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Sex Pheromones of the Hair Crab *Erimacrus isenbeckii*. Part 1: Isolation and Structures of Novel Ceramides

Naoki Asai,[a] Nobuhiro Fusetani,[a,*] Shigeki Matsunaga[a] and Jun Sasaki[b]

[a]*Laboratory of Aquatic Natural Products Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan*

[b]*Hokkaido Abashiri Fisheries Experimental Station, Abashiri, Hokkaido 099-3319, Japan*

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Abstract—Possible sex pheromones of the brachyuran crab *Erimacrus isenbeckii* have been isolated by using a unique sponge assay from the water in which postmolt females had been maintained. Their structures were elucidated to be new ceramides by spectroscopic and chemical degradation studies.

Introduction

Sex pheromones mediate mating behaviors of conspecifics of the opposite sex. They have been well documented in terrestrial animals, particularly insects. By comparison, sex pheromones of crustaceans have received relatively little attention. Some crustacean females, however, are known to emit pheromones that elicit precopulatory behavior in conspecific males, including mate searching, courtship display, and guarding.[1–4] In many marine brachyuran crabs, the male detects the premolt female, grasps and guards her under his abdomen until molting.[5] Mating takes place shortly after ecdysis. Sex pheromones released into the female urine elicit the precopulatory behaviors in males.[2–4] However, no crustacean sex pheromones have been fully elucidated.

The hair crab *Erimacrus isenbeckii*, a commercially important species exhibits a series of typical mating behaviors; precopulatory guarding (male grasps female), molting of female, copulation, and postcopulatory guard. The involvement of a pheromone released from pre- and postmolt females in these behavior was shown by 'sponge assay'.[5] A sexually competent male exhibits the typical mating behaviors toward a bath sponge containing water conditioned with pre- or postmolt females.

By using this unique assay system, we attempted to isolate the pheromone from aquarium water inhabited by pre- and postmolt females, which resulted in isolation of 8 new ceramides; a mixture of ceramides elicited the guard behavior in males. This paper describes isolation and structure elucidation of these ceramides (Fig. 1).

Results and Discussion

The water from an aquarium in which postmolt females (stage of molting cycle: A_1–A_2) were maintained was passed through a column packed with polystyrene gel (TSK-G3000S). The column was washed with water and successively eluted with H_2O/EtOH solvent systems. The fraction eluted with 100% EtOH which induced the guard behavior in male crabs in the sponge assay was fractionated by ODS flash chromatography (aq MeOH) followed by silica gel HPLC ($CHCl_3$/MeOH, 95:5) to obtain an active substance which showed a single peak in the silica gel HPLC and elicited guard behavior in male hair crabs at a dose of 2.0 mg/sponge. This substance was never detected in the water from a tank in which intermolt females (not attractive to males) were kept.

The active substance appeared to be pure judging from the

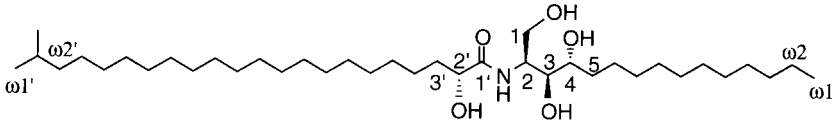

Figure 1. Structure of a hair crab ceramide (**1**).

Keywords: marine metabolites; biologically active compounds; pheromones; lipids.

* Corresponding author. Tel.: +81-3-5841-5299; fax: +81-3-5841-8166; e-mail: anobu@mail.ecc.u-tokyo.ac.jp

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Table 1. ^{1}H and ^{13}C NMR data for the hair crab ceramide ($CDCl_3/CD_3OD$ 1:1) (data were obtained for the mixture of analogues)

Position	δ_H (mult., J=Hz)	δ_C
1	3.76 (1H, dd, 11.4, 4.5)	61.5
	3.70 (1H, dd, 11.4, 4.7)	
2	4.08 (1H, ddd, 4.7, 4.5, 3.8)	52.0
3	4.00 (1H, dd, 8.0, 3.8)	72.5
4	3.50 (1H, m)	75.7
5	1.65 (1H, m)	33.0
	1.52 (1H, m)	
6-ω3	1.50-1.10 (brm)	
ω2	1.25 (2H, m)	24.0
ω1	0.84 (3H, t, 6.7)	14.2
1′		176.5
2′	3.50 (1H, m)	72.7
3′	1.76 (1H, m)	34.8
	1.57 (1H, m)	
4′-ω3′	1.50–1.10 (brm)	
ω2′[a]	1.48 (1H, m)	29.5
ω1′[a]	0.82 (6H, d, 6.6)	22.7

[a] Data for *iso*-form terminus.

