Constituents of Holothuroidea, 12.[1] Isolation and Structure of Glucocerebrosides from the Sea Cucumber *Holothuria pervicax*

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Ten glucocerebrosides, HPC-3-A—HPC-3-J, have been isolated from their obtained parent glucocerebroside molecular species HPC-3, together with other glucocerebroside molecular species HPC-1 and HPC-2, from the less polar lipid fraction of a chloroform/methanol extract of the sea cucumber *Holothuria pervicax*. The structures of these glucocerebrosides have been determined on the basis of chemical and spectroscopic evidence. Reversed-phase HPLC, including a recycling system, was effective in isolating these glucocerebrosides, revealing a very close resemblance in structure, though the problem due to regio-isomers remains.

Key words glycosphingolipid; glucocerebroside; sea cucumber; *Holothuria pervicax*; 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—8] In the study of the GSLs of the sea cucumber *Holothuria pervicax* (Torafunamako in Japanese), we reported the isolation and structure of four new ganglioside molecular species.[5,7] Continuing the preceding studies, the isolation and characterization of cerebrosides from *H. pervicax* was conducted. In this paper, we report the isolation and characterization of glucocerebrosides from the whole bodies of *H. pervicax*.

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

HPC-1, HPC-2, and HPC-3 exhibit strong hydroxy and amide absorptions in their IR spectra, and a series of molecular ion peaks in their positive FAB mass spectra, respectively. In their ^{13}C-NMR spectra (Fig. 1, Table 1), they reveal characteristic signals of a sphingosine-type β-glucocerebroside possessing an unsubstituted fatty acid (HPC-1), a sphingosine-type β-glucocerebroside possessing a 2-hydroxy fatty acid (HPC-2), and a phytosphingosine-type β-glucocerebroside possessing a 2-hydroxy fatty acid (HPC-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 long-chain base (LCB), as shown in Fig. 2 and the Experimental section. The absolute configuration of their glucose moiety (D-form) was determined by the Hara method[9] (Experimental section).

Based on the considerable interest in and importance of determining the molecular species composition of GSLs, the isolation and structural elucidation of glucocerebroside components in the most polar molecular species, HPC-3, was conducted at this time. HPC-3 could be separated by reversed-phase HPLC into eighteen peaks (Fig. 3a), and could be recovered to give six fractions, 5, 8, 9, 11, 14 and 16. They behaved as pure compounds in HPLC. However, fractions 11, 14 and 16 were still regarded as heterogeneous compounds, respectively, since they afforded plural FAMs upon methanolysis. Each fraction, 11, 14 and 16, was successively separated into plural peaks by using recycling reversed-phase (C30) HPLC, and seven fractions could be isolated as shown in Fig. 3b and c. Thus, ten components, designated as HPC-3-A—HPC-3-J, were obtained from the parent glucocerebroside mixture, HPC-3. In the negative FAB mass spectrum, they reveal single quasi-molecular ion peaks $[M+Na]^+$ at *m*/*z*: 838 (HPC-3-A), 852 (HPC-3-B), 826 (HPC-3-C), 840 (HPC-3-D and HPC-3-E), 854 (HPC-3-F, HPC-3-G and HPC-3-H), 868 (HPC-3-I and HPC-3-J). These were confirmed as being the glucocerebroside component of HPC-3, since their ^{13}C-NMR spectra are identical to that of HPC-3 (Table 1).

Upon methanolysis, they yielded methyl 2-hydroxytricosenoate (HPC-3-A), methyl 2-hydroxytetracosenoate (HPC-3-B), methyl 2-hydroxydocosanoate (HPC-3-C, HPC-3-D and HPC-3-F), methyl 2-hydroxytricosanoate (HPC-3-E, HPC-3-G and HPC-3-I), methyl 2-hydroxytetracosanoate (HPC-3-H and HPC-3-J) and 2-amino-1,3,4-trihydroxy-heptadecane (HPC-3-A, HPC-3-B, HPC-3-C, HPC-3-E and HPC-3-H), 2-amino-1,3,4-trihydroxy-octadecane (HPC-3-D, HPC-3-G and HPC-3-J), 2-amino-1,3,4-trihydroxy-nonadecane (HPC-3-F and HPC-3-I) as their fatty acid and LCB components, respectively. The location and geometry of the double bond in the fatty acyl moiety of HPC-3-A and HPC-3-B were determined as follows.

