N-Type Calcium Channel Blockers from a Marine Bacterium, *Cytophaga* sp. SANK 71996

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N-(3-Acyloxyacyl)glycines were isolated as N-type calcium channel blockers from a marine bacterium *Cytophaga* sp. SANK 71996. The identification and fermentation of the producing strain and structure characterization of *N*-(3-acyloxyacyl)glycines by spectral analyses and chemical syntheses are described together with their antagonistic activities.

Intracellular calcium ion plays an important role in the expression of many physiological functions in cells. The increase of Ca^{2+} concentration mainly depends on the release of Ca^{2+} from intracellular cytoplasmic reticula and the influx of extracellular Ca^{2+} *via* voltage-dependent calcium channels on cell membranes, which are classified as T-, L-, N-, Q-, or P-type calcium channels based on their ligand affinities, activation and inactivation potentials, inactivation rates, *etc.* In the nervous system, the N-type calcium channels[1] with irreversibly potent and selective affinity release and transmembrane signalling. Therefore selective inhibitors thereof may be useful for the prevention and treatment of neuropathy.

In an attempt to obtain N-type calcium channel blockers, we screened the extracts of marine organisms for inhibition against the specific binding of ω-conotoxin to calcium channels[2], and found that a lipophilic mycelial extract of a marine bacterium, *Cytophaga* sp., produced some active compounds. We herein report the taxonomy of *Cytophaga* sp. and the fermentation, isolation, characterization, chemical syntheses, and structure-activity relationship of active principles, *N*-(3-acyloxy)acylglycines (**1a*** ~ **c***).

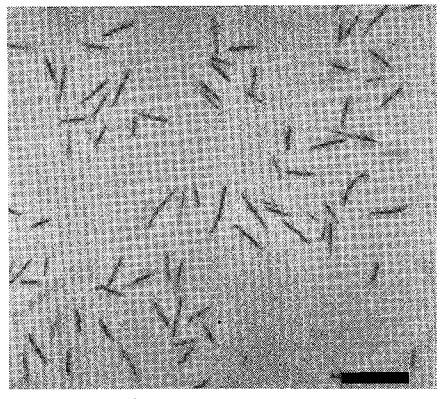
Identification of Producing Strain

The producing strain SANK 71996 was isolated from seawater collected at Ukusu, Kamo-mura, Shizuoka Pref., Japan. SANK 71996 was non-spore forming, Gram-negative, rod-shaped, 0.3 ~ 0.4 μm in diameter, and 7 ~ 12 μm long (Fig. 1). It produced a water-insoluble pink to salmon pigment in the cells. It required sea water for growth and moved by sliding, but did not produce resting cells. It produced catalase and oxidase, but did not oxidize glucose under either aerobic or anaerobic conditions. Its DNA base composition was 40.9 mol% G+C. Its isoprenoid quinone was menaquinone MK-7. Based upon these phenotypic and chemotaxonomical characteristics, the strain SANK 71996 was identified as *Cytophaga* sp.[3].

Fermentation and Isolation

Cytophaga sp., SANK 71996, was cultured at 23°C for 48 hours in a tank fermenter. The cells filtered from the culture broth, were extracted with acetone at room temperature. After removal of acetone, the residue was extracted with EtOAc. The EtOAc extract was fractionated by silica gel chromatography followed by HPLC of the UV-active derivatives of the active principles and acidic hydrolysis, because the active compounds could not be monitored effectively even by UV absorption at

Fig. 1. Cells of the producing strain SANK 71996 on marine agar, 27°C, 48 hours (bar = 10 μm).



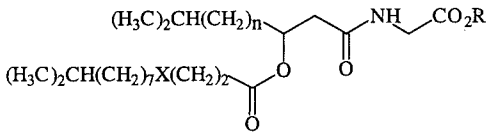
210 nm in HPLC. They were therefore esterified by diphenyldiazomethane to a mixture of UV-active benzhydryl esters (**2a*** ~ **c***), which was separated by HPLC. The purified esters were easily hydrolyzed by trifluoroacetic acid (TFA) to two novel compounds (**1a***, **b***) and a known compound (**1c***), respectively.

Structural Elucidation

Compound **1a***, colorless leaflets, mp 71 ~ 72°C, $[\alpha]_D^{25}$ +0.45° (*c* 7.92, $CHCl_3$), was analyzed for $C_{34}H_{63}NO_5$ by elemental analysis and HR-FAB-MS ([M^+ + Na] *m/z* 588.4591, Δ −1.3 mmu). In the ^1H and ^{13}C NMR spectra (Fig. 3), two isopropyls [δ_H 0.86 (12H, d, J=6.7 Hz, 1.51 (2H, m); δ_C 22.7 (4×q), 28.0 (2×d)] were observed as well as many methylenes characteristic of branched fatty acids. The amide proton [δ 6.39 (1H, br t, J=5.2 Hz)] coupled to methylene protons [δ 4.08 (2H, d, J=5.2 Hz)] adjacent to a carboxyl [δ_C 172.6 (s)], constituted a glycine moiety (–$NHCH_2CO_2H$). The glycine moiety was confirmed by a fragment ion (**III**: $[NH_2CH_2CO_2]^-$ *m/z* 74) in the negative FAB-MS/MS. The proton [δ_H 5.17 (1H, m)] at a carbon [δ_C 71.4 (d)] bearing an acyloxy substituent, coupled to two methylenes [δ_H 1.63 (2H, d, J=6.7 Hz), 2.54 (2H, m); δ_C 34.2 (t), 41.3 (t)]. The HMBC experiment showed the presence of a –CH_2CH-(OCOR)CH_2CONH– group with R=–CH_2CH_2CH=$CHCH_2R'$. The geometry of the double bond was *Z* considering the NOE and coupling constant (J=11.2 Hz) between the olefinic protons. The other partial structures could not be determined from the NMR data.

In the negative FAB-MS, two other diagnostic fragment ions [**Ia** (*m/z* 324), **IIa** (*m/z* 239)] were observed, whose negative FAB-MS/MS spectrum showed two sets

Fig. 2. The structures of *N*-(3-Acyloxyacyl)glycines.



1a*: R=H; X=*cis*-CH=CH–; n=11.
1b*: R=H; X=*cis*-CH=CH–; n=10.
1c*: R=H; X=–CH_2CH_2–; n=11.
2a*: R=benzhydryl; X=*cis*-CH=CH–; n=11.
2b*: R=benzhydryl; X=*cis*-CH=CH–; n=10.
2c*: R=benzhydryl; X=–CH_2CH_2–; n=11.

Fig. 3. The structure of **1a** correlated by HMBC and NOESY.

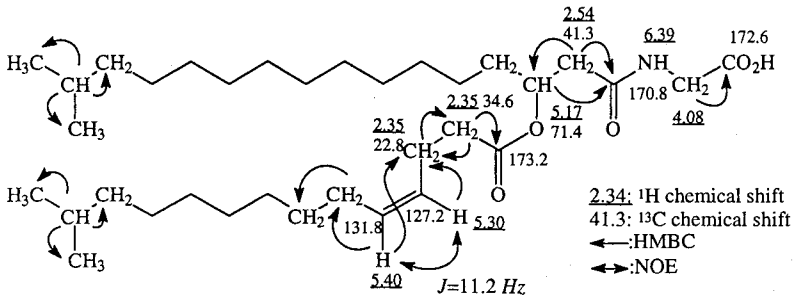
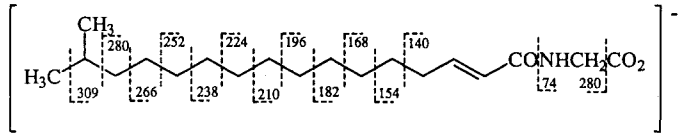
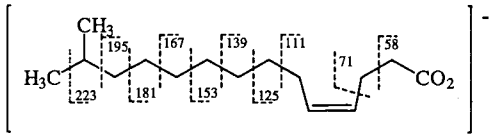


Fig. 4. The fragment ions in negative FAB-MS of **1a*** ~ **1c***.



Ia:*m/z* 324



IIa:*m/z* 239

$[(CH_3)_2CH(CH_2)_{10}CH{=}CHCONHCH_2CO_2]^-$ **Ib**: *m/z* 310

$[(CH_3)_2CH(CH_2)_{11}CO_2]^-$ **IIc**: *m/z* 241

of reasonable fragment ions from **Ia** and **IIa** shown in Fig. 4. The absence of *m/z* 294 and 209 in respective **Ia** and **IIa**, was consistent with the branched fatty acid moieties.

Alkaline hydrolysis clove **1a*** to (−)-*N*-(3-hydroxy-15-methylhexadecanoyl)glycine (**3***) and methyl 13-methyl-4*Z*-tetradecenoate (**4**). The former was identified by unambiguous synthesis of its racemate and comparison of optical rotations of **3a*** ($[\alpha]_D^{25}$ −6.3° [*c* 0.75, MeOH]) and *R*-(−)-*N*-(3-hydroxymyristoyl)glycine (**3k***: $[\alpha]_D^{25}$ −7.0° [*c* 1.0, MeOH]) prepared from commercially available *R*-(−)-3-hydroxymyristic acid. The latter was also identified by unambiguous synthesis described later. Therefore, **1a*** could be depicted as *R*-(+)-*N*-[15-methyl-3-(13-methyl-4*Z*-tetradecenoyloxy)-hexadecanoyl]glycine, identified by total synthesis.

Compound **1b***, a colorless amorphous solid, $[\alpha]_D^{25}$ −3.4° (*c* 0.87, MeOH), was analyzed for $C_{33}H_{61}NO_5$ by elemental analysis and HR-FAB-MS ($[M+Na]^+$ *m/z* 574.4423, Δ −2.5 mmu). The molecular formula was smaller by CH_2 than that of **1a***. The negative FAB-MS contained the important fragment ions [**Ib** (*m/z* 310), **IIa** (*m/z* 239), and **III** (*m/z* 74)] as well as **1a***. The former fragments were analyzed by negative FAB-MS. The MS data indicated, together with its negative optical rotation, that **1b*** could be depicted as *S*-(−)-*N*-[14-methyl-3-(13-methyl-4*Z*-tetradecenoyloxy)pentadecanoyl]glycine, identified by total synthesis.