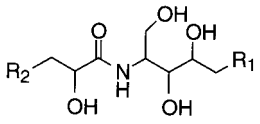

Figure 2. Partial structure A.

^{1}H NMR spectrum. However, the FAB mass spectrum revealed multiple parent ions separated by 14 amu at *m/z* 628, 642, 656, and 670, thus indicating that the active substance was a mixture of homologous compounds different in their aliphatic portions. The major molecular peak at *m/z* 642 matched $C_{39}H_{79}NO_5$ (*m/z* 642.5977, $[M+H]^+$, Δ −5.9 mmu) in HRFABMS data. The ^{1}H NMR spectrum exhibited a large methylene envelope (δ 1.50–1.10) and terminal methyl signals (δ 0.84 and 0.82) indicating the presence of long alkyl chains (Table 1). Relatively deshielded six proton signals at δ 4.08, 4.00, 3.76, 3.70, 3.50, and 3.50 were assigned to three oxygenated methine protons, a pair of oxymethylene protons, and a nitrogen-bearing methine proton, respectively, from analysis of the HMQC spectrum. Concomitant analysis of the COSY, HMQC, and HMBC spectra led to partial structure A (Fig. 2). In addition to the partial structure A, there were signals for long methylene chains and terminal methyl groups, thereby suggesting that the pheromone was a mixture of ceramides consisting of phytosphingosines and 2-hydroxy fatty acids.

The ceramide mixture was then methanolyzed according to the method of Gaver–Sweeley[6] to afford long chain bases (LCBs) and methyl esters of 2-hydroxy fatty acid (FAMs). LCBs were acetylated with acetic anhydride and pyridine to furnish triacetate monoacetamides. The ^{1}H NMR spectrum of the triacetate monoacetamides exhibited a triplet methyl signal at δ 0.86 (3H, t, J=7.0 Hz), suggesting the unbranched nature of LCBs. Absolute stereochemistry of LCBs was determined as 2*S*,3*S*,4*R* by comparing ^{1}H NMR and optical rotation data of the triacetate monoacetamides with those of four authentic diastereomers.[7] The terminal structures of FAMs are mainly isopropyl, while the existence of small amounts of primary methyl group was detected in the ^{1}H NMR spectrum. The 2*R*-stereochemistry was determined from the negative Cotton effect at 215 nm in the CD spectrum of FAMs.[8]

In order to determine composition of the ceramides, the mixture was further separated by ODS-HPLC to yield eight peaks, ceramides A to H (Fig. 3). The structure of each ceramide was then determined by a combination of FAB-MS/MS and ^{1}H NMR experiments.

The molecular formula of ceramide A (**1**) was established to be $C_{38}H_{77}NO_5$ from pseudomolecular ions at *m/z* 628 $[M+H]^+$ and 650 $[M+Na]^+$ in the FAB mass spectrum. In FAB-MS/MS experiments (Fig. 4), the $[M+Na]^+$ ion species gave an intense fragment ion at *m/z* 298, from which the size of sphingosine and fatty acid moieties were established as C_{15} and C_{23}, respectively. In addition to the primary methyl proton signal of sphingosine terminus (δ 0.86, 3H, t, J=6.6 Hz), a characteristic proton signal of an isopropyl group (δ 0.84, 6H, d, J=6.6 Hz) was observed. Thus, ceramide A (**1**) was (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxy-21-methyldocosanoylamino]-1,3,4-pentadecanetriol.