The mass spectra of the dimethyl disulfide (DMDS) derivatives[10,11] of the FAM from HPC-3-A [(a) in Fig. 4] and HPC-3-B [(b) in Fig. 4] show remarkable fragment-ion peaks at *m*/*z*: 173 and 303 for (a) and *m*/*z*: 173 and 317 for (b) due to cleavage of the bonds between the carbons bearing the methylthio groups (Fig. 4). These data indicate that the double bonds in the fatty acyl moieties of HPC-3-A and HPC-3-B are located at C-14 and C-15, respectively. Furthermore, the geometry (*Z*) of the double bonds of both compounds were determined from the δ value (27.6) of the allylic carbon

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atoms obtained from their ^{1}H-detected heteronuclear multiple-bond connectivity (HMBC) spectra (Fig. 5), since allylic carbon signals of *Z*- and *E*-isomer are observed at δ *ca.* 26—27 and δ *ca.* 31—32, respectively.[12)]

On the basis of the above data, the structures of HPC-3-A—HPC-3-J are proposed to be β-D-glucopyranosyl ce-

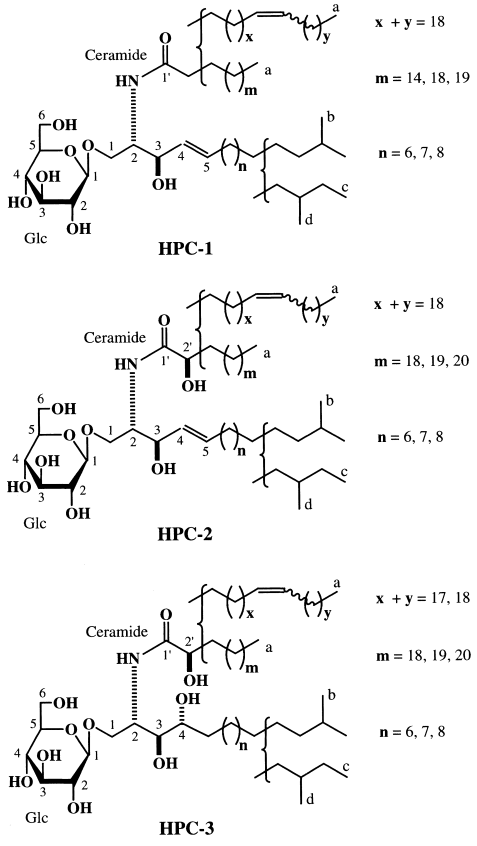


Fig. 1. Structures of HPC-1, HPC-2 and HPC-3

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

C		HPC-1	HPC-2	HPC-3	HPC-3-A	HPC-3-C
Ceramide						
1	(t)	70.6	70.2	70.5	70.5	70.5
2	(d)	55.1	54.6	51.8	51.8	51.8
3	(d)	72.7	72.4[e)]	75.8	75.9	75.9
4	(d)	132.2	131.7	72.6[f)]	72.6[g)]	72.6[h)]
5	(d)	132.6	132.8			
1′	(s)	173.4	175.7	175.7	175.7	175.7
2′	(d)		72.5[e)]	72.5[f)]	72.5[g)]	72.5[h)]
=CH	(d)	130.3	130.3	130.3	130.3	
=CH$\underline{C}$H$_2$	(t)				27.6	
CH$_3$[a)]	(q)	14.3	14.3	14.3	14.3	14.3
CH$_3$[b)]	(q)	22.8	22.8	22.8	22.8	22.8
CH$_3$[c)]	(q)	11.6	11.6	11.6	11.6	11.6
CH$_3$[d)]	(q)	19.4	19.4	19.4	19.4	19.4
Glc						
1	(d)	106.0	105.6	105.6	105.6	105.6
2	(d)	75.3	75.1	75.2	75.2	75.2
3	(d)	78.4	78.4	78.4	78.5	78.5
4	(d)	71.6	71.6	71.5	71.5	71.6
5	(d)	78.5	78.5	78.5	78.5	78.5
6	(t)	62.8	62.7	62.7	62.7	62.7

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

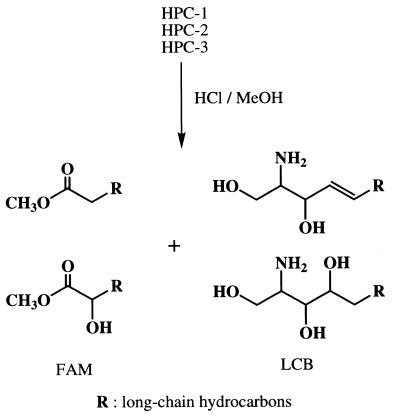


Fig. 2. Methanolysis Products of Cerebrosides
FAM: fatty acid methyl ester, LCB: long-chain base.

