Constituents of Holothuroidea, 15.[1] Isolation of *Ante*-iso Type Regio-isomer on Long Chain Base Moiety of Glucocerebroside from the Sea Cucumber *Holothuria leucospilota*

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An *ante*-iso type regio-isomer on the long chain base moiety of a glucocerebroside, HLC-2-A, has been isolated from its parent glucocerebroside molecular species HLC-2 composed of iso and *ante*-iso isomers, from the less polar lipid fraction of a chloroform/methanol extract of the sea cucumber *Holothuria leucospilota*. Reverse-phase HPLC that included a recycling system was effective in separating the regio-isomer from its counterpart, revealing a very close resemblance in structure. Other typical glucocerebroside molecular species HLC-1 and HLC-3 were obtained together with HLC-2. The structures of these glucocerebrosides were determined on the basis of chemical and spectroscopic evidence.

Key words glycosphingolipid; glucocerebroside; sea cucumber; *Holothuria leucospilota*; recycling HPLC

In our continuing research on biologically active glycosphingolipids (GSLs) from echinoderms, a series of studies on the isolation and structural elucidation of the GSLs from sea cucumber species have been performed in our laboratory.[2—11] In the study of the GSLs of the sea cucumber *Holothuria pervicax*, we reported the isolation and structure of ten glucocerebrosides revealing a very close resemblance in structure.[9] However, all these compounds still exist as a mixture of regio-isomers for terminal methyl groups in the side chain of the long-chain base (LCB) moiety, namely a mixture of iso and *ante*-iso isomers as shown in Fig. 1. Continuing the previous studies, the separation of the mixture of the regio-isomers was conducted. In this paper, we report on the isolation of an *ante*-iso type regio-isomer of glucocerebroside from its parent glucocerebroside molecular species HLC-2, an abundant compound obtained from the whole bodies of the sea cucumber *Holothuria leucospilota* (Nisekuronamako in Japanese).[12]

The less polar lipid fraction, which was obtained from the chloroform/methanol extract of the whole bodies of *H. leucospilota*, was subjected to repeated silica gel column chromatography to give three cerebroside molecular species, HLC-1, HLC-2, and HLC-3, each showing a single spot on silica gel thin-layer chromatography (TLC).

In the IR and positive-ion FAB mass spectra of HLC-1, HLC-2, and HLC-3, strong hydroxy and amide absorptions and a series of molecular ion peaks are observed. Their ^{13}C-NMR spectra (Fig. 1, Table 1) exhibit the characteristic signals of a sphingosine-type β-glucocerebroside possessing an unsubstituted fatty acid (HLC-1), a sphingosine-type β-glucocerebroside possessing a 2-hydroxy fatty acid (HLC-2), and a phytosphingosine-type β-glucocerebroside possessing a 2-hydroxy fatty acid (HLC-3), respectively. Therefore, they are suggested to be the molecular species of three typical types of glucocerebrosides. Their structures shown in Fig. 1 were characterized by comparison of their ^{13}C-NMR spectral data with those of known glucocerebrosides[2—4] hitherto obtained, and by means of the results of their chemical degradations, namely methanolysis followed by the GC-MS analysis of the methanolysis products, fatty acid methyl ester (FAM), and LCB, as shown in Fig. 2 and Experimental. The geometry (*Z*) of the double bond in the unsaturated fatty acyl moiety of HLC-1—HLC-3 was determined from the δ value (27.4, 27.5) of the allylic carbon atoms obtained from their ^{1}H-detected heteronuclear multiple-bond connectivity (HMBC) spectra (Fig. 3), since allylic carbon signals of *Z*-