Compound **1c*** was identified as *R*-(+)-*N*-[15-methyl-3-(13-methyltetradecanoyloxyhexadecanoyl)]glycine by total synthesis as described later. It has been reported that **1c*** was already isolated from a gliding bacterium, *Cytophaga johnsone*[4], though it was about 80% pure and its optical rotation was unknown. Also, its racemate (**1c**)[5] was chemically prepared previously. Pure **1c*** was first obtained as colorless leaflets.

N-(*β*-Acyloxyacyl)amino acids have been frequently isolated from bacteria. *N*-(*β*-acyloxyacyl)serines, WB-3559A, B, C, and D[6~8], obtained from *Flavobacterium* sp., are potent fibrolytic agents, and *Flavobacterium meningosepticum* produces *N*-(*β*-acyloxyacyl)ornithine[9] and WB-3559D[6~8], which are macrophage activators.

Preparation of *N*-(3-Acyloxyacyl)glycines

In order to identify these natural products and to study the structure-activity relationship against the inhibition of *ω*-conotoxin binding to the N-type calcium channel, many derivatives were prepared using diethyl phosphorocyanidate (DEPC)[10] or *via* a succinimide ester[11].

3-Hydroxy-15-methylhexadecanoic acid (**5a**) was treated with *t*-butyl glycine hydrochloride in the presence of DEPC to give *t*-butyl *N*-(3-hydroxy-15-methylhexadecanoyl)glycine (**7a**) in good yield. **7a** was easily acylated by 13-methyl-4*Z*-tetradecenoyl chloride to a

Fig. 5. The alkaline hydrolysis of **1a***, **1c***.

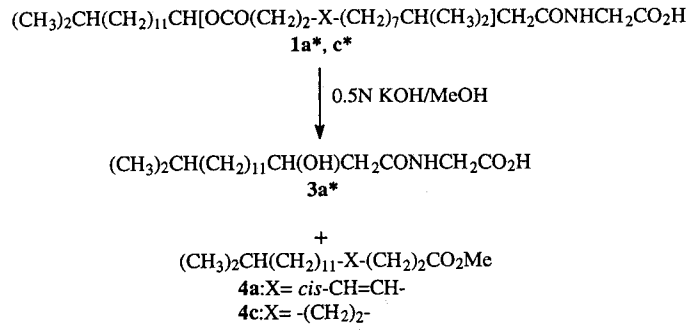


Fig. 6. The Preparation of **1**.

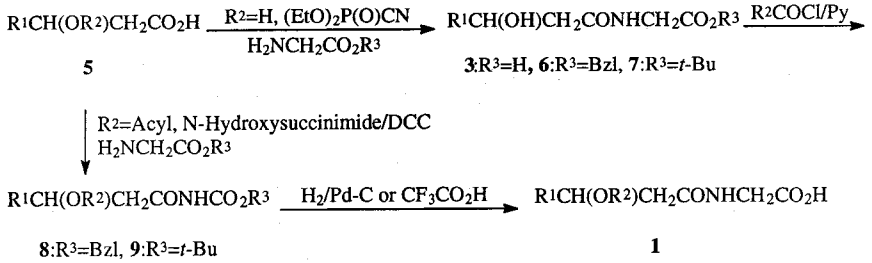


Table 1. The inhibitory activities of *N*-(3-acyloxyacyl)- (**1**) and *N*-(3-hydroxyacyl)glycines (**3**) against the N- and L-type calcium channels.

	Compound (**1**, **3**)		Inhibitory activities (μM)	
	R^1	R^2	N-Type	L-Type
1a*	$(CH_3)_2CH(CH_2)_{11}$	$(CH_3)_2CH(CH_2)_7CH=CH(CH_2)_2CO$	1.8	>100
1a	$(CH_3)_2CH(CH_2)_{11}$	$(CH_3)_2CH(CH_2)_7CH=CH(CH_2)_2CO$	2.5	>100
1b*	$(CH_3)_2CH(CH_2)_{10}$	$(CH_3)_2CH(CH_2)_7CH=CH(CH_2)_2CO$	10.9	>100
1b	$(CH_3)_2CH(CH_2)_{10}$	$(CH_3)_2CH(CH_2)_7CH=CH(CH_2)_2CO$	4.0	>100
1c*	$(CH_3)_2CH(CH_2)_{11}$	$(CH_3)_2CH(CH_2)_{11}CO$	4.9	>100
1c	$(CH_3)_2CH(CH_2)_{11}$	$(CH_3)_2CH(CH_2)_{11}CO$	5.1	>100
1d	$(CH_3)_2CH(CH_2)_{11}$	n-$C_{13}H_{27}CO$	>100	>100
1e	$(CH_3)_2CH(CH_2)_{10}$	$(CH_3)_2CH(CH_2)_{10}CO$	4.0	>100
3b	$(CH_3)_2CH(CH_2)_{10}$	H	>100	>100
1f	n-$C_{14}H_{29}$	$(CH_3)_2CH(CH_2)_{12}CO$	>100	>100
1g	n-$C_{14}H_{29}$	$(CH_3)_2CH(CH_2)_{11}CO$	52.9	>100
1h	n-$C_{14}H_{29}$	$(CH_3)_2CH(CH_2)_{10}CO$	4.7	>100
3f	n-$C_{14}H_{29}$	H	>100	>100
1i	n-$C_{13}H_{27}$	$(CH_3)_2CH(CH_2)_{12}CO$	>100	>100
1j	n-$C_{13}H_{27}$	$(CH_3)_2CH(CH_2)_{11}CO$	>100	>100
3i	n-$C_{13}H_{27}$	H	>100	>100
3k*	(3*R*)-n-$C_{11}H_{23}$	H	>100	>100

corresponding ester (**9a**), which was converted, on treatment with TFA at room temperature, to racemic **1a** in good yield. The synthetic compound (**1a**) was identical to **1a*** in all respects except for optical rotation. **1b** prepared in the same fashion was also identified as **1b***. In the case of hydrogenation-resistant compounds, such as **1c**, or acid-labile compounds, benzyl glycine is a preferred reagent to *t*-butyl glycine, because hydrogenolytic removal of the benzyl group usually gave better yields than acidic removal of the *t*-butyl group. The other homologs were mainly prepared *via* benzyl esters. Some homologs were also prepared by condensation of the corresponding *N*-hydroxysuccinimide esters[11].

Structure-activity Relationship

The inhibitory activities of natural and synthetic *N*-(3-acyloxyacyl)glycines against the N-[2] and L-type[12] calcium channels are summarized in Table 1. The inhibitory activities indicated the following: natural products (**1a*** ~ **c***) are as active as their racemates (**1a** ~ **c**) with a high selectivity for the N-type calcium channel over the L-type one, indicating that optical activity does not always contribute to inhibitory activity; the double bond of R^2 seems to potentiate activity slightly; higher and lower homologs, even if they are branched or linear (data not shown), lose activity; the 3-hydroxy derivatives (**3**) are also inactive; and both $C_{12\sim13}$ branched alkyl groups with terminal isopropyl are favored.

Experimental

General Procedures

Melting points were measured on a Yanaco melting point apparatus. ^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-GX400 spectrometer with TMS as an internal standard in $CDCl_3$, unless otherwise mentioned. In the ^{13}C NMR spectra, chemical shifts in brackets contain some unresolved methylene carbon signals. IR spectra were recorded on a Nicole 5SXC and JASCO Valor-III spectrometers. UV spectra were recorded in EtOH on a Shimadzu UV-265FW spectrophotometer. Mass spectra were recorded on a JEOL JMS-D300 spectrometer. Optical rotations were recorded in $CHCl_3$ on a JASCO DIP-370 spectrometer. Silica gel (230 ~ 400 mesh, Merck), Si-60 (normal phase Lobar column, Merck), and RP-18 (reverse phase Lobar column, Merck) were used for column chromatography and thin layer chromatography. A packed ODS column (ODS-H, 20 i.d. × 250 mm, Senshu) was used for HPLC.

Fermentation and Isolation

Cytophaga sp. was inoculated in a 500-ml Erlenmyer flask containing medium (100 ml) composed of bacto-peptone (10 g), sodium succinate (1.0 g), yeast extract (1.0 g), $(NH_4)_2SO_4$ (1.0 g), $MgSO_4 \cdot 7H_2O$ (1.0 g), $FeCl_2 \cdot nH_2O$ (2 mg), and $MnSO_4 \cdot nH_2O$ (2 mg) in Jamarine S artificial sea water (1 liter), and was pre-cultured at 23°C for 48 hours. The pre-cultured mixture was transferred to a jar fermenter containing a medium (15 liters) [bacto-

peptone (16 g), yeast extract (1.0 g), sodium succinate (1.0 g), glycerol (2.0 g) in Jamarine S artificial sea water (1 liter)], and again pre-cultured at 23°C for 24 hours. The pre-cultured mixture was transfered to two tank fermenters, each containing the same medium (150 liters), and cultured at 23°C for 48 hours.

The cells were extracted twice with acetone (100 liters × 2) at room temperature. The combined acetone layer was concentrated to dryness, and the residue was extracted with EtOAc. The EtOAc layer was successively washed with water and satd NaCl solution, and dried over anhydr Na_2SO_4. Removal of the solvent under reduced pressure left a red oil (19.02 g). The oil was subjected to repeated silica gel chromatography (SiO_2 400 g). Elution with MeOH - $CHCl_3$, 1 : 9 (v/v), finally gave an active fraction (3.11 g).

To a solution of the active fraction in EtOAc (40 ml), was added diphenyldiazomethane (1.5 g), and the reaction mixture was kept at room temperature overnight. After evaporation of the solvent, the residue was fractionated to a mixture of benzhydryl esters (2.688 g) by silica gel chromatography (SiO_2 100 g) with *n*-hexane - EtOAc, 9 : 1 ~ 2 : 1 (v/v), as eluent. The recycled HPLC (ODS, MeOH) of the mixture gave **2a*** (1.665 g), **2b*** (85.9 mg), and **2c*** (623.2 mg) as colorless oily substances.