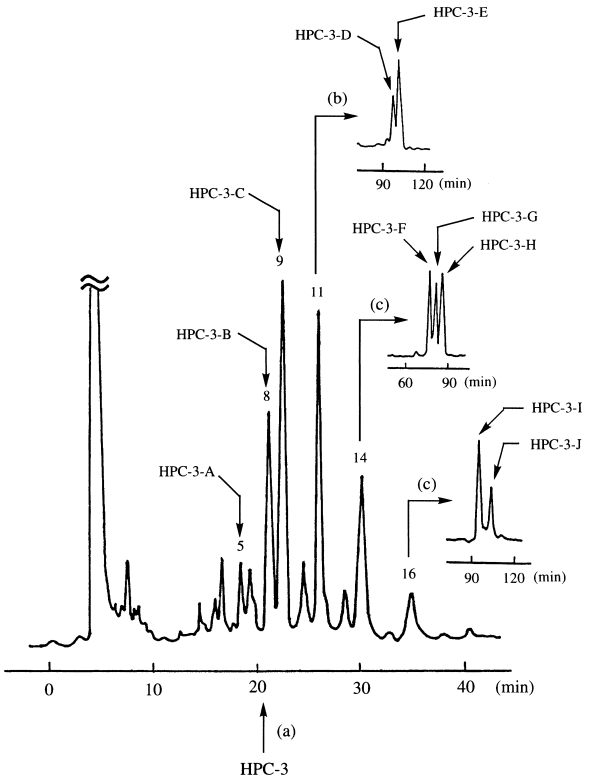


Fig. 3. HPLC (a) and Recycling HPLC (b, c) Chromatograms of the Components of HPC-3

HPLC conditions: (a) Column, Cosmosil 5C18 AR-II (10×300 mm); solvent, 100% MeOH; flow rate, 3.0 ml/min; detector, RI. (b), (c) Column, Develosil C30-UG-5 (4.6×250 mm); solvent, 100% MeOH; flow rate, 1.0 ml/min; number of cycles, 3 for (b) and 2 for (c); detector, RI.

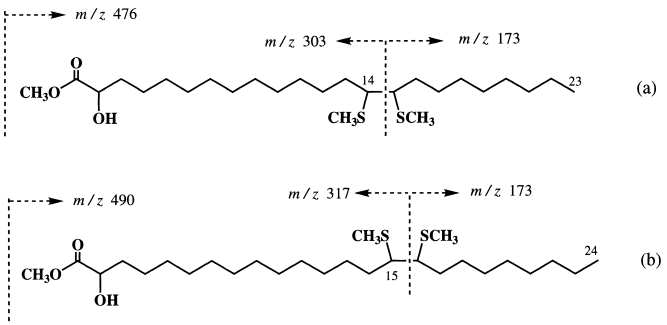


Fig. 4. Mass Fragmentation of DMDS Derivatives of HPC-3-A FAM (a) and HPC-3-B FAM (b)

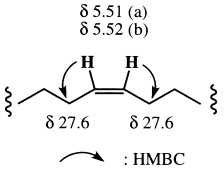
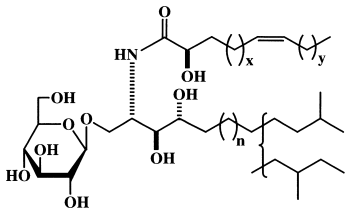
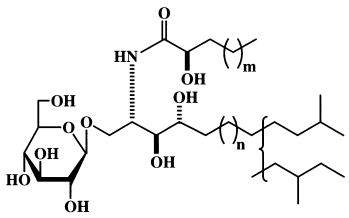


Fig. 5. Partial HMBC Correlations of the Fatty Acyl Moiety of HPC-3-A (a) and HPC-3-B (b)



HPC-3-A : **x** = 10, **y** = 7, **n** = 6
HPC-3-B : **x** = 11, **y** = 7, **n** = 6



HPC-3-C : **m** = 18, **n** = 6
HPC-3-D : **m** = 18, **n** = 7
HPC-3-E : **m** = 19, **n** = 6
HPC-3-F : **m** = 18, **n** = 8
HPC-3-G : **m** = 19, **n** = 7
HPC-3-H : **m** = 20, **n** = 6
HPC-3-I : **m** = 19, **n** = 8
HPC-3-J : **m** = 20, **n** = 7

Fig. 6. Structures of HPC-3-A—HPC-3-J

ramides, as shown in Fig. 6.