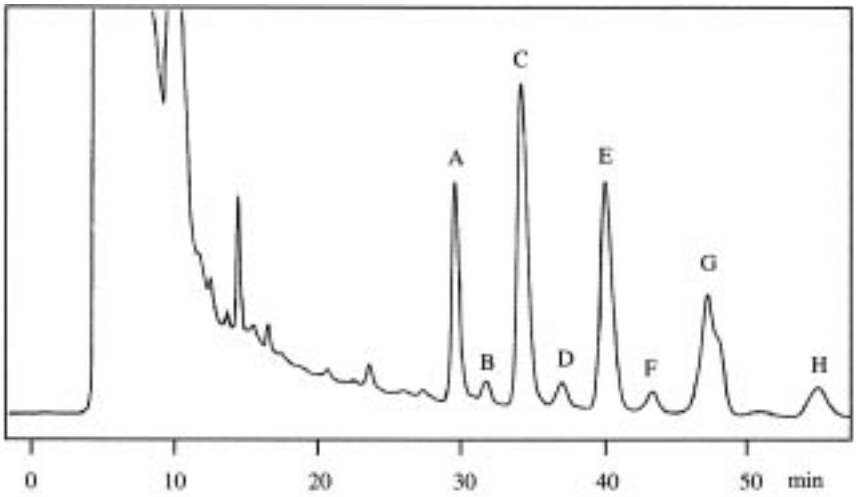

Figure 3. Reverse phase HPLC chromatogram on the hair crab ceramide.

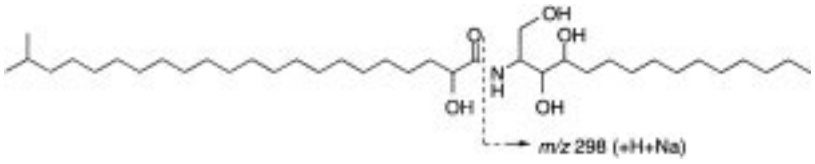

Figure 4. Fragment ion observed for **1** in FAB-MS/MS.

The FAB mass spectrum indicated that ceramides in fraction B had the same molecular formula as **1** ($C_{38}H_{77}NO_5$). In the ^{1}H NMR spectrum, an overlapping primary methyl signal (δ 0.86, 6H, t, J=6.9 Hz) of both sphingosine and fatty acid termini was observed. FAB-MS/MS data showed two fragment peaks at *m*/*z* 298 and 312, which indicated that ceramide B was a mixture of C_{15}/C_{24} and C_{16}/C_{23} sphingosine/fatty acid combinations in a ratio of approximately 5:6. Therefore, this fraction was composed of (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxytetracosanoylamino]-1,3,4-pentadecanetriol (**2**) and (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxytricosanoyl amino]-1,3,4-hexadecanetriol (**3**).

Similarly, structures of ceramides C (**4**, **5**) to H (**12**, **13**) were determined. Structures and relative abundance of the hair crab ceramides are summarized in Table 2. Ceramides A (**1**), F (**10**), and G (**11**) were composed of single components, while ceramides B (**2**, **3**), C (**4**, **5**), D (**6**, **7**), E (**8**, **9**), and H (**12**, **13**) were inseparable mixtures of two compounds having the same molecular formulas. The relative abundance of **1**–**13** (Table 2) was roughly estimated from integration of HPLC peaks and relative intensity of FAB-MS/MS ion peaks.

Ceramides of marine invertebrates are generally composed of branched phytosphingosines and linear fatty acids, whereas the mode of branching in a majority of the ceramides obtained in this study is reversed. There have been only a few report on ceramides consisting of linear phytosphingosines and branched fatty acids.[9] In the sponge assay, four of the five males exhibited guard or copulatory behavior to sponges treated with the isolated ceramide mixture. Although the response was less distinctive as compared to a positive control (postmolt female water), detection of significant amount of ceramides in the 'sexually attractive water' implies their potential role in the reproductive events of *E. isenbeckii*.

Table 2. Structures of ceramide components

Fraction[a]		Sphingosine/fatty acid combination[b]	Relative abundance (%)
A	**1**	*n*-$C_{15:0}$/*i*-$C_{23:0}$	18
B	**2**	*n*-$C_{15:0}$/*n*-$C_{23:0}$	2
	3	*n*-$C_{16:0}$/*n*-$C_{22:0}$	2
C	**4**	*n*-$C_{15:0}$/*i*-$C_{23:0}$	12
	5	*n*-$C_{16:0}$/*i*-$C_{22:0}$	16
D	**6**	*n*-$C_{15:0}$/*n*-$C_{25:0}$	1
	7	*n*-$C_{16:0}$/*n*-$C_{25:0}$	2
E	**8**	*n*-$C_{15:0}$/*i*-$C_{25:0}$	6
	9	*n*-$C_{16:0}$/*i*-$C_{24:0}$	15
F	**10**	*n*-$C_{16:0}$/*n*-$C_{25:0}$	2
G	**11**	*n*-$C_{16:0}$/*i*-$C_{25:0}$	19
H	**12**	*n*-$C_{16:0}$/*i*-$C_{26:0}$	2
	13	*n*-$C_{17:0}$/*i*-$C_{25:0}$	4

