

Two Amphoteric Galactocerebrosides Possessing a Tri-Unsaturated Long-Chain Base from the Leech (*Hirudo nipponica*)

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Six amphoteric galactocerebrosides were isolated from the land annelid (*Hirudo nipponica*). Two of them have a tri-unsaturated long-chain base, *D*-erythro-(4*E*,8*Z*,11*Z*)-docosasphingatriene. The position and geometry of the double bonds in the long-chain base unit were determined on the bases of chemical and spectral data.

Key words amphoteric galactosylceramide; tri-unsaturated long-chain base; *D*-erythro-(4*E*,8*Z*,11*Z*)-docosasphingatriene; annelid; leech; *Hirudo nipponica*

As described in our previous papers, members of the phylum Annelida, such as *Pheretima asiatica*,¹⁾ and *Neanthes diversicolor*,^{2b)} contain amphoteric galactosylceramides (AGSLs). In a continuation of our systematic survey of the constituents in this phylum, we have examined the lipid composition, especially AGSLs, of the land annelid, the leech (*Hirudo nipponica*), and have isolated and characterized two new AGSLs together with four known ones. This paper deals with the isolation and structure determination of these compounds.

The CHCl₃-MeOH extract (72.2 g) of the dried bodies (1 kg) of the leech (sold as a crude drug "Suitetsu")³⁾ was treated with CHCl₃-MeOH-H₂O (1:1:1), and the lower phase was concentrated to give a total lipid fraction. It was subjected to silica gel and Cosmosil 75C₁₈-OPN column chromatographies with various solvents to yield a phospholipid fraction. This was further separated by preparative HPLC in a recycling mode to furnish six AGSLs 1–6. Among them, 3, 4 and 5 were respectively identified as *N*-hexadecanoyl-, *N*-heptadecanoyl-^{2a)} and

N-octadecanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)-phosphinate-β-*D*-galactopyranosyl]-(4*E*)-sphingene,^{2b)} and 6 as *N*-tetracosanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)phosphinate-β-*D*-galactopyranosyl]-(4*E*)-17-methylsphingene¹⁾ by comparison of the physical and spectroscopic data with those of corresponding authentic samples.

The ¹³C-NMR spectrum of 1 (*m/z*: 1030 [M+H]⁺, fast atom bombardment mass spectrum (FAB-MS)) gave six olefinic carbon signals together with typical signals due to a phosphocholine group and a monogalactosylceramide moiety (Fig. 1).^{1,2)} The ¹H-NMR spectrum showed signals ascribable to olefinic protons (4H, δ 5.3–5.4), two methylenes next to a double bond (4H, δ 2.0–2.1), a methylene (2H, δ 2.78) lying between two double bonds in addition to the signals arising from the common AGSL. These findings indicated that 1 has a 2,5-heptadiene-1,7-diyl (–CH=CH–CH₂–CH=CH–) group.

Methanolysis of 1 gave a fatty acid methyl ester, which was identified as methyl *n*-tetracosanoate by gas chro-