To a solution of **2a*** (1.660 g) and a catalytic amount of anisole $CHCl_3$ (16 ml) was added TFA (1.6 ml), and the mixture was stirred at room temperature for 7 hours. After removal of the solvent, the residue was subjected to silica gel chromatography (SiO_2 50 g). Elution with MeOH - $CHCl_3$ - TFA, 2.5 : 97.5 : 0.2 (v/v), gave a crystalline compound which was recrystallized from *n*-hexane-CH_2Cl_2 gave **1a*** (494.3 mg) as colorless leaflets. mp 71 ~ 72°C; $[\alpha]_D^{25}$ +0.45° (*c* 7.92, $CHCl_3$); HR-FAB-MS ($[M+Na]^+$ *m/z* 588.4591, $C_{34}H_{63}NO_5Na$, Δ −1.3 mmu); IR ν_{max} (KBr) cm^{-1} 3362, 2955, 2920, 2850, 2596, 1728, 1719, 1626, 1549, 1469, 1437, 1403, 1384, 1366, 1334, 1246, 1203, 1185, 1168, 1135, 1101, 1070, 1036, 1008, 975, 950, 886, 787, 760, 721, 670, 509; *Anal* Calcd for $C_{34}H_{63}NO_5$: C 72.17, H 11.22, N 2.48. Found: C 71.89, H 11.52, N 2.48.

According to the same treatment of **2a***, **1b*** was obtained as a colorless amorphous solid (43.6 mg) from **2b*** (54.7 mg). $[\alpha]_D^{25}$ −3.4° (*c* 0.87, $CHCl_3$); HR-FAB-MS $[M+Na]^+$ *m/z* 574.4423, $C_{33}H_{61}NO_5Na$, Δ −2.5 mmu; IR ν_{max} cm^{-1} (KBr) 3400 ~ 3200, 2910, 1725, 1650, 1600, 1463, 1170.

According to the same treatment of **2a***, **1c*** was obtained as colorless leaflets (258.1 mg) from **2c*** (620 mg). $[\alpha]_D^{25}$ +0.77° (*c* 11.85, $CHCl_3$); HR-FAB-MS $[M+Na]^+$ *m/z* 590.4739, $C_{34}H_{65}NO_5Na$, Δ +2.1 mmu; IR ν_{max} (KBr) cm^{-1} 3365, 2920, 2850, 2593, 1724, 1628, 1549, 1470, 1401, 1383, 1367, 1334, 1316, 1293, 1250, 1238, 1171, 1136, 1103, 1091, 1070, 1037, 1012, 954, 877, 722, 666, 600, 559, 511, 495; *Anal* Calcd for $C_{34}H_{65}NO_5$: C 71.91, H 11.54, N 2.47. Found: C 71.73, H 11.54, N, 2.46.

Alkaline Hydrolysis of **1a***

To a solution of **1a*** (28.4 mg) in MeOH (2 ml), was added 0.2 N methanolic KOH (2 ml), and the mixture was stirred at room temperature for 4 hours. After the reaction was complete, the mixture was concentrated to dryness, and the residue was dissolved into 90% MeOH (20 ml). This solution was extracted with *n*-hexane after acidification with 1 N HCl. The *n*-hexane layer, after washing with water and drying over anhydrous Na_2SO_4, gave **4a** (9.8 mg) as a colorless oil. The 90% MeOH layer was concentrated to dryness, and the residual solid was dissolved into $CHCl_3$. The $CHCl_3$ layer, after usual work-up, gave **3a*** (18.0 mg). **3a***: mp 98 ~ 100°C; $[\alpha]_D^{25}$ −6.3° (*c* 0.75, MeOH); EI-MS *m/z* 343, 325, 146, 128, 117, 76; ^1H NMR (δ, CD_3OD) 0.88 (6H, d, *J* = 6.6 Hz), 1.17 (2H, m), 1.29 (12H, br s), 1.45 ~ 1.55 (3H, m), 2.35 (1H, dd, *J* = 7.4, 14.1 Hz), 2.38 (1H, dd, *J* = 5.2, 14.1 Hz), 3.89 (1H, d, *J* = 17.8 Hz), 3.94 (1H, d, *J* = 17.8 Hz), 3.96 (1H, m). **4a**: oil; EI-MS *m/z* 254, 240, 222, 199, 180, 167, 76; ^1H NMR δ 0.86 (6H, d, *J* = 6.6 Hz), 1.15 (2H, m), 1.26 (10H, br s), 1.51 (1H, m), 2.04 (2H, m), 2.3 ~ 2.4 (6H, m), 3.67 (3H, s), 5.3 ~ 5.5 (2H, m).

Alkaline Hydrolysis of **1c***

According to the same treatment of **1a***, **1c*** (14.0 mg) gave **3a*** (5.5 mg) and **4c** (5.5 mg). **4c**: a colorless oil; EI-MS *m/z* 256, 241, 225, 213, 199, 171, 157, 143, 129, 87, 76; ^1H NMR δ 0.86 (6H, d, *J* = 6.6 Hz), 1.15 (2H, m), 1.20 ~ 1.35 (16H, m), 1.51 (1H, m), 1.62 (2H, m), 2.30 (2H, t, *J* = 7.5 Hz), 3.67 (3H, s).

Preparation of **6** and **7** from 3-Hydroxyacids (**5**) by DEPC

Benzyl *N*-(3-Hydroxy-15-methylhexadecanoyl)glycine (**6a**)

To an ice-cooled solution of 3-hydroxy-15-methylhexadecanoic acid (**5a**, 1.00 g), glycine benzylester *p*-toluenesulfonate (1.20 g) and triethylamine (1.46 ml) in dry THF (60 ml), was added DEPC (0.68 g). After the addition was complete, the reaction mixture was stirred at room temperature for 3 hours. After removal of THF,

the residue was dissolved into EtOAc. The EtOAc layer was successively washed with water, dil HCl, satd $NaHCO_3$ solution, and satd NaCl solution, and dried over anhydr Na_2SO_4. Removal of EtOAc gave a crystalline compound, which was recrystallized from *n*-hexane-EtOAc, 9:1~5:1 (v/v) to yield **6a** (1.30 g). mp 77~78°C; EI-MS *m/z* 433, 415, 368, 342, 324, 299, 298, 236, 91; IR ν_{max} (KBr) cm^{-1} 3323, 2955, 2920, 2851, 1743, 1645, 1554, 1498, 1468, 1455, 1426, 1398, 1356, 1211, 1134, 1076, 1028, 987, 949, 734, 696; ^1H NMR δ 0.86 (6H, d, J=6.6 Hz), 1.15 (2H, m), 1.26 (18H, br s), 1.4~1.6 (2H, m), 1.51 (1H, m), 2.33 (1H, dd, J=9.2, 15.2 Hz), 2.44 (1H, dd, J=2.7, 15.2 Hz), 4.00 (1H, m), 4.08 (1H, dd, J=5.4, 18.7 Hz), 4.12 (1H, dd, J=5.4, 18.7 Hz), 5.20 (2H, s), 6.36 (1H, br s), 7.3~7.4 (5H, m); ^{13}C NMR δ 22.7 (2×q), 25.5 (t), 27.4 (t), 27.9 (d), [29.55 (t), 29.60 (t), 29.7 (t)], 30.0 (t), 36.9 (t), 39.1 (t), 41.3 (t), 42.7 (t), 67.3 (t), 68.7 (d), 128.4 (2×d), 128.6 (d), 128.7 (2×d), 135.1 (s), 170.0 (s), 172.8 (s); *Anal* Calcd for $C_{26}H_{43}NO_4$: C 72.02, H 10.00, N 3.23. Found: C 71.92, H 9.94, N 3.02.

t-Butyl *N*-(3-Hydroxy-15-methylhexadecanoyl)glycine (**7a**)

According to the same method of preparation of **6a**, **7a** (137.5 mg) was obtained from **5a** (105 mg), glycine *t*-butylester HCl (65.7 mg), triethylamine (0.16 ml), DEPC (76.7 mg) and dry THF (5 ml). mp 49~50°C; EI-MS *m/z* 400, 344, 326, 299, 298, 146, 117, 102, 76, 57; ^1H NMR δ 0.86 (6H, d, J=6.6 Hz), 1.15 (2H, m), 1.25 (18H, br s), 1.4~1.6 (3H, m), 1.46 (9H, s), 2.30 (1H, dd, J=9.2, 15.0 Hz), 2.42 (1H, dd, J=2.7, 15.0 Hz), 3.48 (1H, d, J=3.2 Hz), 3.92 (1H, dd, J=5.1, 18.1 Hz), 3.97 (1H, dd, J=5.1, 18.1 Hz), 4.00 (1H, m), 6.25 (1H, br s); ^{13}C NMR (δ) 22.7 (2×q), 25.5 (t), 27.4 (t), 27.98 (d), 28.03 (3×q), [29.60 (t), 29.67 (t), 29.72 (t)], 30.0 (t), 36.9 (t), 39.1 (t), 42.0 (t), 42.8 (t), 68.7 (d), 82.5 (s), 169.3 (s), 172.6 (s); IR ν_{max} (KBr) cm^{-1} 3351, 3291, 2920, 2850, 1744, 1719, 1669, 1560, 1468, 1413, 1382, 1371, 1246, 1222, 1175, 1077, 1035, 873, 851; *Anal* Calcd. for $C_{23}H_{45}NO_4$: C 69.13, H 11.35, N 3.51. Found: C 68.93, H 11.18, N 3.68.

t-Butyl *N*-(3-Hydroxy-14-methylpentadecanoyl)glycine (**7b**)

According to the same method of preparation of **6a**, **7b** (137.5 mg) was obtained from 3-hydroxy-14-methylpentadecanoic acid (**5b**) (106.6 mg), glycine *t*-butylester HCl (61.5 mg), triethylamine (0.16 ml), DEPC (77.7 mg) and dry THF (5 ml). mp 39~40°C; EI-MS *m/z* 386, 330, 312, 285, 284, 146, 117, 102, 76, 57; IR ν_{max} (KBr) cm^{-1} 3349, 3286, 2921, 2851, 1744, 1720, 1668, 1557, 1467, 1414, 1382, 1369, 1314, 1247, 1223, 1170, 1076, 1035, 869, 851; ^1H NMR δ 0.86 (6H, d, J=6.6 Hz), 1.15 (2H, m), 1.26 (16H, br s), 1.4~1.6 (3H, m), 1.48 (9H, s), 2.30 (1H, dd, J=9.2, 15.0 Hz), 2.42 (1H, dd, J=2.7, 15.0 Hz), 3.48 (1H, d, J=3.2 Hz), 3.92 (1H, dd, J=5.1, 18.1 Hz), 3.97 (1H, dd, J=5.1, 18.1 Hz), 4.00 (1H, m), 6.25 (1H, br s); ^{13}C NMR δ 22.7 (2×q), 25.5 (t), 27.4 (t), 28.00 (d), 28.08 (3×q), [29.6 (t), 29.7 (t), 30.0 (t)], 36.9 (t), 39.1 (t), 42.0 (t), 42.8 (t), 68.8 (d), 82.5 (s), 169.3 (s), 172.6 (s); *Anal* Calcd for $C_{22}H_{43}NO_4$: C 68.53, H 11.24, N 3.63. Found: C 68.58, H 11.26, N 3.68.