[a] Corresponding to each peak in Fig. 3.
[b] *n*-$C_{15:0}$ /*i*-$C_{23:0}$ indicates this ceramide consists of C_{15} straight chain saturated sphingosine and C_{23} branched chain (isopropyl terminus) saturated fatty acid.

Experimental

General

NMR spectra were recorded either on a Bruker AC-300 (300 MHz for ^{1}H, 75 MHz for ^{13}C), a JEOL JMN-α600 (600 MHz for ^{1}H, 150 MHz for ^{13}C), or a JEOL JMN-α500 (500 MHz for ^{1}H, 125 MHz for ^{13}C) NMR spectrometer at 303 K. ^{1}H chemical shifts were referenced to residual solvent peaks: δ 7.24 for $CHCl_3$ in $CDCl_3$; δ 3.30 for CD_2HOD in CD_3OD; δ 3.30 for CD_2HOD in $CDCl_3$/CD_3OD (1:1). ^{13}C chemical shifts were referenced to ^{13}C signals of $CDCl_3$ (δ 77.0) or CD_3OD (δ 49.0). FAB mass spectra were measured on a JEOL JMX-SX102/SX102 tandem mass spectrometer. Glycerol or *m*-nitrobenzyl alcohol (NBA) was used as a matrix.

Collection and maintenance of crabs

Individuals of *E. isenbeckii* were collected off Kushiro, Hokkaido by a ground net or crab-pots. The stock male and female crabs were maintained in separate tanks with recirculating seawater system at 8±2°C, and fed on pieces of shrimp. Premolt and postmolt females were kept in separate tanks with recirculating seawater system and were not fed.

Pheromonal assay

Test solutions, which were prepared by dissolving samples either in ether, EtOH, H_2O, or a combination of solvents (2 mL), were pipetted onto a ball-shaped bath sponge (7–12 cm in diameter). The sponge was air-dried and placed in a beaker containing 200 mL of seawater. Well-soaked sponges were picked up with a pair of tweezers and placed in front of test male crabs. Males were either placed together in a 1,000 L tank or individually in a small aquarium, and the behaviors of males were recorded for several min. The criteria used in defining sexual responses in test males were as follows: (1) Guard behavior—a male grasped and then held a sponge against his ventral side (carrying position) with the chelae and first walking legs. If a sponge was picked up only by the chelae and not held tightly, this response was evaluated as 'weak guard behavior'. (2) Copulatory behavior—a male grasped and held a sponge tightly below his abdomen with the chelae and first to third walking legs. The abdomen was opened and the copulatory appendages are inserted into the sponge. Each test sample was

examined on five male crabs. Water in which premolt or postmolt females had kept was used as a positive control.

Purification of the ceramide

Ten individuals of postmolt female crabs (stages of molting cycle: A_1–A_2) were kept in a 57 L aquarium for several days. The aquarium water (12.6 L) was passed through TSK G3000S column (1000 cm^3); no activity was found in the effluent. The polystyrene gel was washed with three void volumes of water to remove salts, and eluted with a solvent series of 30% EtOH, 50% EtOH, 70% EtOH, 100% EtOH, and $CHCl_3$/MeOH/H_2O (7:3:0.5). The 100% EtOH fraction, which exhibited pheromonal activity, was subjected to ODS flash chromatography (aq MeOH, stepwise gradient). The fraction eluted with 100% MeOH was then purified by silica gel HPLC (YMC-Pak, ϕ 1.0 × 25 cm, $CHCl_3$/MeOH 95:5) to afford an active ceramide mixture (7.7 mg). A portion of the ceramide mixture (1.0 mg) was separated by reversed phase HPLC (Cosmosil-MS, ϕ 0.46 × 25 cm, MeOH) to yield ceramides A–H (each fraction <0.1 mg).