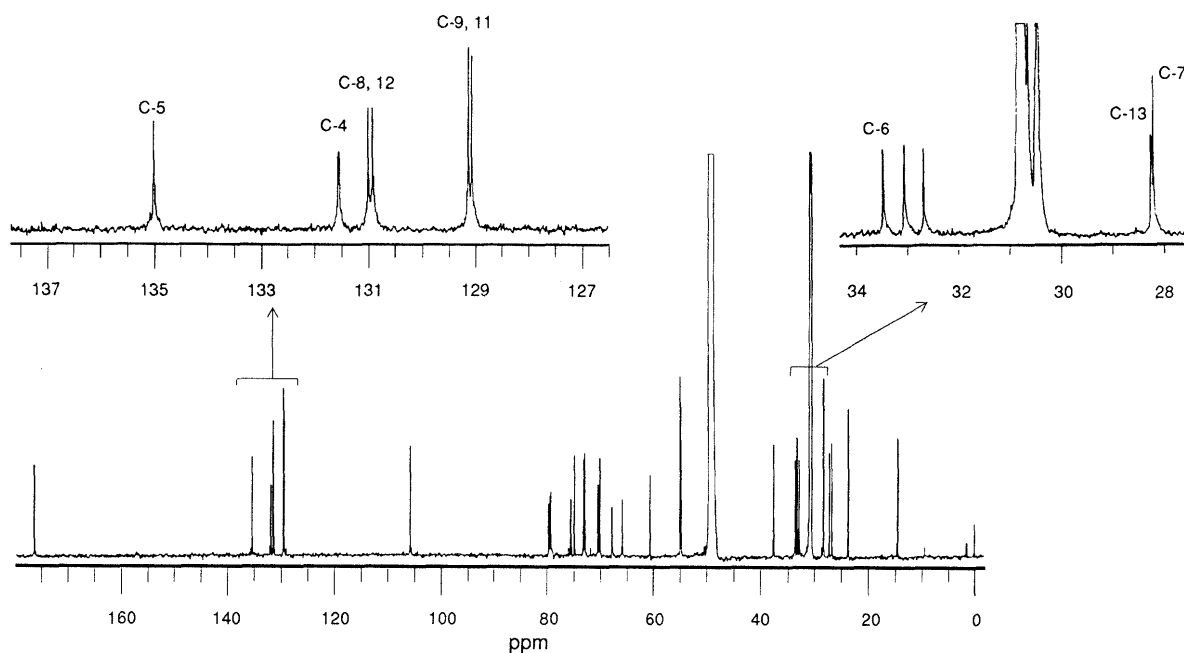


Fig. 1. ¹³C-NMR Spectrum of 1 (150 MHz, CD₃OD:CDCl₃=4:1)

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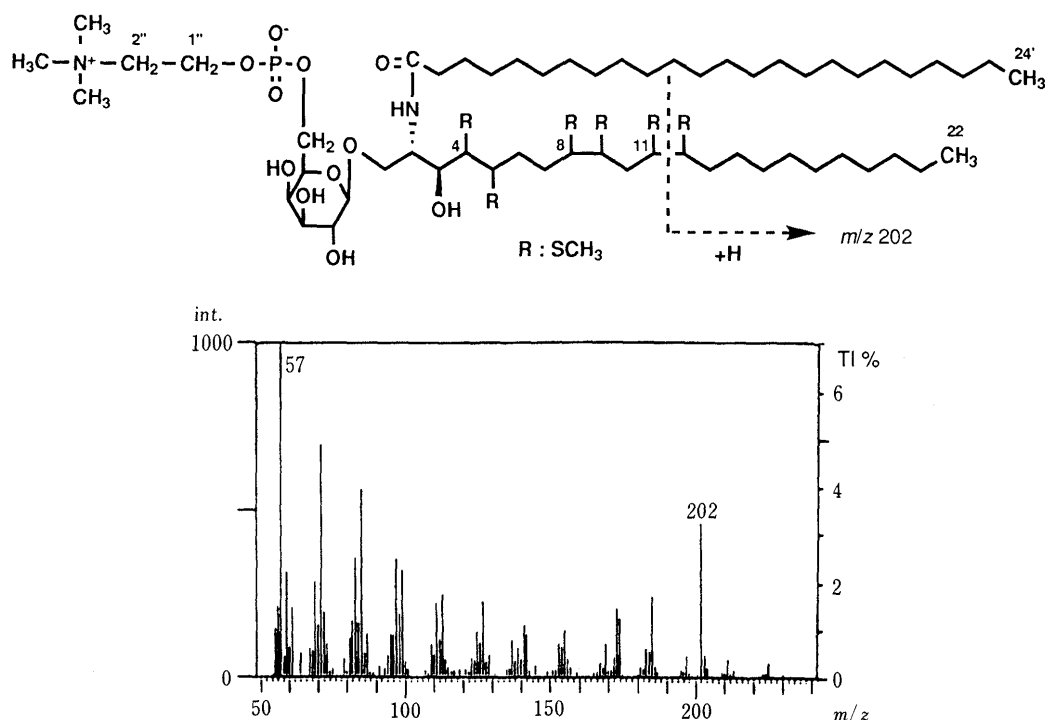


Fig. 2. EI-MS of the Dimethyl Disulfide Derivative of **1**

matography (GC), electron impact mass spectrometry (EI-MS) and ^1H -NMR spectroscopy. In view of the molecular weight of **1** (M.W. 1029) and its components (galactose 6-phosphocholine and *n*-tetracosanoyl units), the long-chain base (LCB) was considered to have a $\text{C}_{22:3}$ tri-unsaturated carbon chain.