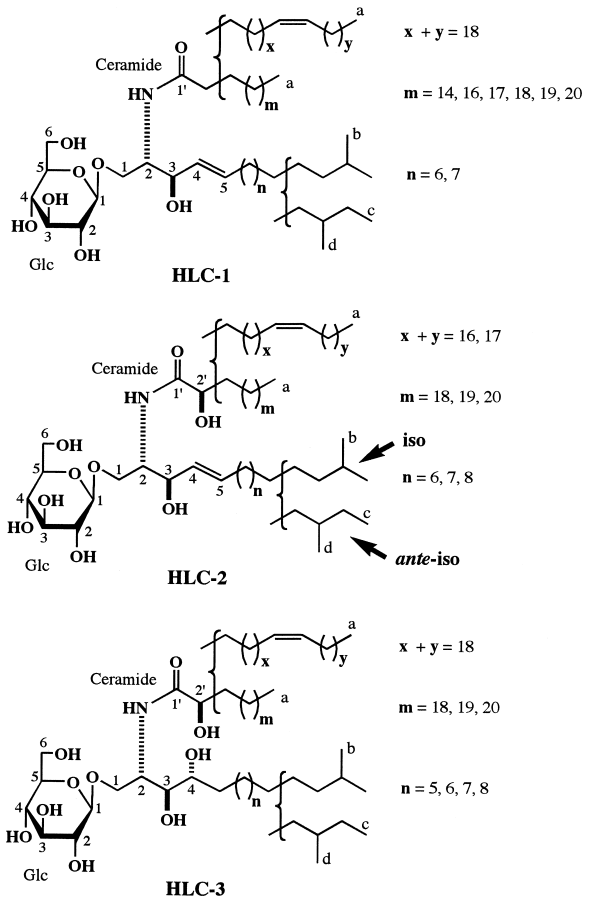

Fig. 1. Structures of HLC-1, HLC-2 and HLC-3

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Table 1. ^{13}C-NMR Spectral Data (δ Values) of Glucocerebrosides in C_5D_5N

C		HLC-1	HLC-2	HLC-3	HLC-2-A
Ceramide					
1	(t)	70.5	70.2	70.4	70.1
2	(d)	55.0	54.6	51.7	54.6
3	(d)	72.7	72.4[e)]	75.8	72.3[g)]
4	(d)	131.1	131.7	72.5[f)]	131.6
5	(d)	132.5	132.8		132.8
1′	(s)	173.4	175.7	175.6	175.7
2′	(d)		72.5[e)]	72.4[f)]	72.5[g)]
=CH	(d)	130.2	130.3	130.5	
=CHC̲H$_2$	(t)	27.5	27.4	27.5	
CH$_3$[a)]	(q)	14.2	14.3	14.2	14.2
CH$_3$[b)]	(q)	22.7	22.8	22.7	
CH$_3$[c)]	(q)	11.5	11.6	11.5	11.5
CH$_3$[d)]	(q)	19.3	19.4	19.3	19.3
Glc					
1	(d)	105.8	105.6	105.5	105.5
2	(d)	75.2	75.1	75.1	75.1
3	(d)	78.5	78.5	78.4	78.4
4	(d)	71.6	71.6	71.5	71.5
5	(d)	78.5	78.5	78.4	78.5
6	(t)	62.7	62.7	62.6	62.6

a—*d*) Terminal methyl groups in the normal, iso and *ante*-iso type of side chain (see Fig. 1). *e*—*g*) Assignments may be interchanged in each vertical column.

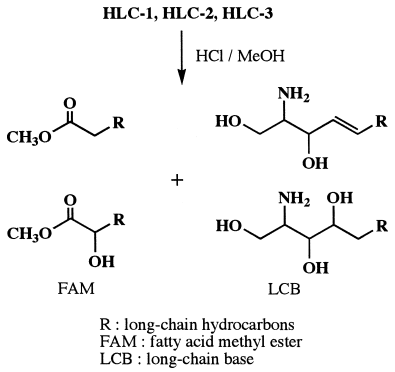

Fig. 2. Methanolysis Products of Cerebrosides

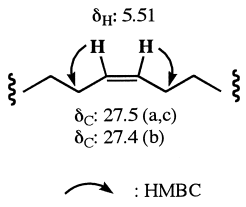

Fig. 3. HMBC Correlations of the Fatty Acyl Moieties of HLC-1 (a), HLC-2 (b) and HLC-3 (c)

and *E*-isomers are observed at δ *ca.* 26—27 and δ *ca.* 31—32, respectively.[13)] The absolute configuration of their glucose moiety (D-form) was determined by the Hara method[14)] (Experimental).

The isolation of a pure glucocerebroside possessing only the *ante*-iso type of an LCB moiety was conducted using the main molecular species, HLC-2, as follows.