At this time, we were able to isolate ten glucocerebrosides, revealing the close resemblance in structure by the aid of reversed-phase HPLC including a recycling system. However, all these compounds are still a mixture of regio-isomers for terminal methyl groups in the side chain of the LCB moiety, namely a mixture of iso and *ante*-iso isomers, since the carbon signals for the terminal methyl groups in LCB are observed at δ: 22.8 (iso form), 11.6 and 19.4 (*ante*-iso form) in the ^{13}C-NMR spectra of HPC-3-A—HPC-3-J, the same as the molecular species HPC-3 (Fig. 1, Table 1). We hope to try to separate these isomers in the future.

Experimental

Melting points were determined on a micro melting point apparatus (Yanako MP-3) without correction. 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 and 67.8 MHz) or a Varian Unity-500 spectrometer (500 and 125 MHz). Positive-ion FAB-MS spectra were acquired with a Jeol DX-300 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 (A), CBP10-W12-100 (0.53 mm×12 m, Shimadzu); column (B), 2% OV-1 (4.2 mm×1.2 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 HPC-1, HPC-2 and HPC-3 For the extraction and fractionation of the less polar fraction from the whole bodies of the sea cucumber *Holothuria pervicax* (126 kg), refer to the previous paper.[5] The less polar fraction, namely the AcOEt/*n*-BuOH soluble fraction (339.9 g), was washed with cold acetone to give an acetone-insoluble fraction (less polar lipid fraction, 192.2 g). The less polar lipid fraction was chromatographed on silica gel (solvent $CHCl_3$–MeOH–H_2O, 95 : 5 : 0 to 3 : 7 : 1) to give eleven fractions. Successive column chromatography of fraction 5 and 7 (silica gel, solvent $CHCl_3$–MeOH, 8 : 2) afforded HPC-1 (276 mg) (*Rf*=0.67) and HPC-2 (1446 mg) (*Rf*=0.58), respectively. Fraction 9 was further chromatographed on silica gel (solvent $CHCl_3$–MeOH–H_2O, 88 : 12 : 0 to 6 : 4 : 1) to afford HPC-3 (975 mg) (*Rf*=0.53) [silica gel TLC, solvent $CHCl_3$–MeOH (8 : 2)].

HPC-1: Amorphous powder. IR (KBr) cm^{-1}: 3310 (OH), 1640, 1540 (amide). Positive-ion FAB-MS *m/z*: 750—850 $[M+Na]^+$ series. ^{1}H-NMR (C_5D_5N) δ: 0.88 (9H, m, terminal methyl groups), 4.97 (1H, d, *J*=7.8 Hz, glucose H-1). ^{13}C-NMR: See Table 1.

HPC-2: Amorphous powder. IR (KBr) cm^{-1}: 3350 (OH), 1650, 1540 (amide). Positive-ion FAB-MS *m/z*: 770—870 $[M+Na]^+$ series. ^{1}H-NMR (C_5D_5N) δ: 0.88 (9H, m, terminal methyl groups), 4.92 (1H, d, *J*=7.8 Hz, glucose H-1). ^{13}C-NMR: See Table 1.

HPC-3: Amorphous powder. IR (KBr) cm^{-1}: 3360 (OH), 1630, 1540 (amide). Positive-ion FAB-MS *m/z*: 820—880 $[M+Na]^+$ series. ^{1}H-NMR (C_5D_5N) δ: 0.88 (9H, m, terminal methyl groups), 4.96 (1H, d, *J*=7.6 Hz, glucose H-1). ^{13}C-NMR: See Table 1.

Methanolysis of HPC-1 HPC-1 (10.4 mg) was heated with 5% HCl in MeOH (3 ml) at 70 °C for 22 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.

FAM: ^{13}C-NMR ($CDCl_3$) δ: 14.1 (terminal methyl group), 52.4 (OCH_3), 175.9 (CO).

GC-MS Analysis of FAM from HPC-1 A FAM mixture from HPC-1 was subjected to GC-MS [column (A), column temp. 180—250 °C (rate of temp. increase 4 °C/min)]. The results were as follows: methyl octadecanoate, t_R[min]=1.4, *m/z*: 298 (M^+), 255 $(M-43)^+$; methyl docosanoate, t_R=4.9, *m/z*: 354 (M^+), 311 $(M-43)^+$; methyl tricosanoate, t_R=6.2, *m/z*: 368 (M^+), 325 $(M-43)^+$; methyl tetracosenoate, t_R=7.2, *m/z*: 380 (M^+), 337 $(M-43)^+$.

