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*)-docosasphingatrienine. 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 $CHCl_3$–MeOH extract (72.2 g) of the dried bodies (1 kg) of the leech (sold as a crude drug "Suitetsu")[3] was treated with $CHCl_3$–MeOH–H_2O (1 : 1 : 1), and the lower phase was concentrated to give a total lipid fraction. It was subjected to silica gel and Cosmosil 75C_{18}-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*)-sphingenine,[2b] and **6** as *N*-tetracosanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)phosphinate-β-D-galactopyranosyl]-(4*E*)-17-methylsphingenine[1] by comparison of the physical and spectroscopic data with those of corresponding authentic samples.

The ^{13}C-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 ^{1}H-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–CH_2–CH=CH–) group.

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

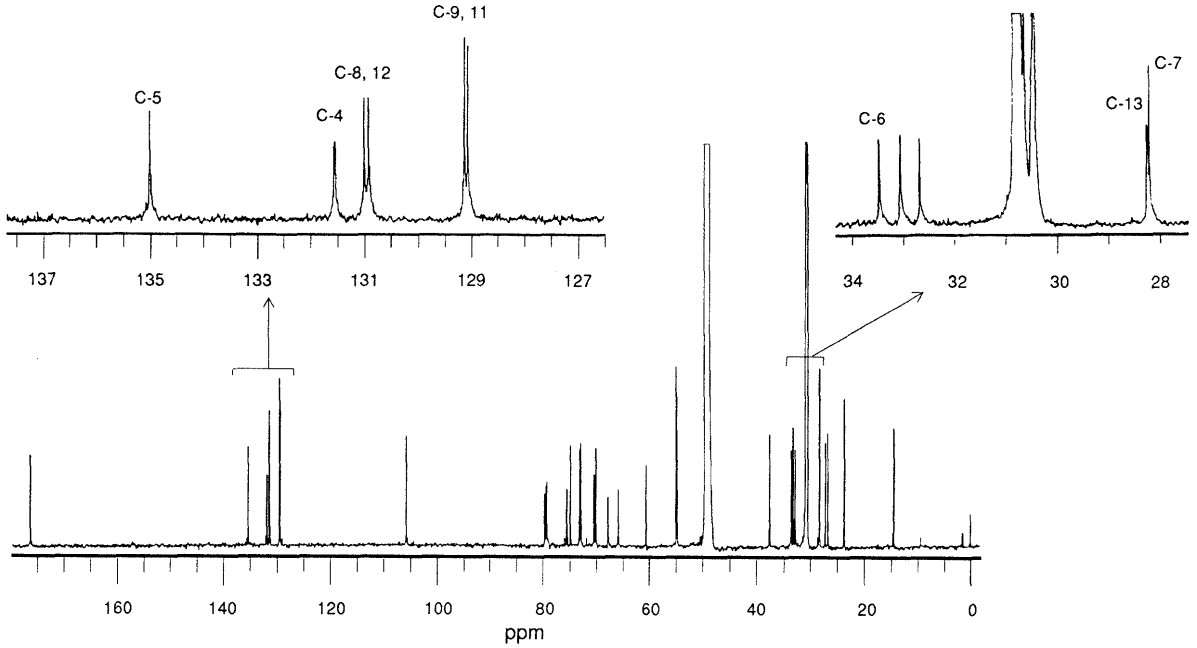

Fig. 1. ^{13}C-NMR Spectrum of **1** (150 MHz, CD_3OD : $CDCl_3$ = 4 : 1)