The reverse-phase (C18) column chromatography of HLC-2 gave seventeen fractions, each showing a single spot on reverse-phase TLC as shown in Fig. 4A. The ^{13}C-NMR spectra of these fractions indicate fraction 12, the major fraction,

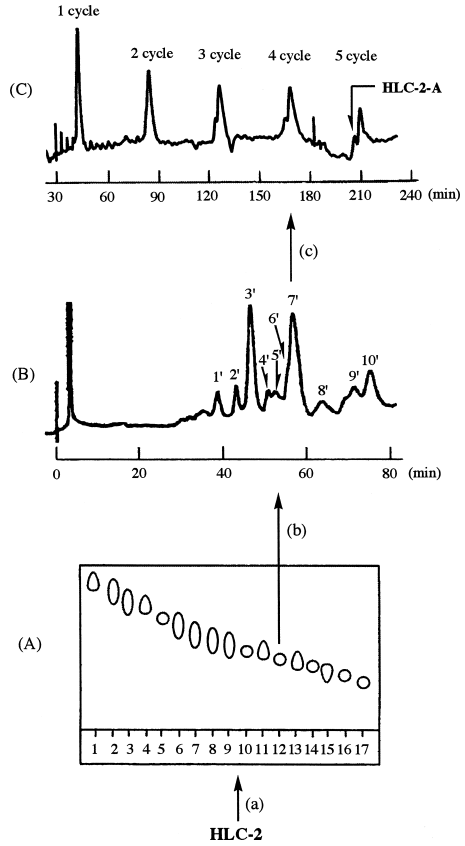

Fig. 4. CC (A), HPLC (B) and Recycling HPLC (C) Chromatograms of the Components of HLC-2

Conditions: (a) Column, Cosmosil 140C18-PREP; solvent, 98% MeOH. (b) Column, Develosil C30-UG-5 (10×250 mm); solvent, 98% MeOH; flow rate, 4.7 ml/min; detector, RI. (c) Column, Develosil C30-UG-5 (10×250 mm); solvent, 99% MeOH; flow rate, 4.7 ml/min; detector, RI; number of cycles, 5. (TLC of A) Plate, $RP_8F_{254}S$; solvent, 100% MeOH; detection, 5% H_2SO_4–MeOH.

contain the *ante*-iso isomer on the basis of the signals due to methyl groups at δ_C: 11.6 and 19.4. When the fraction 12 was analyzed by reverse-phase (C30) HPLC, it was separated into ten peaks (Fig. 4B), and could be recovered to give ten fractions (fr. 1′—fr. 10′). The measurements of the ^{13}C-NMR spectra of the four major fractions, fr. 3′, 7′, 9′ and 10′, each behaving as pure compounds in HPLC, reveal only fr. 7′ contains *ante*-iso isomer, and further the fraction is still a mixture containing an iso isomer. Fraction 7′ was successively separated using recycling reverse-phase (C30) HPLC, and a fraction, HLC-2-A, showing the ^{13}C-NMR signals ascribable to only *ante*-iso isomer (see Table 1), could be isolated as shown in Fig. 4C.

In the positive-ion FAB mass spectrum, HLC-2-A revealed a single quasi-molecular ion peak $[M+Na]^+$ at *m*/*z*: 808. Upon methanolysis, HLC-2-A yielded methyl 2-hydroxydocosanoate and 2-amino-1,3-dihydroxy-4-heptadecene as its fatty acid and LCB components, respectively.

On the basis of the above data, the structure of HLC-2-A is proposed to be β-D-glucopyranosyl ceramides, as shown in Fig. 5. HLC-2-A was found to be identical with CE-2b isolated from the sea cucumber *Cucumaria echinata*.[2)]

At this time, we were able to separate the *ante*-iso type regio-isomer of the glucocerebroside from its counterpart iso type isomer by the aid of reverse-phase HPLC that included a recycling system. The present study is, to the best of our

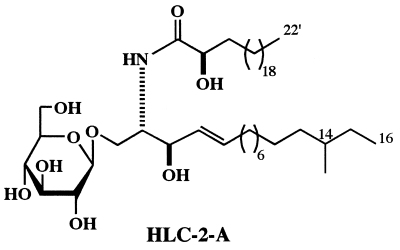

Fig. 5. Structure of HLC-2-A

knowledge, the first regarding the separation of such regioisomers of a cerebroside. The separation of glucocerebrosides revealing the very close resemblance in structure is worth noting. As for the absolute configuration of the branched methyl group of the LCB moiety of HLC-2-A, we plan to attempt its determination in the future.