Benzyl *N*-(3-Hydroxyheptadecanoyl)glycine (**6f**)

According to the same method of preparation of **6a**, **6f** (3.79 g) was obtained from 3-hydroxyheptadecanoic acid (**5f**) (3.15 g), glycine benzylester *p*-toluenesulfonate (4.32 g), triethylamine (5.1 ml), DEPC (2.39 g) and dry THF (60 ml). mp 86~87°C; EI-MS *m/z* 433, 415, 342, 299, 251, 236, 91; IR ν_{max} (KBr) cm^{-1} 3401, 3320, 2919, 2850, 1746, 1646, 1549, 1211, 1195, 731, 696; ^1H NMR δ 0.88 (3H, t, J=7.2 Hz), 1.25 (22H, br s), 1.4~1.6 (4H, m), 2.32 (1H, dd, J=9.0, 15.0 Hz), 2.43 (1H, dd, J=2.5, 15.0 Hz), 4.00 (1H, m), 4.08 (1H, dd, J=5.4, 18.3 Hz), 4.12 (1H, dd, J=5.4, 18.3 Hz), 5.19 (2H, s), 6.34 (1H, br s), 7.3~7.4 (5H, m); ^{13}C NMR δ 14.1 (q), 22.7 (t), 25.5 (t), [29.4 (t), 29.54 (t), 29.61 (t), 29.7 (t)], 31.9 (t), 36.9 (t), 41.3 (t), 42.7 (t), 67.4 (t), 68.8 (d), 128.4 (2×d), 128.6 (d), 128.7 (2×d), 135.1 (s), 170.0 (s), 172.8 (s); *Anal* Calcd for $C_{26}H_{43}NO_4$:C 72.02, H 10.00, N 3.23. Found: C 71.86, H 10.05, N 3.22.

Benzyl *N*-(3-Hydroxyhexadecanoyl)glycine (**6i**)

According to the same method of preparation of **6a**, **6i** (7.04 g) was obtained from 3-hydroxyhexadecanoic acid (**5i**) (5.44 g), glycine benzylester *p*-toluenesulfonate (7.85 g), triethylamine (8.2 ml), DEPC (3.60 g), and dry THF (60 ml). mp 80.5~81.5°C; EI-MS *m/z* 419, 401, 328, 310, 285, 236, 91; IR ν_{max} (KBr) cm^{-1} 3406, 3316, 2919, 2850, 1748, 1647, 1548, 1210, 1197, 732, 696; ^1H NMR δ 0.88 (3H, t, J=7.2 Hz), 1.25 (20H, br s), 1.4~1.6 (4H, m), 2.32 (1H, dd, J=9.0, 15.1 Hz), 2.43 (1H, dd, J=2.5, 15.1 Hz), 4.00 (1H, m), 4.08 (1H, dd, J=5.2, 18.4 Hz), 4.12 (1H, dd, J=5.4, 18.4 Hz), 5.19 (2H, s), 6.34 (1H, br s), 7.3~7.4 (5H, m); ^{13}C NMR δ 14.1 (q), 22.7 (t), 25.5 (t), [29.4 (t), 29.55 (t), 29.60 (t), 29.7 (t)], 31.9 (t), 36.9 (t), 41.3 (t), 42.7 (t), 67.4 (t), 68.8 (d), 128.4 (2×d), 128.6 (d), 128.7 (2×d), 135.1 (s), 170.0 (s), 172.8 (s); *Anal* Calcd for $C_{25}H_{41}NO_4$: C 71.56, H 9.85, N 3.34.

Found: C 71.35, H 9.65, N 3.39.

Benzyl *N*-(3*R*-Hydroxytetradecanoyl)glycine (**6k***)

According to the same method of preparation of **6a**, **6k*** (4.10 g), after recrystallization from *n*-hexane-CH_2Cl_2, was prepared from commercially available 3*R*-hydroxy-myristic acid (3.66 g), triethylamine (6.2 ml), and DEPC (2.93 g) in dry THF (30 ml). mp 86~87°C; *Anal* Calcd for $C_{23}H_{37}NO_4$: C 70.55, H 9.53, N 3.58. Found: C 70.42, H 9.52, N 3.52.

Acylation of **6** and **7**

t-Butyl *N*-[15-Methyl-3-(13-methy-4*Z*-tetradecenoyloxy)hexadecanoyl]glycine (**9a**)

To an ice-cooled solution of **7a** (150 mg) and dry pyridine (0.3 ml) in dry CH_2Cl_2 (10 ml), was added 13-methyl-4Z-tetradecenoyl chloride (74 mg) in dry CH_2Cl_2 (5 ml). The mixture was stirred at room temperature for 1 hour. After the reaction was complete, CH_2Cl_2 was evaporated under reduced pressure, and the residue was dissolved in EtOAc. The EtOAc layer was successively washed with 10% $CuSO_4$ solution, water, and satd NaCl solution, and dried over anhydyr Na_2SO_4. Elution with *n*-hexane-EtOAc, 9:1 (v/v) on silica gel chromatography of the EtOAc extract, gave **9a** (162.3 mg) as a colorless oil. $C_{38}H_{71}NO_5$; EI-MS *m/z* 622, 566, 565, 345, 344, 326, 251, 91; IR ν_{max} (liquid film) cm^{-1} 3309, 3005, 2954, 2926, 2855, 1739, 1656, 1545, 1467, 1393, 1367, 1160, 722; ^1H NMR δ 0.86 (12H, d, J=6.6 Hz), 1.1~1.2 (4H, m), 1.25 (34H, br s), 1.47 (9H, s), 1.51 (2H, m), 1.6~1.7 (2H, m), 2.03 (2H, m), 2.3~2.4 (4H, m), 2.48 (1H, dd, J=5.3, 14.7 Hz), 2.52 (1H, dd, J=6.6, 14.7 Hz), 3.92 (2H, d, J=4.9 Hz), 5.17 (1H, m), 5.2~5.5 (2H, m), 6.18 (1H, br s); ^{13}C NMR δ 22.7 (4×q), 22.8 (t), 25.3 (t), 27.3 (t), 27.4 (t), 28.0 (d), 28.1 (3×q) [29.4 (t), 29.5 (t), 29.68 (t), 29.74 (t), 29.9 (t)], 34.1 (t), 34.5 (t), 39.1 (t), 41.5 (t), 42.1 (t), 71.4 (d), 82.3 (s), 127.3 (d), 127.8 (d), 169.0 (s), 169.7 (s), 172.8 (s).

Benzyl *N*-[15-Methyl-3-(13-methyltetradecanoyloxy)hexadecanoyl]glycine (**8c**)

According to the same method of preparation of **6a**, **8c** (1.30 g), mp 35~38°C, was obtained from **6a** (1.00 g) and 13-methyltetradecanoyl chloride (650 mg). $C_{41}H_{71}NO_5$; EI-MS *m/z* 657, 642, 615, 550, 493, 433, 415, 372, 324, 281, 251, 242, 209, 248, 91; IR ν_{max} (KBr) cm^{-1} 3309, 2954, 2926, 2851, 1737, 1729, 1640, 1549, 1470, 1216, 1198, 751, 694; ^1H NMR δ 0.86 (12H, d, J=6.8 Hz), 1.15 (4H, m), 1.25 (34H, br s), 1.51 (2H, m), 1.62 (4H, m), 2.50 (1H, dd, J=5.3, 14.7 Hz), 2.52 (2H, t, J=6.0 Hz), 2.54 (1H, dd, J=6.6, 14.7 Hz), 4.07 (2H, d, J=5.2 Hz), 5.16 (1H, br s), 5.19 (2H, s), 6.26 (1H, m), 7.36 (5H, m); ^{13}C NMR δ 22.7 (4×q), 22.8 (t), 25.3 (t), 27.3 (t), 28.0 (2×d), [29.2 (t), 29.3 (t), 29.5 (t), 29.6 (t), 29.7 (t)], 30.0 (t), 34.1 (t), 34.5 (t), 39.1 (t), 41.4 (2×t), 67.2 (t), 71.1 (d), 128.4 (2×d), 128.6 (d), 128.7 (2×d), 135.1 (s), 169.7 (s), 169.9 (s), 173.5 (s).