Ceramide mixture

^1H and ^{13}C NMR, see Table 1; (pos.) FABMS (glycerol) *m/z* (relative intensity) 670 (7), 656 (14), 642 (20), 628 (11); (pos.) HRFABMS (glycerol) *m/z* 642.6036 calcd for $C_{39}H_{80}NO_5$ $[M+H]^+$ found 642.5977.

Ceramide fraction A: (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxy-21-methyldocosanoylamino]-1,3,4-pentadecanetriol (1). ^1H NMR (600 MHz, $CDCl_3$) δ 0.86 (3H, t, 6.9), 0.84 (6H, d, 6.5), other signals were identical with those for the ceramide mixture. This is also true for **2**–**12**; (pos.) FABMS (NBA/NaCl) *m/z* (relative intensity, %) 650 $[M+Na]^+$ (28), 628 $[M+H]^+$ (11); (pos.) FAB-MS/MS (NBA/NaCl) *m/z* 650.4 $[M+Na]^+$, 298.3 $[C_{15}H_{32}NO_3+H+Na]^+$.

Ceramide fraction B: (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxytetracosanoylamino]-1,3,4-pentadecane-triol (2) and (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxytricosanoylamino]-1,3,4-hexadecanetriol (3). ^1H NMR (600 MHz, $CDCl_3$) δ 0.86 (6H, t, 7.1); (pos.) FABMS (NBA/NaCl) *m/z* (relative intensity, %) 650 $[M+Na]^+$ (32), 628 $[M+H]^+$ (10); (pos.) FAB-MS/MS (NBA/NaCl) *m/z* (relative intensity, %) 650.3 $[M+Na]^+$, 312.3 $[C_{16}H_{34}NO_3+H+Na]^+$ (5.7), 298.4 $[C_{15}H_{32}NO_3+H+Na]^+$ (4.9).

Ceramide fraction C: (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxy-22-methyltricosanoylamino]-1,3,4-pentadecanetriol (4) and (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxy-21-methyldocosanoylamino]-1,3,4-hexadecanetriol (5). ^1H NMR (600 MHz, $CDCl_3$) δ 0.86 (3H, t, 6.9), 0.84 (6H, d, 6.9); (pos.) FABMS (NBA/NaCl) *m/z* (relative intensity, %) 664 $[M+Na]^+$ (16), 628 $[M+H]^+$ (4); (pos.) FAB-MS/MS (NBA/NaCl) *m/z* (relative intensity, %) 664.4 $[M+Na]^+$, 312.3 $[C_{16}H_{34}NO_3+H+Na]^+$ (42), 298.4 $[C_{15}H_{32}NO_3+H+Na]^+$ (30).

Ceramide fraction D: (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxypentacosanoylamino]-1,3,4-pentadecanetriol (6) and (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxytetracosanoylamino]-1,3,4-hexadecanetriol (7). ^1H NMR (600 MHz, $CDCl_3$) δ 0.86 (6H, t, 6.9); (pos.) FABMS (NBA/NaCl) *m/z* (relative intensity, %) 664 $[M+Na]^+$ (22), 642 $[M+H]^+$ (5); (pos.) FAB-MS/MS (NBA/NaCl) *m/z* (relative intensity, %) 664.4 $[M+Na]^+$, 312.3 $[C_{16}H_{34}NO_3+H+Na]^+$ (5.8), 298.3 $[C_{15}H_{32}NO_3+H+Na]^+$ (3.8).

Ceramide fraction E: (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxy-23-methyltetracosanoylamino]-1,3,4-pentadecanetriol (8) and (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxy-22-methyltricosanoylamino]-1,3,4-hexadecanetriol (9). ^1H NMR (600 MHz, $CDCl_3$) δ 0.87 (3H, t, 6.9), 0.84 (6H, d, 6.5); (pos.) FABMS (NBA/NaCl) *m/z* (relative intensity, %) 678 $[M+Na]^+$ (18), 656 $[M+H]^+$ (2); (pos.) FAB-MS/MS (NBA/NaCl) *m/z* (relative intensity, %) 678.4 $[M+Na]^+$, 312.3 $[C_{16}H_{34}NO_3+H+Na]^+$ (32), 298.4 $[C_{15}H_{32}NO_3+H+Na]^+$ (14).