Experimental

Melting points were determined on a micro melting point apparatus (Yanako MP-3) without correction. Optical rotations were measured with a Jasco Dip-370 digital polarimeter at 25 °C. IR spectra were obtained on a Jasco FT/IR-410 infrared spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Jeol GX-270 spectrometer (270, 67.8 MHz) or a Varian Unity-500 spectrometer (500, 125 MHz). Positive-ion FAB-MS spectra were acquired with a Jeol JMS-SX102 mass spectrometer (xenon atom beam; matrix, *m*-nitrobenzyl alcohol). GC-MS were taken with a Shimadzu QP-1000 [EI mode; ionizing potential, 70 eV; separator and ion-source temperature 250 °C; column, CBP10-W12-100 (0.53 mm×12 m, Shimadzu); carrier gas, He]. GC were run on a Shimadzu GC-14B [FID mode; column, Fused Silica Capillary Column DB-17 (0.32 mm×30 m, J & W Scientific); carrier, N_2]. HPLC was performed with BIP-I and RID-300 (Jasco) as a pump and an RI detector, respectively. A Type 70 Switching Valve Model 7000 (Rheodyne, L. P.) was used for the recycling HPLC system.

Separation of HLC-1, HLC-2 and HLC-3 Whole bodies of the sea cucumber *Holothuria leucospilota* (wet weight 49.9 kg, collected at Ushibuka, Kumamoto Prefecture, Japan) were homogenized and extracted with $CHCl_3$/MeOH (1 : 2, 27.5 l, three times). The combined extracts were concentrated *in vacuo* to give an aqueous suspension, which was extracted three times with AcOEt/*n*-BuOH (2 : 1, 12.5 l). The organic layer was concentrated *in vacuo*, and the residue (495.2 g) was washed with cold acetone to give an acetone-insoluble fraction (less polar lipid fraction, 83.0 g). The less polar lipid fraction was chromatographed on silica gel (solvent $CHCl_3$–MeOH–H_2O, 95 : 5 : 0 to 5 : 5 : 1) to give fifteen fractions. Successive column chromatography of fraction 11, 12 and 13 (silica gel, solvent $CHCl_3$–MeOH–H_2O, 95 : 5 : 0 to 5 : 5 : 1) afforded HLC-1 (0.38 g) (*Rf*=0.63), HLC-2 (2.64 g) (*Rf*=0.56), and HLC-3 (0.32 g) (*Rf*=0.47), respectively [silica gel TLC, solvent $CHCl_3$–MeOH (8 : 2)].

HLC-1: Amorphous powder, $[\alpha]_D$ −2.1° (*c*=0.91, 1-PrOH). IR (KBr) cm^{-1}: 3390 (OH), 1640, 1540 (amide). Positive-ion FAB-MS *m/z*: 770—850 $[M+Na]^+$ series. ^{1}H-NMR (C_5D_5N) δ: 0.88 (9H, m, terminal methyl groups), 4.97 (1H, d, *J*=7.6 Hz, glucose H-1), 5.51 (m, olefinic H of fatty acyl moiety). ^{13}C-NMR: see Table 1.

HLC-2: Amorphous powder, $[\alpha]_D$ +6.2° (*c*=0.95, 1-PrOH). IR (KBr) cm^{-1}: 3400 (OH), 1635, 1535 (amide). Positive-ion FAB-MS *m/z*: 800—870 $[M+Na]^+$ series. ^{1}H-NMR (C_5D_5N) δ: 0.88 (9H, m, terminal methyl groups), 4.92 (1H, d, *J*=7.6 Hz, glucose H-1), 5.51 (m, olefinic H of fatty acyl moiety). ^{13}C-NMR: see Table 1.

HLC-3: Amorphous powder, $[\alpha]_D$ +8.8° (*c*=0.29, 1-PrOH). IR (KBr) cm^{-1}: 3400 (OH), 1630, 1540 (amide). Positive-ion FAB-MS *m/z*: 790—870 $[M+Na]^+$ series. ^{1}H-NMR (C_5D_5N) δ: 0.88 (9H, m, terminal methyl groups), 4.96 (1H, d, *J*=7.9 Hz, glucose H-1), 5.51 (m, olefinic H of fatty acyl moiety). ^{13}C-NMR: see Table 1.

Methanolysis of HLC-1 HLC-1 (2.1 mg) was heated with 5% HCl in MeOH (1.6 ml) at 70 °C for 4 h. The reaction mixture was then extracted with *n*-hexane, and the extract was concentrated *in vacuo* to yield a mixture of FAM. The MeOH layer was neutralized with Ag_2CO_3, filtered, and the filtrate was concentrated *in vacuo* to give a mixture of LCB and methyl glycoside.