Benzyl *N*-[3-(14-Methyl-11-pentadecenoyloxy)heptadecanoyl]glycine (**8f**)

According to the same method of preparation of **8a**, **8f** (203.5 mg), a colorless oil, was prepared from **6f** (217 mg) and 14-methyl-11-pentadecenoyl chloride (270 mg). $C_{42}H_{71}NO_5$; EI-MS *m/z* 669, 578, 416, 344, 326, 251, 91; IR ν_{max} (liquid film) cm^{-1} 3445, 2900, 2825, 1740, 1675, 1515, 1465, 1380, 1355, 1190, 950, 695; ^1H NMR δ 0.88 (3H, t, J=7.2 Hz), 0.89 (6H, d, J=6.8 Hz), 1.2~1.4 (34H, br s), 1.55~1.70 (7H, m), 2.00 (2H, m), 2.30 (2H, t, J=7.4 Hz), 2.51 (1H, dd, J=5.4, 14.7 Hz), 2.55 (1H, dd, J=6.6, 14.7 Hz), 2.55 (1H, m), 4.07 (2H, d, J=5.1 Hz), 5.1~5.3 (2H, m), 5.19 (2H, s), 5.38 (2H, m), 6.26 (1H, br s), 7.3~7.5 (5H, m); ^{13}C NMR δ 14.1 (q), 22.4 (2×q), 22.7 (t), 25.0 (t), 25.3 (t), 27.3 (t), 28.7 (d), [29.2 (t), 29.3 (t), 29.5 (t), 29.7 (t)], 32.0 (t), 34.1 (t), 34.5 (t), 36.4 (t), 41.5 (2×t), 67.3 (t), 71.1 (d), 127.5 (d), 128.4 (2×d), 128.6 (2×d), 128.7 (2×d), 130.6 (d), 135.1 (s), 137.5 (d), 169.8 (s), 169.9 (s), 173.5 (s); *Anal* Calcd for $C_{42}H_{71}NO_5$: C 75.29, H 10.68, N 2.09. Found: C 74.99, H 10.87, N 2.01.

Benzyl *N*-[3-(13-Methyl-11-tetradecenoyloxy)heptadecanoyl]glycine (**8g**)

According to the same method of preparation of **8a**, **8g** (498.7 mg), a colorless oil, was prepared from **6g** (350 mg) and 13-methyl-11-tetradecenoyl chloride (400 mg). $C_{41}H_{69}NO_5$; EI-MS *m/z* 655, 564, 434, 416, 344, 326, 251, 91; IR ν_{max} (liquid film) cm^{-1} 3445, 2920, 2850, 1735, 1675, 1570, 1463, 1393, 1355, 1193, 696; ^1H NMR δ 0.88 (3H, t, J=7.2 Hz), 0.93 (6H, d, J=6.6 Hz), 1.2~1.4 (36H, br s), 1.55~1.70 (4H, m), 2.20 (2H, m), 2.30 (2H, t, J=7.4 Hz), 2.51 (1H, dd, J=5.6, 14.9 Hz), 2.55 (1H, dd, J=6.7, 14.9 Hz), 2.57 (1H, m), 4.08 (2H, d, J=5.1 Hz), 5.1~5.3 (2H, m), 5.19 (2H, s), 6.25 (1H, br s), 7.3~7.5 (5H, m); ^{13}C NMR δ 14.1 (q), 22.7 (t), 23.3 (2×q), 25.0 (t), 26.4 (d), 27.3 (t), [29.2 (t), 29.3 (t), 29.5 (t), 29.7 (t)], 30.0 (t), 32.0 (t), 34.1 (t), 34.5 (t), 41.4 (2×t), 67.2 (t), 71.1 (d), 127.5 (d), 128.4 (2×d), 128.6 (d), 128.7 (2×d), 135.1 (s), 137.5 (d), 169.8 (s), 169.9 (s), 173.5 (s).

Benzyl *N*-[3-(12-Methyl-11-tridecenoyloxy)heptadecanoyl]glycine (**8h**)

According to the same method of preparation of **8a**, **8h** (338.5 mg), a colorless iol, after chromatographic purification, was obtained from **6h** (300 mg) and 12-methyl-11-tridecenoyl chloride (320 mg). $C_{40}H_{67}NO_5$; EI-MS *m/z* 641, 551, 550, 416, 415, 281, 251, 226, 153, 91; IR ν_{max} (liquid film) cm^{-1} 3450, 2925, 2850, 1735, 1686, 1515, 1466, 1385, 1370, 1355, 1190, 695; ^1H NMR δ 0.88 (3H, t, J=7.2 Hz), 1.56 (6H, s), 1.2~1.4 (34H, br s), 1.4~1.5 (2H, m), 1.5~1.7 (4H, m), 1.7~1.8 (2H, m), 2.30 (2H, t, J=7.4 Hz), 2.51 (1H, dd, J=5.7, 14.8 Hz), 2.55 (1H, dd, J=6.7, 14.8 Hz), 4.08 (2H, d, J=5.1 Hz), 5.1~5.3 (2H, m), 5.19 (2H, s), 6.27 (1H, br s), 7.3~7.5 (5H, m); ^{13}C NMR δ 14.1 (q), 22.7 (t), 25.0 (t), 25.3 (t), [29.2 (t), 29.3 (t), 29.4 (t), 29.5 (t), 29.7 (t)], 32.0 (t), 32.4 (2×q), 34.1 (t), 34.5 (t), 41.35 (t), 41.42 (t), 67.3 (t), 71.1 (d), 128.4 (2×d), 128.6 (d), 128.7 (2×d), 135.1 (s), 169.7 (s), 170.1 (s), 173.5 (s).

Benzyl *N*-[3-(13-Methyl-11-tetradecenoyloxy)hexadecanoyl]glycine (**8i**)

According to the same method of preparation of **8a**, **8i** (265 mg), a colorless oil, after chromatographic purification, was obtained from **6i** (243.4 mg) and 13-methyl-11-tridecenoyl chloride (300 mg). $C_{40}H_{67}NO_5$; EI-MS *m/z* 641, 550, 420, 402, 330, 312, 237, 91; IR ν_{max} (liquid film) cm^{-1} 3307, 2925, 2855, 1736, 1655, 1541, 1380, 1360, 1186, 736; ^1H NMR δ 0.88 (3H, t, J=7.2 Hz), 1.56 (6H, s), 1.2~1.4 (32H, br s), 1.40~1.80 (12H, m), 2.30 (2H, t, J=7.5 Hz), 2.51 (1H, dd, J=5.4, 14.7 Hz), 2.55 (1H, dd, J=6.6, 14.7 Hz), 4.08 (2H, d, J=5.1 Hz), 5.16 (1H, m), 5.19 (2H, s), 5.1~5.2 (2H, m), 6.26 (2H, br t, J=5.1 Hz), 7.3~7.4 (5H, m). ^{13}C NMR (δ) 14.1 (q), 22.7 (t), 25.0 (t), 25.1 (t), 25.3 (t), [29.2 (t), 29.3 (t), 29.4 (t), 29.5 (t), 29.7 (t)], 32.0 (2×t), 32.4 (2×q), 34.1 (t), 34.5 (t), 41.4 (2×t), 46.1 (t), 67.2 (t), 71.1 (d), 128.4 (2×d), 128.6 (2×d), 128.7 (2×d+s), 130.6 (d), 135.1 (s), 169.8 (s), 169.9 (s), 173.5 (s).

Benzyl *N*-[3-(12-Methyl-11-tridecenoyloxy)hexadecanoyl]glycine (**8j**)

According to the same method of preparation of **8a**, **8j** (284 mg), a colorless oil, after chromatographic purification, was obtained from **6i** (276 mg) and 12-methyl-11-tridecenoyl chloride (242 mg). $C_{39}H_{65}NO_5$; EI-MS *m/z* 627, 536, 420, 402, 330, 312, 237, 91; IR ν_{max} (liquid film) cm^{-1} 3345, 2925, 2850, 1735, 1685, 1515, 1465, 1385, 1370, 1355, 1190, 695; ^1H NMR δ 0.88 (3H, t, J=7.2 Hz), 1.56 (6H, s), 1.2~1.4 (32H, br s), 1.40~1.80 (12H, m), 2.30 (2H, t, J=7.5 Hz), 2.51 (1H, dd, J=5.4, 14.7 Hz), 2.55 (1H, dd, J=6.6, 14.7 Hz), 4.08 (2H, d, J=5.1 Hz), 5.16 (1H, m), 5.19 (2H, s), 5.1~5.2 (2H, m), 6.26 (2H, br t, J=5.1 Hz), 7.3~7.4 (5H, m); ^{13}C NMR δ 14.1 (q), 22.7 (t), 25.0 (t), 25.1 (t), 25.3 (t), [29.2 (t), 29.3 (t), 29.4 (t), 29.5 (t), 29.7 (t)], 32.0 (2×t), 32.4 (2×q), 34.1 (t), 34.5 (t), 41.4 (2×t), 46.1 (t), 67.2 (t), 71.1 (d), 128.4 (2×d), 128.6 (2×d), 128.7 (2×d+s), 130.6 (d), 135.1 (s), 169.8 (s), 169.9 (s), 173.5 (s).

Preparation of **2** *via* Succinimide Ester

t-Butyl *N*-[14-Methyl-3-(13-methyl-4*Z*-tetradecenoyl)pentadecanoyl]glycine (**9b**)

To an ice-cooled solution of 14-methyl-3-(13-methyl-4*Z*-tetradecenoyloxy)pentadecanoic acid (**5b'**, 103 mg) in dry EtOAc (5 ml), was successively added *N*-hydroxysuccinimide (26.5 mg) and DCC (47.2 mg), and the reaction mixture was stirred at room temperature overnight. After filtration of precipitate formed during the reaction, the filtrate was concentrated to dryness, and the residue was dissolved in dry DMF (5 ml). To the DMF solution, was added glycine *t*-butylester HCl (34.9 mg) and $NaHCO_3$ (19.5 mg), and the mixture was stirred at room temperature for 5 hours. After the reaction was compete, water and EtOAc were added to the reaction mixture, and then the EtOAc layer was successively washed with water and satd NaCl solution, and dried over anhydr Na_2SO_4. The residue, after evaporation of EtOAc, was subjected to a Lobar colunm chromatography (Si-60). Elution with EtOAc-$CHCl_3$, 5:95 (v/v) gave **7a** (94.7 mg) as a colorless oil. $C_{38}H_{71}NO_5$; EI-MS *m/z* 636, 566, 565, 345, 344, 326, 251, 91; IR ν_{max} (liquid film) cm^{-1} 3309, 3005, 2954, 2926, 2855, 1739, 1656, 1545, 1467, 1393, 1367, 1160, 722; ^1H NMR δ 0.86 (12H, d, J=6.6 Hz), 1.1~1.2 (4H, m), 1.25 (30H, br s), 1.47 (9H, s), 1.51 (2H, m), 1.6~1.7 (2H, m), 2.03 (2H, m), 2.3~2.4 (4H, m), 2.48 (1H, dd, J=5.3, 14.7 Hz), 2.52 (1H, dd, J=6.6, 14.7 Hz), 3.92 (2H, d, J=4.9 Hz), 5.17 (1H, m), 5.2~5.5 (2H, m), 6.18 (1H, br s); ^{13}C NMR δ 22.7 (4×q), 22.8 (t), 25.3 (t), 27.3 (t), 27.4 (t), 28.0 (d), 28.1 (3×q), [29.4 (t), 29.5 (t), 29.68 (t), 29.74 (t), 29.9 (t)], 34.1 (t), 34.5 (t), 39.1 (t), 41.5 (t), 42.1 (t), 71.4 (d), 82.3 (s), 127.3 (d), 127.8 (d), 169.0 (s), 169.7 (s), 172.8 (s).

