tetradecyl)hexadecanamido]hexyl *β*-D-glucopyranosyluronic acid-(1→6)-*β*-D-galactopyranosyl-(1→6)-*β*-D-galactopyranoside (1) To a solution of compound **5** (5.5 mg, 3.6 μmol) in 2 : 2 : 1 MeOH–1,4-dioxane–H_2O (1 ml) was added NaOMe (15 mg), and the mixture was stirred for 14 h at room temperature and then neutralized with Amberlite IR-120 (H^+) resin. The resin was filtered off and washed with $CHCl_3$–MeOH. The filtrate and washings were combined and concentrated. Column chromatography ($CHCl_3$: MeOH=2 : 1) of the residue on Sephadex LH-20 gave **1** (3.7 mg, 97%). $[\alpha]_D^{23}$ +0.2° (*c*=0.1, $CHCl_3$: MeOH=1 : 1). ^{1}H-NMR (500 MHz, 1 : 1 $CDCl_3$–CD_3OD): δ 4.24—4.20 (2H, br d, *J*=7.9, 7.3 Hz, H-1, H-1′), 4.13 (1H, d, *J*=7.3 Hz, H-1″), 3.41 (3H, s, OCH_3). ^{13}C-NMR (125 MHz, 1 : 1 $CDCl_3$—CD_3OD): δ 178.4, 104.9, 104.3, 103.7, 83.1, 76.8, 74.6, 74.4, 74.3, 74.1, 72.0, 71.9, 70.5, 69.3, 69.04, 68.96, 60.5, 48.1, 39.8, 33.7, 32.6, 30.4, 30.33, 30.30, 30.2, 30.05, 30.00, 28.3, 27.4, 26.3, 23.3, 14.4, 1.4. MALDI-TOF-MS: Calcd for $C_{55}H_{103}NNaO_{18}$: *m/z* 1089.7 [M+Na]$^+$. Found: *m/z* 1089.9 [M+Na]$^+$.

Compound 7 To a solution of **6** (11 mg, 2.3 μmol) and 2-(tetradecyl)-hexadecanoic acid (**4**) (2.1 mg, 4.6 μmol) in DMF (1 ml) were added triethylamine (7.7 μl, 40.4 μmol) and DEPC (6.1 μl, 40.4 μmol). After stirring the reaction mixture for 16 h at room temperature, the mixture was extracted with chloroform, washed with water, dried ($MgSO_4$), and concentrated. The product was purified on silica gel column chromatography (chloroform : methanol=30 : 1) to give **7** (6 mg, 50%); $[\alpha]_D^{23}$ +3.2° (*c*=0.2, $CHCl_3$). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.00—7.38 (40H, m, Ar-H), 5.60 (4H, t, H-3″×4), 5.36—5.28 (12H, m, H-2″×4, H-4×4, H-4′×4), 5.16—5.09 (8H, m, H-2×4, H-2′×4), 5.01—4.92 (8H, m, H-3×4, H-3′×4), 4.77 (4H, d, H-1″), 4.44 (8H, dd, H-1×4, H-1′×4), 4.08 (4H, d, H-5″×4), 3.96—3.40 (72H, m, H-4″×4, H-5×4, H-5′×4, H-6a×4, H-6b×4, H-6a′×4, H-6b′×4, $COOCH_3$×4, NCH_2CO of *β*-alanine×3, NCH_2 of *β*-alanine×3, OCH_2 of sugar unit×4, OCH_3×4), 3.16 (8H, m, NCH_2 of sugar unit×4), 2.10—1.95 (72H, m, OAc×6×4), 1.65—1.25 (84H, m, (CH_2)×42), 0.87 (6H, t, CH_3×2). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 170.1, 170.0, 169.9, 169.3, 168.4, 165.5, 164.9, 133.31, 133.26, 129.8, 129.1, 128.4, 127.6, 101.2, 100.9, 100.7, 78.6, 74.1, 72.0, 71.9, 71.8, 71.0, 70.9, 69.9, 68.7, 67.5, 67.3, 60.4, 52.7, 31.9, 31.1, 29.93, 29.87, 29.7, 29.6, 29.5, 29.4, 29.3, 26.7, 25.61, 25.56, 22.6, 20.8, 20.7, 20.61, 20.57, 14.1. MALDI-TOF-MS: Calcd for $C_{253}H_{341}N_7NaO_{107}$: *m/z* 5212.1 Found: *m/z* 5212.6 [M+Na]$^+$.