GC-MS Analysis of FAM from HLC-1 A FAM mixture from HLC-1 was subjected to GC-MS [column temperature: 180—250 °C (rate of temperature increase 4 °C/min)]. The results were as follows: methyl octadecanoate, t_R [min]=1.6, *m/z*: 298 (M^+), 255 $(M-43)^+$; methyl icosanoate, t_R=3.2, *m/z*: 326 (M^+), 283 $(M-43)^+$; methyl heneicosanoate, t_R=4.3, *m/z*: 340 (M^+), 297 $(M-43)^+$; methyl docosanoate, t_R=5.5, *m/z*: 354 (M^+), 311 $(M-43)^+$; methyl tricosanoate, t_R=6.9, *m/z*: 368 (M^+), 325 $(M-43)^+$; methyl tetracosenoate, t_R=7.7, *m/z*: 380 (M^+), 337 $(M-43)^+$; methyl tetracosanoate, t_R=8.4, *m/z*: 382 (M^+), 339 $(M-43)^+$.

GC-MS Analysis of TMS Ethers of LCB from HLC-1 A mixture of LCB and methyl glycoside from HLC-1 was heated with 1-(trimethylsilyl) imidazole–pyridine (1 : 1) for 10 min at 70 °C, and the reaction mixture (TMS ethers) was analyzed by GC-MS [column temperature: 180—250 °C (rate of temperature increase 4 °C/min)]. The results were as follows: 2-amino-1,3-dihydroxy-4-heptadecene, t_R [min]=3.3, *m/z*: 326 $(M-103)^+$, 297 $(M-132)^+$, 132; 2-amino-1,3-dihydroxy-4-octadecene, t_R=4.1, *m/z*: 340 $(M-103)^+$, 311 $(M-132)^+$, 132.

GC Analysis of TMS Ethers of Methyl Glycoside from HLC-1 The mixture of TMS ethers of LCB and methyl glycoside was analyzed by GC [column temperature: 100—250 °C (rate of temperature increase 5 °C/min)]: t_R [min]=18.0 and 18.1 (methyl α- and β-glucopyranoside).

Determination of Absolute Configuration of Glucose Moiety of HLC-1 (Hara Method) HPC-1 (1 mg) was heated with 4 N H_2SO_4 (0.5 ml) at 100 °C for 18 h in a sealed vial. The reaction mixture was then extracted with *n*-hexane, and the acidic aqueous phase was neutralized with $Ba(OH)_2$, centrifuged, and the clear supernatant solution was concentrated. The residue (sugar fraction) was heated with L-cysteine methyl ester hydrochloride (1.5 mg) and pyridine (0.1 ml) at 60 °C for 1 h. Then, 0.1 ml of 1-(trimethylsilyl) imidazole was added, and the mixture was heated at 60 °C for a further 0.5 h to yield a trimethylsilyl ether of the methyl (4*R*)-thiazolidine-4-carboxylate derivative. The derivative was analyzed by GC [column temperature: 200—250 °C (rate of temperature increase 2.5 °C/min)]; t_R=12.4 min (derivative of D-glucose, 12.4 min; L-glucose, 13.0 min). In the same way, the absolute configuration of the glucose moiety (D-form) of HLC-2 and HLC-3 was determined.

Methanolysis of HLC-2 In the same manner as described for HLC-1, HLC-2 was methanolyzed and the reaction mixture was worked up to give a mixture of FAM and a residue composed of LCB and methyl glycoside.

GC-MS Analysis of FAM from HLC-2 A FAM mixture from HLC-2 was subjected to GC-MS under the same conditions as described for the FAM mixture obtained from HLC-1. The results were as follows: methyl 2-hydroxydocosenoate, t_R [min]=6.2, *m/z*: 368 (M^+), 309 $(M-59)^+$; methyl 2-hydroxydocosanoate, t_R=7.0, *m/z*: 370 (M^+), 311 $(M-59)^+$; methyl 2-hydroxytricosenoate, t_R=7.5, *m/z*: 382 (M^+), 323 $(M-59)^+$; methyl 2-hydroxytricosanoate, t_R=8.4, *m/z*: 384 (M^+), 325 $(M-59)^+$; methyl 2-hydroxytetracosanoate, t_R=10.0, *m/z*: 398 (M^+), 339 $(M-59)^+$.