GC-MS Analysis of TMS Ethers of LCB from HPC-1 A mixture of LCB and methyl glycoside from HPC-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 (A), column temp. 180—250 °C (rate of temp. increase 4 °C/min)]. The results were as follows: 2-amino-1,3-dihydroxy-4-heptadecene, t_R[min]=2.6, *m/z*: 326 $(M-103)^+$, 297 $(M-132)^+$, 132; 2-amino-1,3-dihydroxy-4-octadecene, t_R=3.7, *m/z*: 340 $(M-103)^+$, 311 $(M-132)^+$, 132; 2-amino-1,3-dihydroxy-4-nonadecene, t_R=4.8, *m/z*: 354 $(M-103)^+$, 325 $(M-132)^+$, 132.

GC Analysis of TMS Ethers of Methyl Glycoside from HPC-1 The mixture of TMS ethers of LCB and methyl glycoside was analyzed by GC [column temp.: 100—250 °C (rate of temp. increase 5 °C/min)]: t_R[min]=17.9 and 18.1 (methyl α- and β-glucopyranoside).

Determination of Absolute Configuration of Glucose Moiety of HPC-1 (Hara method) HPC-1 (1 mg) was heated with 2 N H_2SO_4 (0.5 ml) at 100 °C for 8 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 trimethylsilyl ether of the methyl (4*R*)-thiazolidine-4-carboxylate derivative. The derivative was analyzed by GC [column temp.: 200—250 °C (rate of temp. increase 2.5 °C/min)]; t_R=12.3 min (derivative of D-glucose, 12.3 min; L-glucose, 13.0 min). In the same way, the absolute configuration of the glucose moiety (D-form) of HPC-2 and HPC-3 was determined.

Methanolysis of HPC-2 In the same manner as described for HPC-1, HPC-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.

FAM: ^{13}C-NMR ($CDCl_3$) δ: 14.2 (terminal methyl group), 52.4 (OCH_3), 70.6 (C-2′), 175.9 (CO).

GC-MS Analysis of FAM from HPC-2 A FAM mixture from HPC-2 was subjected to GC-MS under the same conditions as described for the FAM mixture obtained from HPC-1. The results were as follows: methyl 2-

hydroxydocosanoate, t_R[min]=15.2, *m/z*: 370 (M^+), 311 $(M-59)^+$; methyl 2-hydroxytricosanoate, t_R=17.0, *m/z*: 384 (M^+), 325 $(M-59)^+$; methyl 2-hydroxytetracosenoate, t_R=18.9, *m/z*: 396 (M^+), 337 $(M-59)^+$; methyl 2-hydroxytetracosanoate, t_R=18.9, *m/z*: 398 (M^+), 339 $(M-59)^+$.

GC-MS and GC Analyses of TMS Ethers of LCB and Methyl Glycoside from HPC-2 The residue (mixture of LCB and methyl glycoside) from HPC-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, and 2-amino-1,3-dihydroxy-4-nonadecene were detected. Methyl glycoside (GC): methyl α- and β-glucopyranoside were detected.

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

FAM: ^{13}C-NMR ($CDCl_3$) δ: 14.1 (terminal methyl group), 52.4 (OCH_3), 70.5 (C-2′), 175.8 (CO).

GC-MS Analysis of FAM from HPC-3 A FAM mixture from HPC-3 was subjected to GC-MS [column (B), column temp. 180—250 °C (rate of temp. increase 5 °C/min)]. The results were as follows: methyl 2-hydroxydocosanoate, t_R[min]=9.3, *m/z*: 370 (M^+), 311 $(M-59)^+$; methyl 2-hydroxytricosenoate, t_R=10.3, *m/z*: 382 (M^+), 323 $(M-59)^+$; methyl 2-hydroxytricosanoate, t_R=10.6, *m/z*: 384 (M^+), 325 $(M-59)^+$; methyl 2-hydroxytetracosenoate, t_R=11.4, *m/z*: 396 (M^+), 337 $(M-59)^+$; methyl 2-hydroxytetracosanoate, t_R=12.1, *m/z*: 398 (M^+), 339 $(M-59)^+$.