The dimethyl disulfide derivative of **1** gave a fragment ion peak at m/z 202 $[\text{C}_{12}\text{H}_{25}\text{S} + \text{H}]^+$, which was regarded as being due to a fragment ion produced by cleavage between the C-11 and C-12 sulfided carbons,^{2b,4)} suggesting that the 2,5-heptadiene-1,7-diyl group is located at C-7 (Fig. 2).

To confirm the above suggestion as well as the geometry of the double bonds, the proton and carbon signals of **1** were assigned by two-dimensional shift correlation spectroscopy (^1H - ^1H and ^{13}C - ^1H COSY) and two-dimensional nuclear Overhauser effect spectroscopy (NOESY) experiments. The ^1H - ^1H COSY spectrum exhibited a series of correlation peaks from H-3 to H₂-13, showing the locations of three double bonds (C4, C8 and C11). Furthermore, in the NOESY spectrum, the signals of the methylene (H₂-10) lying between two double bonds (C8 and C11) were correlated with those of H₂-7 and H₂-13, and an nuclear Overhauser effect (NOE) correlation between H-4 and H₂-6 appeared (Fig. 3). These observations revealed that the geometry of the double bond at C4 is *trans* and that of the double bonds at C8 and C11 is *cis*. The chemical shifts of the carbon signals next to the double bonds, C-6 (δ 33.47), C-7 (δ 28.20), C-10 (δ 26.60) and C-13 (δ 28.24), supported⁵⁾ the above conclusion.

With regard to the configuration of the LCB unit, all AGSLs so far isolated from members of Annelida contain the *D*-*erythro*-sphingenine moiety, so it was presumed that the LCB part of **1** also has the *erythro* form. Hydrogenation

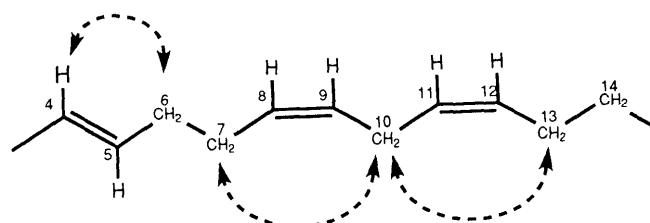


Fig. 3. NOE Correlations of **1**

over palladium carbon of **1** followed by methanolysis gave a saturated LCB, which was acetylated to give an LCB (docosasphinganine) triacetate (**1a**). When the ^1H -NMR spectral data of **1a** were compared with those of two pairs of *D,L*-*erythro*- and *D,L*-*threo*-sphinganine triacetates reported by Shibuya *et al.*,⁶⁾ the chemical shifts and splitting patterns of H₂-1, H-2 and H-3 of **1a** were in good accord with those of the former. Moreover, the sign of the specific rotation was the same as that of *D*-*erythro*-sphinganine triacetate. From the above findings, the configuration was concluded to be *D*-*erythro*, and hence the LCB of **1** was assigned the structure *D*-*erythro*-(4*E*,8*Z*,11*Z*)-docosasphingatrienine.

On the basis of all the above results, the structure of **1** is defined as *N*-tetracosanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)phosphinate- β -*D*-galactopyranosyl]- (4*E*,8*Z*,11*Z*)-docosasphingatrienine (Fig. 4).