Benzyl *N*-[14-Methyl-3-(13-methyl-4*Z*-tetradecenoyl)pentadecanoyl]glycine (**8b**)

According to the method for preparation of **8a**, **8b** (109.0 mg) as a colorless oil was prepared from **5b'**

(109 mg), *N*-hydroxysuccinimide (28.0 mg), DCC (50.1 mg), benzyl glycine *p*-toluenesufonate (78.2 mg), and $NaHCO_3$ (19.5 mg). $C_{40}H_{69}NO_5$; EI-MS *m/z* 643, 536, 416, 324, 251, 211, 91; IR ν_{max} (liquid film) cm^{-1} 3346, 2958, 2918, 2850, 1736, 1645, 1532, 1469, 1404, 1357, 1231, 1162, 969, 746, 721, 696; ^1H NMR δ 0.86 (6H, d, $J=6.7$ Hz), 0.86 (3H, t, $J=7.2$ Hz), 1.0~1.2 (2H, m), 1.25 (42H, br s), 1.57 (1H, m), 1.5~1.7 (42H, br s), 2.30 (2H, t, $J=7.7$ Hz), 2.50 (1H, dd, $J=5.3$, 14.7 Hz), 2.54 (1H, dd, $J=6.6$, 14.7 Hz), 4.07 (1H, d, $J=5.2$ Hz), 5.16 (1H, m), 5.19 (2H, s), 6.26 (1H, br s), 7.3~7.4 (5H, m); ^{13}C NMR δ 14.1 (q), 22.7 (2×q+t), 25.0 (t), 25.3 (t), 27.4 (t), 28.0 (d), [29.2 (t), 29.3 (t), 29.5 (t), 29.6 (t), 29.7 (t), 30.0 (t)], 32.0 (t), 34.1(t), 34.5 (t), 39.1 (t), 41. (t), 68.1 (t), 71.1 (d), 128.4 (2×d), 128.6 (d), 128.7 (2×d), 135.2 (s), 169.8 (s), 169.9 (s).

Preparation of **1** and **3**

N-[15-Methyl-3-(13-methyl-4*Z*-tetradecenoyloxy)-hexadecanoyl]glycine (**1a**)

To a solution of **9a** (140 mg) in CH_2Cl_2 (5 ml), was added TFA (1 ml), and the mixture was stirred at room temperature overnight. Removal of the solvent gave a crystalline compound, which was recrystallized from *n*-hexane-CH_2Cl_2 to yield **1a** (57 mg). mp 84~85°C; $C_{34}H_{63}NO_5$; EI-MS *m/z* 566, 565, 345, 326, 251, 117, 76; IR ν_{max} (KBr) cm^{-1} 3348, 2956, 2922, 2851, 2604, 1747, 1715, 1621, 1569, 1468, 1418, 1383, 1344, 1249, 1203, 1163, 722; ^1H NMR δ 0.86 (12H, d, $J=6.7$ Hz), 1.1~1.2 (4H, m), 1.25 (34H, br s), 1.51 (2H, m), 1.6~1.7 (2H, m), 2.03 (2H, m), 2.3~2.4 (4H, m), 2.52 (1H, dd, $J=5.6$, 14.7 Hz), 2.56 (1H, dd, $J=6.7$, 14.7 Hz), 4.08 (2H, d, $J=5.2$ Hz), 5.17 (1H, m), 5.2~5.5 (2H, m), 6.39 (1H, br t, $J=4.9$ Hz); ^{13}C NMR δ 22.7 (4×q), 22.8 (t), 25.3 (t), 27.3 (t), 27.4 (t), 28.0 (d), [29.4 (t), 29.5 (t), 29.6 (t), 29.7 (t), 29.9 (t)], 30.0 (t), 34.1 (t), 34.5 (t), 39.1 (2×t), 41.4 (t), 71.4 (d), 127.2 (d), 131.1 (d), 170.7 (s), 172.3 (s), 173.2 (s); *Anal* Calcd for $C_{34}H_{63}NO_5$: C 72.17, H 11.22, N 2.48. Found: C 71.93, H 12.11, N 2.47.

N-[14-Methyl-3-(13-methyl-4*Z*-tetradecenoyloxy)-pentadecanoyl]glycine (**1b**)

According to the method for preparation of **1a**, **1b** (48.3 mg) was prepared from **9b** (93 mg). mp 74~75°C; $C_{33}H_{61}NO_5$; EI-MS *m/z* 552, 551, 331, 330, 312, 237, 236, 117, 76; IR ν_{max} (KBr) cm^{-1} 3347, 2955, 2922, 2851, 2604, 1746, 1714, 1621, 1570, 1467, 1420, 1383, 1343, 1250, 1191, 1163, 722; ^1H NMR δ 0.86 (12H, d, $J=6.6$ Hz), 1.1~1.2 (4H, m), 1.25 (26H, br s), 1.51 (2H, m), 1.6~1.7 (2H, m), 2.03 (2H, m), 2.3~2.4 (4H, m), 2.52 (1H, dd, $J=5.4$, 14.7 Hz), 2.56 (1H, dd, $J=6.6$, 14.7 Hz), 4.08 (2H, d, $J=5.1$ Hz), 5.17 (1H, m), 5.2~5.5 (2H, m), 6.39 (1H, br s); ^{13}C NMR δ 22.7 (4×q), 22.8 (t), 25.3 (t), 27.3 (t), 27.4 (t), 28.0 (d), [29.4 (t), 29.5 (t), 29.6 (t), 29.7 (t), 29.9 (t)], 30.0 (t), 34.1 (t), 34.5 (t), 39.1 (t), 41.4 (2×t), 71.4 (d), 127.2 (d), 131.8 (d), 170.7 (s), 172.7 (s), 173.2 (s); *Anal* Calcd for $C_{33}H_{61}NO_5$: C 71.82, H 11.14, N 2.54. Found: C 71.63, H 11.05, N 2.57.

N-[15-Methyl-3-(13-methyltetradecanoyloxy)hexadecanoyl]glycine (**1c**)

A solution of **8c** (140 mg) in EtOH (20 ml) was shaken under atmospheric H_2 in the presence of 10% Pd-C (70 mg) at room temperature. After the reaction was complete, filtration of Pd-C and evaporation of the filtrate gave a crystalline solid, which was recrystallized from *n*-hexane-CH_2Cl_2 to yield **1c** (88 mg). mp 92~94°C; $C_{34}H_{65}NO_5$; EI-MS *m/z* 567, 493, 342, 325, 282, 242, 199, 117, 76; IR ν_{max} (KBr) cm^{-1} 3349, 2955, 2921, 2851, 2606, 1746, 1722, 1623, 1568, 1468, 1379, 1248, 1200, 928, 654; ^1H NMR δ 0.86 (12H, d, $J=6.7$ Hz), 1.0~1.2 (4H, m), 1.25 (34H, br s), 1.51 (2H, m), 1.55~1.70 (4H, m), 2.31 (2H, t, $J=7.5$ Hz), 2.52 (1H, dd, $J=5.3$, 14.8 Hz), 2.56 (1H, dd, $J=6.8$, 14.8 Hz), 4.07 (2H, d, $J=5.2$ Hz), 5.16 (1H, m), 6.41 (1H, br s); ^{13}C NMR δ 22.7 (4×q), 25.0 (t), 25.3 (t), 27.5 (t), 28.0 (d), [29.2 (t), 29.4 (t), 29.6 (t), 29.7 (t), 29.8 (t)], 30.0, (t), 34.2 (t), 34.6 (t), 39.1 (t), 41.3 (t), 41.4 (t), 71.2 (d), 170.8 (s), 172.7 (s), 1734.0 (s); *Anal* Calcd. for $C_{34}H_{65}NO_5$: C 71.91, H 11.54, N 2.47. Found: C 71.62, H 11.42, N 2.26.

N-(15-Methyl-3-tetradecanoyloxyhexadecanoyl)glycine (**1d**)

According to the same method of preparation of **1a**, **1d** (66.7 mg) was prepared from **8c** (90 mg), and recrystallized from *n*-hexane-$CHCl_3$. mp 89~90°C; $C_{33}H_{63}NO_5$; EI-MS *m/z* 554, 553, 326, 325, 251, 250, 228, 185, 117, 76. IR ν_{max} (KBr) cm^{-1} 3348, 2956, 2921, 2851, 2605, 1746, 1722, 1622, 1568, 1468, 1419, 1379, 1344, 1249, 1199, 1178, 722; ^1H NMR δ 0.86 (6H, d, $J=6.7$ Hz), 0.88 (3H, t, $J=6.7$ Hz), 1.1~1.2 (2H, m), 1.25 (28H, br s), 1.51 (1H, m), 1.55~1.70 (4H, m), 2.31 (2H, t, $J=7.4$ Hz), 2.52 (1H, dd, $J=5.6$, 14.7 Hz), 2.56 (1H, dd, $J=6.7$, 14.7 Hz), 4.08 (2H, d, $J=5.3$ Hz), 5.16 (1H, m), 6.40 (1H, br t, $J=5.1$ Hz); ^{13}C NMR δ 14.1(q), 22.7 (2×q+t), 25.0 (t), 25.3 (t), 27.5 (t), 28.0 (d), [29.2 (t), 29.4 (t), 29.5 (t), 29.6 (t), 29.7 (t)], 30.0 (t), 32.0 (t), 34.2 (t), 34.6 (t), 39.1 (t), 41.38 (t), 41.43 (t), 71.2 (d), 170.7 (s), 172.7 (s), 173.9 (s); *Anal* Calcd for $C_{33}H_{63}NO_5$:

C 71.56, H 11.37, N 2.53. Found: C 71.22, H 11.67, N 2.56.