Ceramide fraction F: (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxypentacosanoylamino]-1,3,4-hexadecanetriol (10). ^1H NMR (600 MHz, $CDCl_3$) δ 0.86 (6H, t, 6.9); (pos.) FABMS (NBA/NaCl) *m/z* (relative intensity, %) 678 $[M+Na]^+$ (12), 656 $[M+H]^+$ (2.5); (pos.) FAB-MS/MS (NBA/NaCl) *m/z* 678.4 $[M+Na]^+$, 312.4 $[C_{16}H_{34}NO_3+H+Na]^+$.

Ceramide fraction G: (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxy-23-methyltetracosanoylamino]-1,3,4-hexadecanetriol (11). ^1H NMR (600 MHz, $CDCl_3$) δ 0.86 (3H, t, 6.9), 0.84 (6H, d, 6.5); (pos.) FABMS (NBA/NaCl) *m/z* (relative intensity, %) 692 $[M+Na]^+$ (10), 670 $[M+H]^+$ (1); (pos.) FAB-MS/MS (NBA/NaCl) *m/z* 692.5 $[M+Na]^+$, 312.3 $[C_{16}H_{34}NO_3+H+Na]^+$.

Ceramides fraction H: (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxy-24-methylpentacosanoylamino]-1,3,4-hexadecanetriol (12) and (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxy-23-methyltetracosanoylamino]-1,3,4-heptadecanetriol (13). ^1H NMR (600 MHz, $CDCl_3$) δ 0.86 (3H, t, 6.9), 0.84 (6H, d, 6.9); (pos.) FABMS (NBA/NaCl) *m/z* (relative intensity, %) 706 (6), 692 (4), 660 (8), 638 (11); (pos.) FAB-MS/MS (NBA/NaCl) *m/z* (relative intensity, %) 706.4 $[M+Na]^+$, 326.3 $[C_{17}H_{36}NO_3+H+Na]^+$ (4.8), 298.4 $[C_{16}H_{34}NO_3+H+Na]^+$ (2.1).

Methanolysis of the ceramide mixture

The ceramide mixture was methanolyzed according to the method of Gaver and Sweeley.[9] Aqueous HCl/MeOH [conc. HCl/MeOH/H_2O, 8.6:9.4:82), 1 mL] was added to the ceramide mixture (1.6 mg) and the mixture was heated for 19 h at 70°C in a screw-capped test tube. The reaction mixture was dried in a nitrogen stream and partitioned between ether and water to afford organic (0.7 mg) and aqueous fractions (1.1 mg).

Esterification of the fatty acids

In the course of methanolysis, 2-hydroxy fatty acids were partially hydrolyzed and not completely esterified. Therefore, the organic layer was methylated with diazomethane. The organic fraction above (1.6 mg) was treated with CH_2N_2 ethereal solution (1 mL). The reaction mixture was concentrated and chromatographed on silica gel (*n*-hexane/

EtOAc, 9:1) to yield fatty acid methyl esters (0.4 mg). CD λ_{max}(MeOH) $[\theta]_{215}$ −794° (tentatively calculated for the molecular weight 397); ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.17 (1H, dd, 7.4, 4.3), 3.77 (3H, s), 1.76 (2H, m), 1.4–1.2 (br), 0.84 (6H, d, 6.6); (neg.)-FABMS (glycerol) *m/z* (relative intensity, %) 397 (60), 383 (97), 369 (95), 339 (44), 325 (68), 311 (67).

Acetylation of the sphingosine

The aqueous phase of the methanolysis product was acetylated with acetic anhydride (0.5 mL) and pyridine (0.5 mL) at rt for 12 h, and the reaction mixture was purified by preparative TLC on silica gel with *n*-hexane/EtOAc, 7:3) to afford phytosphingosine triacetate monoacetamide (0.5 mg). $[\alpha]_D$=+20.0° (c=0.025, $CHCl_3$) (calculated for the molecular weight of 458); ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.94 (1H, d, 9.4), 5.08 (1H, dd, 8.2, 3.0), 4.92 (1H, dt, 9.3, 3.7), 4.45 (1H, m), 4.27 (1H, dd, 11.7, 4.8), 3.98 (dd, 11.7, 3.1), 2.06 (3H, s), 2.03 (6H, s), 2.01 (3H, s), 1.4–1.2 (br), 0.86 (3H, t, 7.0); (pos.) FABMS (glycerol) *m/z* (relative intensity, %) 458 (3), 444 (2.8), 398 (7.5), 384 (6), 236 (5.5), 222 (5).

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