GC-MS and GC Analyses of TMS Ethers of LCB and Methyl Glycoside from HPC-3 A mixture of LCB and methyl glycoside from HPC-3 was trimethylsilylated in the same way as HPC-1, and the reaction mixture was analyzed by GC-MS and GC. LCB (GC-MS) [column (A), column temp. 200—250 °C (rate of temp. increase 3 °C/min)]: 2-amino-1,3,4-trihydroxy-heptadecane, t_R[min]=3.2, *m/z*: 326 $(M-193)^+$, 285 $(M-234)^+$, 132; 2-amino-1,3,4-trihydroxy-octadecane, t_R=4.1, *m/z*: 340 $(M-193)^+$, 299 $(M-234)^+$, 132; 2-amino-1,3,4-trihydroxy-nonadecane, t_R=5.4, *m/z*: 354 $(M-193)^+$, 313 $(M-234)^+$, 132. Methyl glycoside (GC, same condition as in HPC-1): methyl α- and β-glucopyranoside were detected.

Isolation of Glucocerebroside Components HPC-3-A—HPC-3-J from HPC-3 HPLC of HPC-3 [Fig. 3. condition (a)] showed eighteen peaks. Using this condition, 585 mg of HPC-3 was separated by HPLC to give six fractions, 5 (HPC-3-A, 3 mg), 8 (HPC-3-B, 25 mg), 9 (HPC-3-C, 38 mg), 11, 14 and 16. Fractions 11, 14 and 16 were successively separated, respectively, by using recycling HPLC [Fig. 3. conditions (b) and (c)] to yield seven compounds, HPC-3-D (11 mg), HPC-3-E (25 mg), HPC-3-F (6 mg), HPC-3-G (6 mg), HPC-3-H (9 mg), HPC-3-I (3 mg) and HPC-3-J (2 mg).

HPC-3-A—HPC-3-J (abbreviate as A—J): Amorphous powder, mp 138—140 (A), 134—137 (B), 141—142 (C), 152—154 (D), 151—153 (E), 150—152 (F), 152—154 (G), 151—153 (H), 150—152 (I), 152—154 (J) °C. Positive-ion FAB-MS *m/z*: 838 (A), 852 (B), 826 (C), 840 (D and E), 854 (F, G and H), 868 (I and J) $[M+Na]^+$. ^{1}H-NMR (C_5D_5N) δ: 5.51 (2H, m, HC=CH) (A), 5.52 (2H, m, HC=CH) (B). ^{13}C-NMR (C_5D_5N) δ: 27.6 ($\underline{C}H_2$-CH=CH) (A and B). Other carbon signals are identical with those of HPC-3 (See Table 1).

Methanolysis of HPC-3-A—HPC-3-J (abbreviate as A—J) Experiments were conducted in the same manner as in the case of HPC-3, leading to FAM and LCB being derived from each HPC-3 series (A—J). The FAM was subjected to GC-MS under the same conditions as described for HPC-3, and methyl 2-hydroxytricosenoate (from A), methyl 2-hydroxytetracosenoate (from B), methyl 2-hydroxydocosanoate (from C, D and F), methyl 2-hydroxytricosanoate (from E, G and I), methyl 2-hydroxytetracosanoate (from H and J) were detected. The LCB was trimethylsilylated and analyzed by GC-MS in the same way as in the case of HPC-3. The results were as follows: 2-amino-1,3,4-trihydroxy-heptadecane (from A, B, C, E and H), 2-amino-1,3,4-trihydroxy-octadecane (from D, G and J), 2-amino-1,3,4-trihydroxy-nonadecane (from F and I).

DMDS Derivatives of FAM from HPC-3-A and HPC-3-B Each FAM (0.7 mg) from HPC-3-A and HPC-3-B (methyl 2-hydroxytricosenoate and methyl 2-hydroxytetracosenoate) was dissolved in carbon disulfide (0.2 ml), and DMDS (0.2 ml) and iodine (1 mg) were added to the solution. The resulting mixture was kept at 60 °C for 40 h in a small-volume sealed vial. The reaction was subsequently quenched with aqueous $Na_2S_2O_3$ (5%), and the mixture was extracted with *n*-hexane (0.3 ml). The extract was concentrated and the residue (DMDS derivative) was analyzed by GC-MS [column (B), column temp. 250 °C]: DMDS derivative of HPC-3-A FAM, t_R[min]=12.0, *m/z*: 476 (M^+), 303, 173; DMDS derivative of HPC-3-B FAM, t_R=15.9, *m/z*: 490 (M^+), 317, 173.

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