N-[14-Methyl-3-(12-methyltridecanoyloxy)pentadecanoyl]glycine (**1e**)

According to the method for preparation of **1c**, **1e** (62.3 mg) was prepared from **8e** (109 mg), and recrystallized from $CHCl_3$/hexane. mp 89~90°C; $C_{33}H_{63}NO_5$; EI-MS *m/z* 554, 553, 312, 311, 237, 236, 199, 117, 76; IR ν_{max} (KBr) cm^{-1} 3350, 2954, 2921, 2851, 2603, 1745, 1721, 1623, 1568, 1467, 1418, 1382, 1343, 1249, 1202, 1178, 722; ^1H NMR δ 0.86 (12H, d, J=6.7 Hz), 1.2~1.4 (4H, m), 1.25 (34H, br s), 1.51 (2H, m), 1.55~1.70 (2H, m), 2.31 (2H, t, J=7.4 Hz), 2.52 (1H, dd, J=5.7, 14.7 Hz), 2.56 (1H, dd, J=6.7, 14.7 Hz), 4.08 (2H, d, J=5.3 Hz), 5.16 (1H, m), 6.39 (1H, br t, J=5.1 Hz); ^{13}C NMR δ 22.7 (4×q), 25.0 (t), 25.3 (t), 27.5 (t), 28.0 (2×d), [29.2 (t), 29.4 (t), 29.5 (t), 29.6 (t), 29.7 (t)], 30.0 (t), 34.2 (t), 34.6 (t), 39.1 (t), 41.4 (t), 41.5 (t), 71.2 (d), 170.7 (s), 172.7 (s), 173.9 (s); *Anal* Calcd for $C_{33}H_{63}NO_5$: C 71.56, H 11.47, N 2.53. Found: C 71.20, H 11.73, N 2.53.

N-[3-(14-Methylpentadecanoyloxy)heptadecanoyl]-glycine (**1f**)

According to the same method of preparation of **1c**, **1f** (149.3 mg) was prepared from **6f** (204 mg), and recrystallized from *n*-hexane-$CHCl_3$. mp 96~97°C; $C_{35}H_{69}NO_5$; EI-MS *m/z* 582, 581, 326, 325, 251, 250, 213, 117, 76; IR ν_{max} (KBr) cm^{-1} 3349, 2955, 2921, 2851, 2611, 1745, 1722, 1623, 1568, 1468, 1418, 1378, 1343, 1248, 1200, 1178, 721; ^1H NMR δ 0.86 (6H, d, J=6.6 Hz), 0.88 (3H, t, J=6.8 Hz), 1.1~1.2 (2H, m), 1.25 (52H, br s), 1.51 (1H, m), 1.55~1.70 (4H, m), 2.31 (2H, t, J=7.6 Hz), 2.52 (1H, dd, J=5.3, 14.7 Hz), 2.57 (1H, dd, J=6.7, 14.7 Hz), 4.08 (2H, d, J=5.0 Hz), 5.16 (1H, m), 6.40 (1H, br t, J=5.0 Hz); ^{13}C NMR δ 14.1 (q), 22.7 (2×q+t), 25.0 (t), 25.3 (t), 27.5 (t), 28.0 (d), [29.2 (t), 29.4 (t), 29.6 (t), 29.7 (t)], 30.0 (t), 32.0 (t), 34.1 (t), 34.6 (t), 39.1 (t), 41.4 (t), 41.5 (t), 71.2 (d), 170.8 (s), 172.8 (s), 173.9 (s); *Anal* Calcd for $C_{35}H_{69}NO_5$: C 72.24, H 11.61, N 2.41. Found: C 71.87, H 11.90, N 2.39.

N-[3-(13-Methyltetradecanoyloxy)heptadecanoyl]-glycine (**1g**)

According to the same method of preparation of **1c**, **1g** (167 mg) was prepared from **6g** (224.1 mg), and recrystallized from *n*-hexane-$CHCl_3$. mp 93~94°C; $C_{34}H_{65}NO_5$; EI-MS *m/z* 568, 567, 398, 385, 326, 325, 251, 250, 117, 76; IR ν_{max} (KBr) cm^{-1} 3349, 2956, 2921, 2851, 2605, 1745, 1721, 1623, 1568, 1468, 1419, 1379, 1342, 1249, 1190, 1177, 939, 722, 657; ^1H NMR δ 0.86 (6H, d, J=6.6 Hz), 0.88 (3H, t, J=7.1 Hz), 1.1~1.2 (2H, m), 1.25 (50H, br s), 1.51 (1H, m), 1.55~1.70 (4H, m), 2.31 (2H, t, J=7.5 Hz), 2.52 (1H, dd, J=5.6, 14.7 Hz), 2.57 (1H, dd, J=6.7, 14.7 Hz), 4.08 (2H, d, J=5.1 Hz), 5.16 (1H, m), 6.40 (1H, br t, J=5.1 Hz); ^{13}C NMR δ 14.1 (q), 22.7 (2×q+t), 25.0 (t), 25.3 (t), 27.5 (t), 28.0 (d), [29.2 (t), 29.4 (t), 29.55 (t), 29.59 (t), 29.7 (t)], 30.0 (t), 32.0 (2×t), 34.1 (t), 34.6 (t), 39.1 (t), 41.3 (t), 41.4 (t), 71.2 (d), 170.8 (s), 172.8 (s), 173.9 (s); *Anal* Calcd for $C_{34}H_{67}NO_5$: C 71.91, H 11.54, N 2.47. Found: C 71.62, H 11.73, N 2.47.

N-[3-(12-Methytridecanoyloxy)heptadecanoyl]glycine (**1h**)

According to the same method of preparation of **1c**, **1h** (196.3 mg) was prepared from **6h** (262 mg), and recrystallized from *n*-hexane-$CHCl_3$. mp 85~86°C; $C_{33}H_{63}NO_5$; EI-MS *m/z* 544, 538, 496, 479, 440, 398, 385, 344, 326, 251, 185, 152, 117, 76; IR ν_{max} (KBr) cm^{-1} 3349, 2955, 2923, 2851, 2607, 1746, 1721, 1623, 1568, 1468, 1378, 1247, 1198, 1180, 935, 722, 659; ^1H NMR δ 0.86 (6H, d, J=6.6 Hz), 0.88 (3H, t, J=7.1 Hz), 1.16 (2H, m), 1.25 (38H, m), 1.51 (1H, m), 1.55~1.70 (4H, m), 2.31 (2H, t, J=7.1 Hz), 2.52 (1H, dd, J=5.5, 14.7 Hz), 2.56 (1H, dd, J=6.7, 14.7 Hz), 4.07 (2H, d, J=5.1 Hz), 5.16 (1H, m), 6.42 (1H, br s); ^{13}C NMR δ 14.1 (q), 22.7 (2×q), 25.0 (t), 25.2 (t), 25.3 (t), 27.5 (t), 28.0 (d), [29.1 (t), 29.4 (t), 29.6 (t), 29.7 (t)], 30.0 (t), 32.0 (2×t), 34.1 (t), 34.6 (t), 39.1 (t), 41.3 (t), 41.4 (t), 71.2 (d), 170.8 (s), 172.7 (s), 173.9 (s); *Anal* Calcd for $C_{33}H_{63}NO_5$: C 71.49, H 11.37, N 2.53. Found: C 71.63, H 11.47, N 2.41.

N-[3-(14-Methylpentadecanoyloxy)hexadecanoyl]-glycine (**1i**)

According to the same method of preparation of **1c**, **1i** (150 mg) was prepared from **6i** (150 mg), and recrystallized from *n*-hexane-$CHCl_3$. mp 95~96°C; $C_{34}H_{67}NO_5$; EI-MS *m/z* 567, 567, 312, 311, 256, 237, 213, 117, 76; IR ν_{max} (KBr) cm^{-1} 3349, 2956, 2921, 2851, 2611, 1746, 1721, 1623, 1568, 1468, 1419, 1378, 1344, 1247, 1200, 1178, 722, 657; ^1H NMR δ 0.86 (6H, d, J=6.6 Hz), 0.88 (3H, t, J=7.1 Hz), 1.25 (50H, br s), 1.55~1.70 (4H, m), 2.31 (2H, t, J=7.4 Hz), 2.52 (1H, dd, J=5.4, 14.7 Hz), 2.55 (1H, dd, J=6.8, 14.7 Hz), 4.08 (2H, d, J=5.1 Hz), 5.16 (1H, m), 6.41 (1H, br t, J=5.1 Hz); ^{13}C NMR δ 14.1 (q), 22.7 (2×q+t), 25.0 (t), 25.3 (t), 27.5 (t), 28.0 (d), [29.2 (t), 29.4 (t), 29.6 (t), 29.7 (t)], 30.0 (t), 32.0 (2×t), 34.1 (t), 34.6 (t), 39.1 (t), 41.3 (t), 41.5 (t), 71.2 (d), 170.8 (s), 172.8 (s), 173.9 (s);

Anal Calcd for $C_{34}H_{67}NO_5$: C 71.91, H 11.54, N 2.47. Found: C 71.70, H 11.45, N 2.42.