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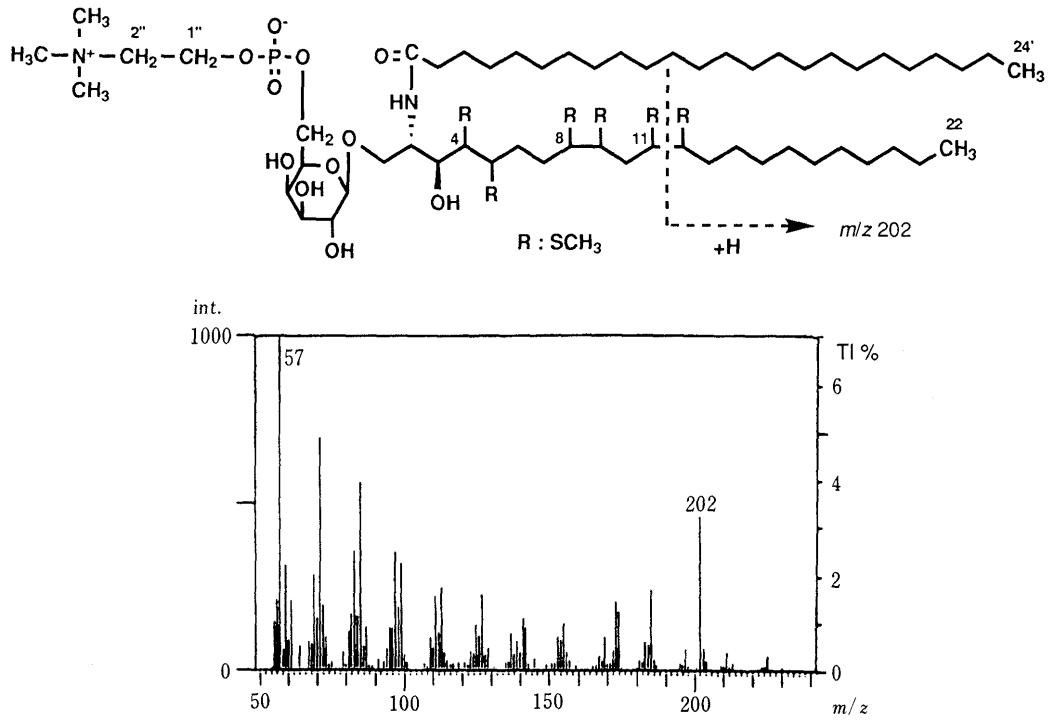

Fig. 2. EI-MS of the Dimethyl Disulfide Derivative of **1**

matography (GC), electron impact mass spectrometry (EI-MS) and ^{1}H-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 $C_{22:3}$ tri-unsaturated carbon chain.

The dimethyl disulfide derivative of **1** gave a fragment ion peak at *m/z* 202 $[C_{12}H_{25}S+H]^+$, 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 (^{1}H–^{1}H and ^{13}C–^{1}H COSY) and two-dimensional nuclear Overhauser effect spectroscopy (NOESY) experiments. The ^{1}H–^{1}H COSY spectrum exhibited a series of correlation peaks from H-3 to H_2-13, showing the locations of three double bonds (C4, C8 and C11). Furthermore, in the NOESY spectrum, the signals of the methylene (H_2-10) lying between two double bonds (C8 and C11) were correlated with those of H_2-7 and H_2-13, and an nuclear Overhauser effect (NOE) correlation between H-4 and H_2-6 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), supported[5] 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

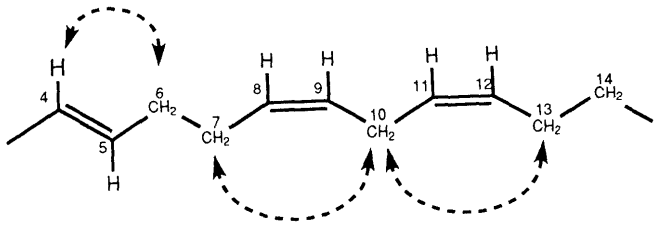

Fig. 3. NOE Correlations of **1**

over palladium carbon of **1** followed by methanolysis gave a saturated LCB, which was acetylated to give an LCB (docosasphinganine) triacetate (**1a**). When the ^{1}H-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 H_2-1, 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 ^{1}H-NMR spectrum to that of **1**, while in the FAB-MS, the $[M+H]^+$ 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