GC-MS and GC Analyses of TMS Ethers of LCB and Methyl Glycoside from HLC-2 The residue (mixture of LCB and methyl glycoside) from HLC-2 was trimethylsilylated, and the reaction mixture was analyzed by GC-MS and GC in the same manner as described for HPC-1. LCB (GC-MS): 2-amino-1,3-dihydroxy-4-heptadecene; 2-amino-1,3-dihydroxy-4-octadecene; 2-amino-1,3-dihydroxy-4-nonadecene, t_R [min]=5.1, *m/z*: 354 $(M-103)^+$, 325 $(M-132)^+$, 132. Methyl glycoside (GC): methyl α- and β-glucopyranoside were detected.

Methanolysis of HLC-3 HLC-3 was methanolyzed and the reaction mixture was worked up in the same way as HLC-1. A mixture of FAM and a residue composed of LCB and methyl glycoside were obtained.

GC-MS Analysis of FAM from HLC-3 In the same conditions as described for the FAM mixture obtained from HLC-2, a FAM mixture from HLC-3 was analyzed using GC-MS. The results were as follows: methyl 2-hydroxydocosanoate; methyl 2-hydroxytricosanoate; methyl 2-hydroxytetracosenoate, t_R [min]=9.4, *m/z*: 396 (M^+), 337 $(M-59)^+$; methyl 2-hydroxytetracosanoate.

GC-MS and GC Analyses of TMS Ethers of LCB and Methyl Glycoside from HLC-3 In the same way as HLC-1, a mixture of LCB and methyl glycoside from HLC-3 was trimethylsilylated, and the reaction mixture was analyzed using GC-MS and GC. LCB (GC-MS): 2-amino-1,3,4-trihydroxy-hexadecane, t_R [min]=4.1, *m/z*: 312 $(M-193)^+$, 271 $(M-234)^+$, 132; 2-amino-1,3,4-trihydroxy-heptadecane, t_R=4.7, *m/z*: 326 $(M-193)^+$, 285 $(M-234)^+$, 132; 2-amino-1,3,4-trihydroxy-octadecane, t_R=6.1, *m/z*: 340 $(M-193)^+$, 299 $(M-234)^+$, 132; 2-amino-1,3,4-trihydroxy-nonadecane, t_R=7.5, *m/z*: 354 $(M-193)^+$, 313 $(M-234)^+$, 132. Methyl glycoside (GC): methyl α- and β-glucopyranoside were detected.

Isolation of HLC-2-A from HLC-2 Reverse-phase column chromatography of HLC-2 (7.3 g) [Fig. 4, condition (a)] afforded seventeen fractions

(Fig. 4A): fr. 1 (10 mg), fr. 2 (17 mg), fr. 3 (23 mg), fr. 4 (34 mg), fr. 5 (97 mg), fr. 6 (114 mg), fr. 7 (137 mg), fr. 8 (147 mg), fr. 9 (68 mg), fr. 10 (173 mg), fr. 11 (85 mg), fr. 12 (3.8 g), fr. 13 (1.1 g), fr. 14 (93 mg), fr. 15 (150 mg), fr. 16 (15 mg), fr. 17 (5 mg). HPLC of the fraction 12 [Fig. 4, condition (b)] showed ten peaks (Fig. 4B). Using this condition, 1.6 g of fraction 12 was separated by HPLC to give ten fractions: fr. 1′ (41 mg), fr. 2′ (46 mg), fr. 3′ (155 mg), fr. 4′ (31 mg), fr. 5′ (37 mg), fr. 6′ (31 mg), fr. 7′ (135 mg), fr. 8′ (44 mg), fr. 9′ (68 mg), fr. 10′ (95 mg). Fraction 7′ (130 mg) was successively separated by using recycling HPLC [Fig. 4, conditions (c)] to yield HLC-2-A (7 mg) (Fig. 4C).

HLC-2-A: Amorphous powder, mp 160 °C. Positive-ion FAB-MS *m*/*z*: 808 $[M+Na]^+$. ^{1}H-NMR (C_5D_5N) δ: 0.85 (9H, m, terminal methyl groups), 4.89 (1H, d, J=7.8 Hz, glucose H-1). ^{13}C-NMR: see Table 1.

Methanolysis of HLC-2-A Experiments were conducted in the same manner as in the case of HLC-2, and methyl 2-hydroxydocosanoate and 2-amino-1,3-dihydroxy- 4-heptadecene were detected as FAM and LCB components, respectively.

FAM: ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=6.6 Hz, terminal methyl group), 3.79 (3H, s, OCH_3), 4.21 (1H, m, H-2).

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