Compound 2 To a solution of compound **7** (6.0 mg, 1.2 μmol) in 2 : 2 : 1 MeOH–1,4-dioxane–H_2O (1 ml) was added NaOMe (20 mg), and the mixture was stirred for 14 h at room temperature and then neutralized with Amberlite IR-120 (H^+) resin. The resin was filtered off and washed with MeOH–H_2O. The filtrate and washings were combined and concentrated. Column chromatography (MeOH : H_2O=1 : 2) of the residue on Sephadex LH-20 gave **2** (3.6 mg, 95%). ^{1}H-NMR (500 MHz, 1 : 1 CD_3OD–D_2O): δ 4.34 (8H, br d, *J*=8.6 Hz, H-1×4, H-1′×4), 4.25 (4H, d, *J*=7.3 Hz, H-1″×4), 3.43 (12H, s, OCH_3×4). ^{13}C-NMR (125 MHz, 1 : 1 CD_3OD–D_2O): δ 103.5, 102.6, 82.1, 77.9, 77.6, 77.4, 75.4, 73.6, 73.2, 72.84, 72.81, 70.7, 70.0, 68.7, 68.5, 59.6, 48.6, 31.4, 29.03, 28.98, 28.9, 28.8, 28.5, 22.2, 13.2. MALDI-TOF-MS: Calcd for $C_{145}H_{253}N_7NaO_{75}$: *m/z* 3315.6. Found: *m/z* 3315.8 [M+Na]$^+$.

AFM Observation GM1, GD1a, GT1b and L-*α*-dipalmitoylphosphatidylcholine (DPPC) were purchased from Sigma Chemical Co. DPPC, ganglioside, compounds **1** and **2** were dissolved in a mixed solvent of chloroform/methanol/water (3 : 3 : 1) taking the solubility of **2** into account. Total concentration of DPPC and ganglioside was 1 mM and the mole fraction of ganglioside to DPPC was 0.4, where relatively clear morphologies are observed in the binary systems. The mole fraction of **1** or **2** to the lipid was 0.1—0.3. After the lipid solution containing **1** or **2** was spread on water without surface disturbances using a microsyringe, the system was allowed to stand for 15 min. Compounds **1** and **2** oriented at the air/water interface and form monolayer with the lipids, where sugar chains of **1** and **2** are in the water phase and the double alkyl chains of **1** and **2** protrude beyond the air phase. The surface pressure of the lipid monolayer at the air/water interface was determined at 37±0.2 °C by the Wilhelmy plate method using a surface pressure meter (HBM-A, Kyowa Interface Science Co., Ltd.). The compression rate was 20 mm/min. Single layer Langmuir–Blodgett films of the lipids for AFM were obtained at 37 °C using a vertical dipping method onto freshly cleaved mica. The hydrophilic groups of the lipid monolayer are faced the mica. Deposition proceeded at 5 mm/min, under surface pressure of 35 mN/m. Surface pressures in biological membranes range from 30 to 45 mN/m.[20,21] Thus we chose 35 mN/m for measurement through this study. Images were captured using a model JSPM-5200 atomic force microscope (JEOL Ltd.). The AFM probe used was a Micro-Cantilever CSC38 (JEOL Ltd.) made of silicon and coated with Au, which had a spring constant of 0.08 N/m, a length of 250 μm, and a thickness of 1.0 μm. AFM observation was carried out with the contact mode in the air.

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