Compound **2** showed a quite similar ^1H -NMR spectrum to that of **1**, while in the FAB-MS, the $[\text{M} + \text{H}]^+$ ion peak was 28 mass units less than that of **1**. Methanolysis of **2** provided methyl *n*-docosanoate. High-resolution NMR spectroscopic analyses (COSY, NOESY) in the same manner as for **1** revealed that **2** differs from **1** only in the fatty acid residue; the *n*-tetracosanoyl group in **1** is replaced by a *n*-docosanoyl group in **2**. Therefore, **2** was

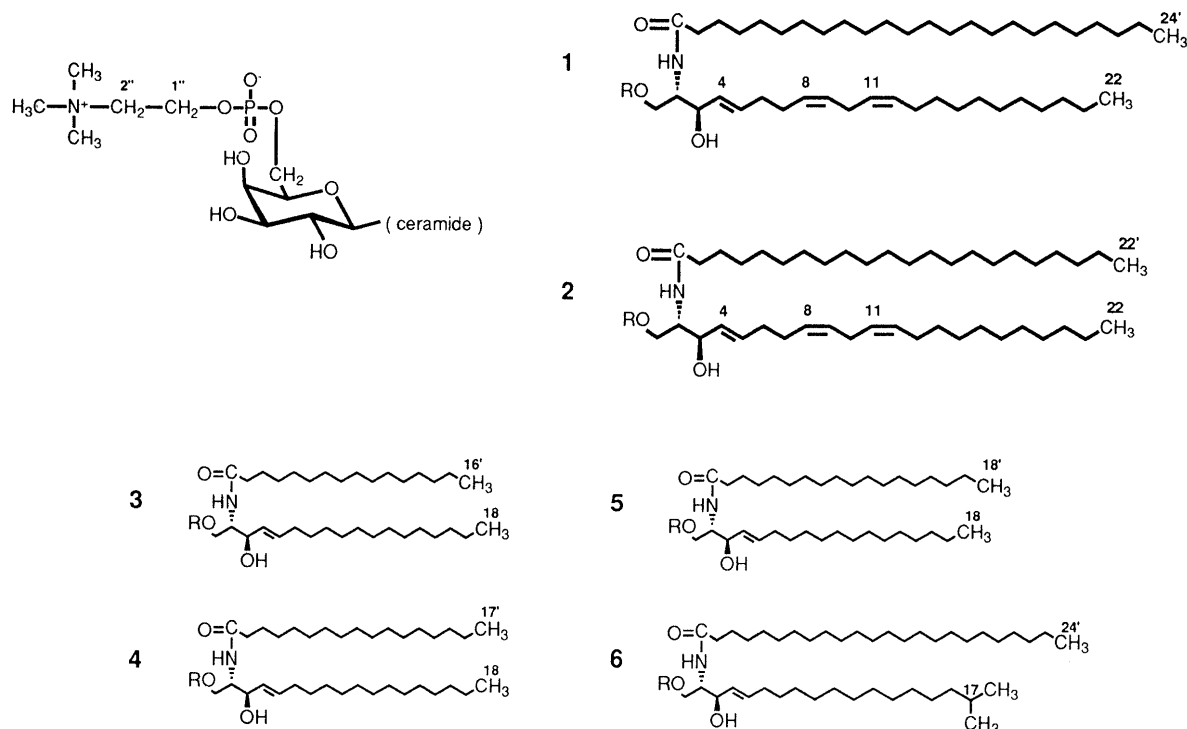


Fig. 4. Structures of Compounds 1–6

characterized as *N*-docosanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)phosphinate- β -D-galactopyranosyl]-(*4E,8Z,11Z*)-docosasphingatrienine (Fig. 4).

Glycosphingolipids obtained so far from natural sources have mainly a mono- or di-unsaturated LCB group, and only two tri-unsaturated LCBs ($C_{18:3}$, $C_{19:3}$) were detected in a methanolized mixture of the glycolipids fraction of the starfish, *Asterias amurensis*.⁷⁾ In regard to intact glycosphingolipids with a tri-unsaturated LCB unit, only four glycosphingolipids have been isolated recently from marine animals.⁸⁾ All of them consist of sphingatrienine or sphingatrienine possessing a branched methyl group, and the geometry and position of the three double bonds in LCB part are *4E,8E,10E*. The two compounds (**1** and **2**) isolated here are the first examples of AGSLs with a $C_{22:3}$ LCB, (*4E,8Z,11Z*)-docosasphingatrienine unit.