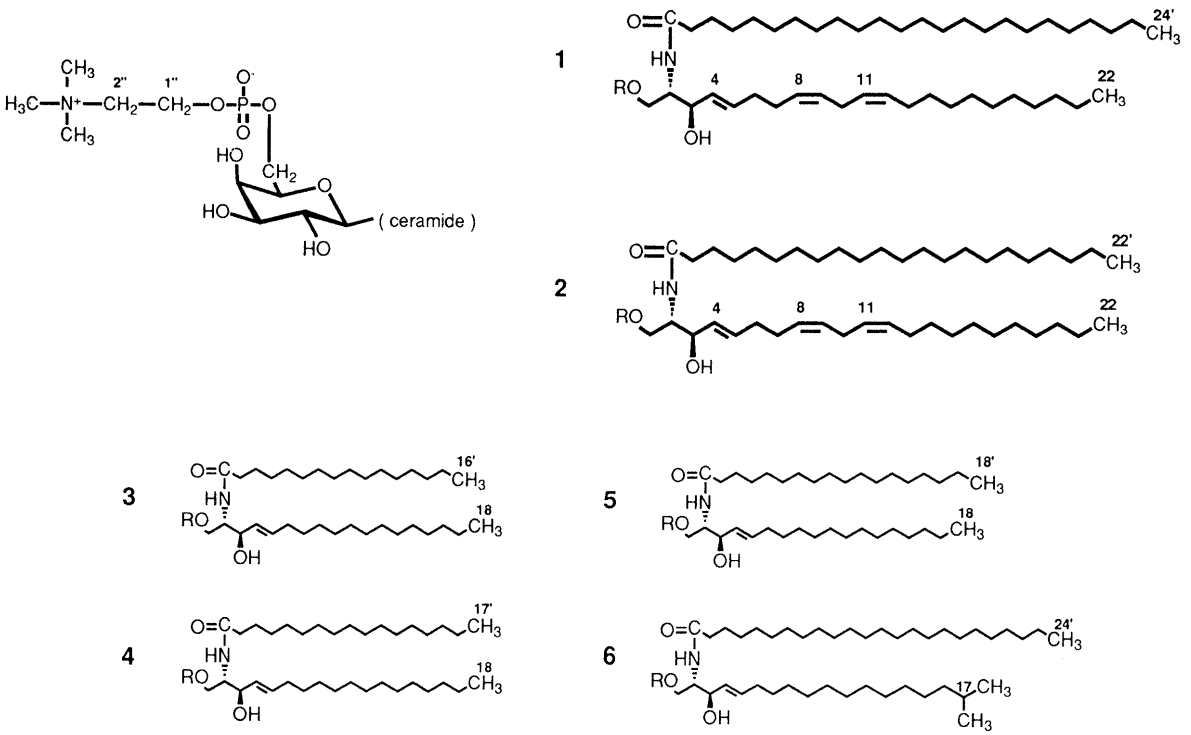

Fig. 4. Structures of Compounds **1**—**6**

characterized as *N*-docosanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)phosphinate-*β*-D-galactopyranosyl]-(4*E*,8*Z*,11*Z*)-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 methanolyzed mixture of the glycolipids fraction of the starfish, *Asterias amurensis*.[7] In regard to intact glycosphingolipids with a tri-unsaturated LCB unit, only four glycocerebrosides 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 4*E*,8*E*,10*E*. The two compounds (**1** and **2**) isolated here are the first examples of AGSLs with a $C_{22:3}$ LCB, (4*E*,8*Z*,11*Z*)-docosasphingatrienine unit.

Experimental

The NMR spectra were recorded on a GE NMR OMEGA 600 instrument at 600 MHz (^{1}H) 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 75C_{18}-OPN (Nacalai Tesque). Preparative HPLC was conducted over an L-column octadecyl silica (ODS) (10 mm × 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 (55.6 g). It was placed on a silica gel column and eluted successively with $CHCl_3$–MeOH (8 : 2→7 : 3)→$CHCl_3$–MeOH–H_2O (7 : 3 : 0.5→6 : 4 : 1→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 reagent[9] 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 75C_{18}-OPN column using MeOH→$CHCl_3$–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 1. ^{1}H-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.

Table 2. ^{13}C-NMR Chemical Shifts (δ) of **1** and **2** (CD_3OD : $CDCl_3$ = 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° (c=0.39, MeOH : $CHCl_3$ = 1 : 1). Positive ion FAB-MS m/z: 1030 $(M+H)^+$. ^{1}H-NMR δ: Table 1. ^{13}C-NMR δ: Table 2. **2**: mp 159—172 °C, $[\alpha]_D^{26}$ +8.62° (c=0.58, MeOH : $CHCl_3$ = 1 : 1). Positive ion FAB-MS m/z: 1002 $(M+H)^+$. ^{1}H-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*)-sphingenine, and **6** as *N*-tetracosanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)phosphinate-β-D-galactopyranosyl]-(4*E*)-17-methylsphingenine 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, ^{1}H-NMR and GC (fused silica capillary column Bonded MPS-50, Quadrex, 0.25 mm × 50 m; column temperature, 230 °C (hold, 12 min)→ 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^+). ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, t, J=7.0 Hz, H_3-22), 1.21—1.72 (–C$\underline{H}_2$–), 2.30 (2H, t, J=7.0 Hz, H_2-2), 3.66 (3H, s, OC$\underline{H}_3$). Methyl *n*-tetracosanoate: EI-MS m/z: 382 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 0.90 (3H, t, J=7.0 Hz, H_3-24), 1.23—1.72 (–C$\underline{H}_2$–), 2.29 (2H, t, J=7.0 Hz, H_2-2), 3.65 (3H, s, OC$\underline{H}_3$).

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 methanolyzed 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° (c=0.13, $CHCl_3$). Positive ion FAB-MS m/z: 484 $[M+H]^+$. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz, H_3-22), 1.26—1.75 (–C$\underline{H}_2$–), 1.99, 2.06, 2.07 (each, s, OCOC$\underline{H}_3$ × 2 and NHCOC$\underline{H}_3$), 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, N$\underline{H}$).

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