N-[3-(13-Methyltetradecanoyloxy)hexadecanoyl]glycine (**1j**)

According to the same method of preparation of **1c**, **1j** (200 mg) was prepared from **6j** (160 mg), and recrystallized from *n*-hexane-CH_2Cl_2. mp 91~92°C; $C_{33}H_{63}NO_5$; EI-MS *m/z* 553, 328, 312, 311, 268, 237, 236, 199, 117, 76; IR ν_{max} (KBr) cm^{-1} 3349, 2956, 2920, 2851, 2607, 1746, 1721, 1623, 1568, 1468, 1419, 1379, 1343, 1249, 1200, 1176, 722, 657; ^1H NMR δ 0.86 (6H, d, J=6.6 Hz), 0.88 (3H, t, J=7.0 Hz), 1.25 (48H, br s), 1.55~1.70 (4H, m), 2.31 (2H, t, J=7.4 Hz), 2.52 (1H, dd, J=5.4, 14.7 Hz), 2.55 (1H, dd, J=6.8, 14.7 Hz), 4.07 (2H, d, J=5.0 Hz), 5.16 (1H, m), 6.40 (1H, br t, J=4.6 Hz); ^{13}C NMR δ 14.1 (q), 22.7 (2×q+t), 25.0 (t), 25.3 (t), 27.5 (t), 28,0 (d), [29.2 (t), 29.4 (t), 29.6 (t), 29.7(t)], 30.0 (t), 32.0 (2×t), 34.1 (t), 34.6 (t), 39.1 (t), 41.4 (t), 41.4 (t), 71.2 (d), 170.8 (s), 172.8 (s), 173.9 (s); *Anal* Calcd for $C_{34}H_{67}NO_5$: C 71.56, H 11.47, N 2.58. Found: C 71.54, H 11.55, N 2.58.

N-(3-Hydroxy-15-methylhexadecanoyl)glycine (**3a**)

According to the same method of preparation of **1a**, **3a** (136.7 mg) was prepared from **6a** (220 mg), and recrystallized from *n*-hexane-EtOAc. mp 84~85°C; $C_{19}H_{37}NO_4$; EI-MS *m/z* 343, 325, 146, 128, 117, 76; IR ν_{max} (KBr) cm^{-1} 3353, 3274, 3071, 2956, 2924, 2870, 1713, 1645, 1557, 1451, 1422, 1261, 1125, 1085, 895; ^1H NMR (CD_3OD) δ 0.88 (6H, d, J=6.6 Hz), 1.17 (2H, m), 1.29 (12H, br s), 1.45~1.55 (3H, m), 2.35 (1H, dd, J=7.4, 14.1 Hz), 2.38 (1H, dd, J=5.2, 14.1 Hz), 3.89 (1H, d, J=17.8 Hz), 3.94 (1H, d, J=17.8 Hz), 3.96 (1H, m); *Anal* Calcd for $C_{19}H_{37}NO_4$: C 66.43, H 10.86, N 4.08. Found: C 66.06, H 10.59, N 4.09.

N-(3-Hydroxyheptadecanoyl)glycine (**3f**)

According to the same method of preparation of **1a**, **3f** (206.4 mg) was prepared from **6f** (300 mg) and recrystallized from MeOH-EtOAc. mp 108~110.5°C; $C_{19}H_{37}NO_4$; EI-MS *m/z* 344, 251, 146, 117, 76; IR ν_{max} (KBr) cm^{-1} 3325, 3268, 2954, 2921, 2849, 1711, 1643, 1557, 1463, 1450, 1422, 1407, 906; ^1H NMR (CD_3OD) δ 0.90 (3H, t, J=7.0 Hz), 1.2~1.4 (24H, br s), 1.40~1.55 (4H, m), 2.35 (1H, dd, J=5.1, 14.3 Hz), 2.38 (1H, dd, J=7.4, 14.3 Hz), 3.88 (1H, d, J=17.8 Hz), 3.93 (1H, d, J=17.8 Hz), 3.96 (1H, m); ^{13}C NMR (CD_3OD) δ 14.5 (q), 23.8 (t), 26.7 (t), 30.5 (2×t), 30.8 (7×t), 33.1 (t), 38.1 (t), 42.0 (t), 44.7 (t), 69.8 (d), 173.3 (s), 174.7 (s); *Anal* Calcd for $C_{19}H_{37}NO_4$: C 66.43, H 10.86, N 4.08. Found: C 66.13, H 11.06, N 4.13.

N-(3-Hydroxyhexadecanoyl)glycine (**3i**)

According to the method for preparation of **1a**, **3i** (100 mg) was prepared from **6i** (168 mg) and recrystallized from MeOH-EtOAc. mp 102~105°C; $C_{18}H_{35}NO_4$; EI-MS *m/z* 330, 146, 128, 117, 76; IR ν_{max} (KBr) cm^{-1} 3336, 3269, 3072, 2956, 2923, 2850, 1711, 1643, 1558, 1462, 1451, 1422, 1407; ^1H NMR (CD_3OD) δ 0.90 (3H, t, J=7.0 Hz), 1.2~1.4 (22H, br s), 1.40~1.55 (4H, m), 2.35 (1H, dd, J=5.1, 14.3 Hz), 2.38 (1H, dd, J=7.4, 14.3 Hz), 3.88 (1H, d, J=17.8 Hz), 3.93 (1H, d, J=17.8 Hz), 3.96 (1H, m); ^{13}C NMR (CD_3OD) δ 14.9 (q), 24.2 (t), 27.1 (t), 31.0 (t), 31.19 (t), 31.22 (6×t), 31.3 (t), 33.6 (t), 38.6 (t), 42.3 (t), 45.1 (t), 70.2 (d), 173.6 (s), 175.2 (s); *Anal* Calcd for $C_{18}H_{35}NO_4$: C 65.62, H 10.71, N 4.25. Found: C 65.87, H 11.08, N 4.20.

N-(3*R*-Hydroxymyristoyl)glycine (**3k***)

According to the same method of preparation of **1c**, **3k*** (215.2 mg) was prepared from **6k*** (301 mg) and recrystallized from EtOAc. mp 95~97°C; $C_{16}H_{31}NO_4$; $[\alpha]_D^{25}$ −7.0° (*c* 1, MeOH). EI-MS *m/z* 301, 283, 146, 117, 76. IR ν_{max} (KBr) cm^{-1} 3445, 3326, 3184, 2956, 2918, 2849, 1732, 1718, 1704, 1647, 1539, 1519, 1470, 1433, 1408, 1347, 1226; ^1H NMR (CD_3OD) δ 0.91 (3H, t, J=6.7 Hz), 1.29 (18H, br s), 1.40~1.55 (2H, m), 2.35 (1H, dd, J=7.7, 14.5 Hz), 2.38 (1H, dd, J=5.1, 14.5 Hz), 3.89 (1H, d, J=17.9 Hz), 3.94 (1H, d, J=17.9 Hz), 3.96 (1H, m); ^{13}C NMR (CD_3OD) δ 14.5 (q), 23.7 (t), 25.7 (t), [30.5 (t), 30.8 (t)], 33.1 (t), 38.1 (t), 41.8 (t), 44.6 (t), 69.7 (d), 173.1 (s), 174.7 (s); *Anal* Calcd for $C_{16}H_{31}NO_4$: C 63.79, H 10.30, N 4.65. Found: C 63.66, H 10.34, N 4.61.

Measurement of [^{125}I]-ω-Conotoxin GVIA Binding to the N-Type Calcium Channel of Guinea Pig Brain Membrane

Guinea pig brain membranes were prepared as follows. Guinea pig cerebrums were homogenized in 5 volumes of an ice-cooled solution of 0.1 mM phenylmethylsulfonyl fluoride and 20 mM $NaHCO_3$ in a teflon-glass homogenizer. The homogenate was centrifuged at 4,000 *g* for 15 minutes, and the pellet was suspended in 20 mM Tris-HCl buffer (pH 7.2) containing 0.1 mM phenylmethylsulfonyl fluoride. After being centrifuged at 4,000 *g* for 15 minutes, the pellet was resuspended in the same buffer and stored at ~80°C. The binding assay was carried out according to ABE's method[2]. The brain

membrane pellet (2 μg protein/tube) was incubated with [^{125}I]-ω-conotoxin GVIA (20 pM final concentration, 2,000 Ci/mM) in a total assay volume (0.2 ml) of 20 mM Tris-HCl buffer (pH 7.2) containing 0.1% BSA and 1 mM EDTA at 4°C for 60 minutes. Incubation was terminated by rapid filtration through a Whatman GF/C glass fiber filter presoaked in 0.6% polyethylenimine. After washing with ice-cooled 20 mM Tris-HCl buffer (pH 7.2), the radioactivity of the dried filter was measured by γ-counter. The non-specific binding was measured in the presence of 1 μM unlabelled ω-conotoxin GVIA.

Measurement of [^{3}H]PN 200-110 Binding to the L-Type Calcium Channel of Rabbit Skeletal Muscle T-Tube Membrane

The preparation of the membrane fraction and the binding assay were carried out by GROSSMAN's method[12]. Rabit skeletal muscle from the back and hind limb was finely minced with scissors in an ice-cooled solution of 0.1 mM phenylmethylsulfonyl fluoride and 20 mM $NaHCO_3$, and was homogenized with a Polytron homogenizer. The homogenate was centrifuged at 1,500 g for 15 minutes, and then the supernatant was filtered through gauze. The filtrate was centrifuged at 48,000 g for 15 minutes. The pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 0.1 mM phenylmethylsulfonyl fluoride, and was layered on a discontinuous sucrose gradient [30 and 25% (w/w) sucrose in 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 mM phenylmethylsulfonyl fluoride]. The gradient was centrifuged at 100,000 g for 17 hours. The membrane fraction at the 25% sucrose layer and overlay interface were collected, diluted in 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 mM phenylmethylsulfonyl fluoride, and were centrifuged at 100,000 g for 30 minutes. The pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 0.1 mM phenylmethylsulfonyl fluoride, stored at ~80°C, and used for the binding assay. The membrane fraction (5 μg protein/tube) was incubated with [^{3}H]PN 200-110 (5 nM final concentration, 87 Ci/mM) in a total volume (0.2 ml) of 50 mM Tris-HCl buffer (pH 7.4) containing 2 mM $CoCl_2$ at 4°C for 60 minutes. Incubation was terminated by rapid filtration through a Whatman GF/C glass fiber filter. The radioactivity of each filter incubated in 10% Triton-X (300 μl) overnight was measured by a liquid scintillation counter. The non-specific binding was measured in the presence of 10 μM unlabelled PN 200-110.

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