Experimental

The NMR spectra were recorded on a GE NMR OMEGA 600 instrument at 600 MHz (^1H) and 150 MHz (^{13}C) at a probe temperature of 35 °C using tetramethylsilane (TMS) as an internal reference. MS were acquired on a JEOL JMS DX-300 spectrometer (EI-MS: ionization voltage, 30 eV; accelerating voltage, 3–10 kV. Positive ion FAB-MS: accelerating voltage, 3 kV; matrix, glycerol; collision gas, Xe). Optical rotations were measured with a JASCO DIP-140 polarimeter. TLC was carried out on silica gel HPTLC with Al sheets (Merck Art. 5556). Spots were visualized with 5% H_2SO_4 in MeOH (by heating). Column chromatography was carried out on Merck silica gel (230–400 mesh, Art. 9385), and Cosmosil 75C₁₈-OPN (Nacalai Tesque). Preparative HPLC was conducted over an L-column octadecyl silica (ODS) (10 mm $\times$ 250 mm, Chemicals Inspection & Testing Ins.) on a JASCO PU-980 equipped with a model 504R unit (GL Sciences). Recycling HPLC was carried out on a JASCO PU-980 equipped with a JASCO preparative recycle valve.

Isolation of AGSLs 1–6 The CHCl_3 -MeOH extractives (72.2 g) of the crushed powder (1 kg) of the leech, *Hirudo nipponica* (sold as a crude drug "Suitetsu," purchased from Tochimoto Tenkaido, 1993), were treated with CHCl_3 -MeOH- H_2O (1:1:1, 600 ml), and the lower soluble portion was collected and concentrated to give a total lipid fraction

Table 1. ^1H -NMR Chemical Shifts (δ) of **1** and **2** (CD_3OD : CDCl_3 = 4:1)

No.	1	2
1	3.53 (1H, dd, $J=8.0, 10.0$ Hz)	3.52 (1H, dd, $J=8.0, 10.0$ Hz)
	4.18 (1H, dd, $J=4.0, 10.0$ Hz)	4.19 (1H, dd, $J=4.0, 10.0$ Hz)
2	3.96 (1H, ddd, $J=4.0, 8.0, 8.0$ Hz)	3.96 (1H, ddd, $J=4.0, 8.0, 8.0$ Hz)
3	4.07 (1H, t, $J=8.0$ Hz)	4.07 (1H, t, $J=8.0$ Hz)
4	5.45 (1H, dd, $J=8.0, 15.0$ Hz)	5.45 (1H, dd, $J=8.0, 15.2$ Hz)
5	5.69 (1H, td, $J=7.0, 15.0$ Hz)	5.69 (1H, td, $J=7.0, 15.2$ Hz)
6	2.03 (2H, m)	2.03 (2H, m)
7	2.07 (2H, m)	2.07 (2H, m)
8	5.35 (1H, m) ^{a)}	5.35 (1H, m) ^{a)}
9	5.34 (1H, m) ^{b)}	5.34 (1H, m) ^{b)}
10	2.78 (2H, t, $J=8.0$ Hz)	2.78 (2H, t, $J=8.0$ Hz)
11	5.34 (1H, m) ^{b)}	5.34 (1H, m) ^{b)}
12	5.35 (1H, m) ^{a)}	5.35 (1H, m) ^{a)}
13	2.04 (2H, m)	2.05 (2H, m)
14	1.37 (2H, m)	1.37 (2H, m)
22	0.90 (3H, t, $J=7.0$ Hz)	0.90 (3H, t, $J=7.0$ Hz)
2'	2.17 (2H, t, $J=7.5$ Hz)	2.17 (2H, t, $J=7.5$ Hz)
3'	1.58 (2H, m)	1.59 (2H, m)
22'		0.90 (3H, t, $J=7.0$ Hz)
24'	0.90 (3H, t, $J=7.0$ Hz)	
Gal-1	4.24 (1H, d, $J=7.5$ Hz)	4.24 (1H, d, $J=7.0$ Hz)
Gal-2	3.55 (1H, dd, $J=7.5, 10.0$ Hz)	3.55 (1H, dd, $J=7.0, 10.0$ Hz)
Gal-3	3.51 (1H, dd, $J=3.0, 10.0$ Hz)	3.50 (1H, dd, $J=3.0, 10.0$ Hz)
Gal-4	3.86 (1H, dd, $J=0.8, 3.0$ Hz)	3.87 (1H, dd, $J=0.7, 3.0$ Hz)
Gal-5	3.71 (1H, td, $J=0.8, 6.5$ Hz)	3.71 (1H, td, $J=0.7, 6.5$ Hz)
Gal-6	4.03 (2H, t, $J=6.5$ Hz)	4.03 (2H, t, $J=6.5$ Hz)
Cho-1''	4.30 (2H, m)	4.30 (2H, m)
Cho-2''	3.65 (2H, t, $J=4.5$ Hz)	3.64 (2H, t, $J=4.5$ Hz)
CH ₃	3.22 (9H, s)	3.23 (9H, s)

a, b) Signals with the same superscripts are overlapping.

(55.6 g). It was placed on a silica gel column and eluted successively with CHCl_3 -MeOH (8:2 $\rightarrow$ 7:3) $\rightarrow$ CHCl_3 -MeOH- H_2O (7:3:0.5 $\rightarrow$ 6:4:1 $\rightarrow$ 5:5:1). The eluates were monitored by TLC (mobile phase: CHCl_3 -MeOH- H_2O , 6:4:1) and those showing a positive spot with Dittmer-Lester's reagent⁹⁾ were combined and evaporated to give six fractions, fr. 1 (38.3 g), fr. 2 (3.3 g), fr. 3 (1.9 g), fr. 4 (4.6 g), fr. 5 (1.8 g) and fr. 6 (4.5 g). Fraction 6 was subjected to chromatography on a 75C₁₈-OPN column using MeOH $\rightarrow$ CHCl_3 -MeOH (1:1) as the eluent to yield four fractions, fr. 7 (570 mg), fr. 8 (2.46 g), fr. 9 (1.28 g) and fr.

Table 2. ^{13}C -NMR Chemical Shifts (δ) of **1** and **2** ($\text{CD}_3\text{OD}:\text{CDCl}_3=4:1$)

No.	1	2
1	70.18	70.20
2	54.68	54.74
3	72.89	72.94
4	131.52	131.54
5	134.97	135.00
6	33.47	33.49
7	28.20	28.24
8	130.98 ^{a)}	131.01 ^{a)}
9	129.12 ^{b)}	129.14 ^{b)}
10	26.60	26.62
11	129.05 ^{b)}	129.08 ^{b)}
12	130.90 ^{a)}	130.92 ^{a)}
13	28.24	28.25
22	14.45	14.45
1'	175.80	175.87
2'	37.41	37.45
3'	27.14	27.17
22'		14.45
24'	14.45	
Gal-1	105.45	105.49
Gal-2	72.70	72.74
Gal-3	74.61	74.67
Gal-4	69.89	69.95
Gal-5	75.25	75.31
Gal-6	65.67	65.70
Cho-1''	60.45	60.48
Cho-2''	67.58	67.64
CH ₃	54.82	54.85

a, b) Assignments with the same superscripts in each column may be interchangeable.

10 (190 mg). Fraction 9 was separated by HPLC (mobile phase: CHCl_3 -MeOH, 1.5:10) in a recycling mode to give compounds **1** (20.5 mg), **2** (54.7 mg), **3** (27.8 mg), **4** (15.5 mg), **5** (19.8 mg) and **6** (23.6 mg). **1**: mp 170–183 °C, $[\alpha]_D^{26} + 8.76^\circ$ ($c=0.39$, MeOH: $\text{CHCl}_3=1:1$). Positive ion FAB-MS m/z : 1030 ($\text{M}+\text{H}$)⁺. ^1H -NMR δ : Table 1. ^{13}C -NMR δ : Table 2. **2**: mp 159–172 °C, $[\alpha]_D^{26} + 8.62^\circ$ ($c=0.58$, MeOH: $\text{CHCl}_3=1:1$). Positive ion FAB-MS m/z : 1002 ($\text{M}+\text{H}$)⁺. ^1H -NMR δ : Table 1. ^{13}C -NMR δ : Table 2. Compounds **3**, **4** and **5** were respectively identified as *N*-hexadecanoyl, *N*-heptadecanoyl- and *N*-octadecanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)phosphinate- β -D-galactopyranosyl]-(4*E*)-sphingene, and **6** as *N*-tetracosanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)phosphinate- β -D-galactopyranosyl]-(4*E*)-17-methylsphingene by comparison with authentic samples obtained previously.^{1,2)}

Analysis of the Fatty Acids Each (ca. 1 mg) of **1**–**6** was treated with 5% methanolic HCl at 90 °C for 1 h. The reaction mixture was extracted with *n*-hexane and the fatty acid liberated was analyzed by EI-MS, ^1H -NMR and GC (fused silica capillary column Bonded MPS-50, Quadrex, 0.25 mm $\times$ 50 m; column temperature, 230 °C (hold, 12 min) $\rightarrow$ 240 °C at 1 °C/min; carrier gas, He at 33.4 ml/min): t_R (min): 6.54 (methyl hexadecanoate) from **3**, 7.63 (methyl heptadecanoate from **4**), 9.11 (methyl octadecanoate from **5**), 21.02 (methyl docosanoate from **2**), 31.75 (methyl tetracosanoate from **1** and **6**). Methyl *n*-docosanoate: EI-MS

m/z : 354 (M^+). ^1H -NMR (CDCl_3) δ : 0.89 (3H, t, $J=7.0$ Hz, H_3 -22), 1.21–1.72 ($-\text{CH}_2-$), 2.30 (2H, t, $J=7.0$ Hz, H_2 -2), 3.66 (3H, s, OCH_3). Methyl *n*-tetracosanoate: EI-MS m/z : 382 (M^+). ^1H -NMR (CDCl_3) δ : 0.90 (3H, t, $J=7.0$ Hz, H_3 -24), 1.23–1.72 ($-\text{CH}_2-$), 2.29 (2H, t, $J=7.0$ Hz, H_2 -2), 3.65 (3H, s, OCH_3).

Preparation of the Dimethyl Disulfide Derivative of 1 Carbon disulfide (0.2 ml) and iodine (1 mg) were added to **1** (2 mg) in dimethyl disulfide (0.2 ml), and the mixture was kept at 60 °C for 40 h. The reaction was quenched with 5% aqueous solution of sodium thiosulfate, then the mixture was shaken with CHCl_3 -MeOH (1:1, 3 ml). The lower soluble portion was separated and concentrated under a nitrogen stream. The residue was subjected to analysis by EI-MS (Fig. 2).

Determination of the LCB Part (1a) of 1 and 2 Each (15 mg) of compounds **1** and **2** was hydrogenated over 10% palladium carbon (mg) in CHCl_3 -MeOH (1:4, 50 ml). The catalyst was filtered off and the filtrate was evaporated to dryness to give a product. It was methanolized with 7.5% methanolic HCl at 90 °C for 2 h. The fatty acid liberated was extracted with *n*-hexane and the methanolic layer was neutralized by adding a small excess of AgCO_3 . After centrifugation, the supernatant was evaporated to dryness to give a residue. The residue was passed through a Sephadex LH-20 column with MeOH, yielding a product. This was acetylated with acetic anhydride-pyridine (1:1, 1 ml) at room temperature for 1 d to give an LCB triacetate (**1a**, 4 mg). **1a**: $[\alpha]_D^{25} + 8.4^\circ$ ($c=0.13$, CHCl_3). Positive ion FAB-MS m/z : 484 [$\text{M}+\text{H}$]⁺. ^1H -NMR (CDCl_3) δ : 0.88 (3H, t, $J=7.0$ Hz, H_3 -22), 1.26–1.75 ($-\text{CH}_2-$), 1.99, 2.06, 2.07 (each, s, $\text{OCOCH}_3 \times 2$ and NHCOCCH_3), 4.06 (1H, dd, $J=3.9$, 12.0 Hz, H-1), 4.24 (1H, dd, $J=6.1$, 12.0 Hz, H-1), 4.39 (1H, m, H-2), 4.90 (1H, ddd, $J=5.4$, 5.4, 7.8 Hz, H-3), 5.81 (1H, m, NH).

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