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Unnarmicins A and C, New Antibacterial Depsipeptides Produced by Marine Bacterium *Photobacterium* sp. MBIC06485

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Abstract Two new antibiotic depsipeptides, unnarmicins C (**1**) and A (**2**), were isolated from the fermentation broth of a marine bacterium, *Photobacterium* sp. strain MBIC06485. The structure of **1** was established by spectroscopic studies and chiral analyses of its chemical degradation/conversion products, and that of **2** by comparing its NMR, MS, and CD data with those of **1**. Both compounds selectively inhibited the growth of two strains belonging to the genus *Pseudovibrio*, one of the most prevalent genera in the marine environments within the class *Alphaproteobacteria*.

Keywords unnarmicin, antibacterial, *Photobacterium*, marine bacterium

Introduction

Gram-negative bacteria, especially those belonging to *Alphaproteobacteria* or *Gammaproteobacteria* are among the most abundant in the marine environment [1]. However, most studies on metabolites from marine bacteria have been made on Actinomycetes, largely as a reflection of historically proven success with their terrestrial counterparts [2～5]. To exploit the greater potential of marine microbial sources for biomedical applications, we have focused our research interest on unexploited/underexploited taxa for their bioactive metabolites. The results include discovery of cytotoxins from two strains belonging to Thermoactinomycetaceae genera [6～8] and from the *Gammaproteobacterium*, *Pelagiobacter variabilis* [9], antibiotic fatty acid derivatives from the *Gammaproteobacteria*, *Pseudoalteromonas* sp. [10] and *Vibrio gazogenes* [11], anti-cyanobacterial peptides from the *Alphaproteobacterium*, *Sphingomonas* sp. [12, 13], hydroxamate siderophores from *Pseudoalteromonas* sp. [14] and the *Bacteroidetes*, *Tenacibaculum* sp. [15], and a xanthine oxidase inhibitor from the *Alphaproteobacterium*, *Agrobacterium aurantiacum* [16].

In our antibacterial screening of marine bacterial fermentation products against several strains of cosmopolitan marine bacteria, a fermentation extract of an isolate identified as a new *Photobacterium* (*Gammaproteobacteria*) showed selective growth inhibition against two strains of *Pseudovibrio*, one of the most common Alphaproteobacterial genera frequently isolated from the marine environments. This strain is closely related to *Photobacterium leiognathi* ATCC25521^T (95% identity based on the 16S rRNA gene sequence), a light organ symbiont for the Leiognathid fish and several other invertebrates [17]. The active components responsible for this unique antibacterial profile were found to be a series of new depsipeptides which were designated as unnarmicins. The taxonomy of the producing organism and fermentation, isolation, and elucidation of the structure study of unnarmicins C (**1**) and A (**2**) (Fig. 1) are described below.

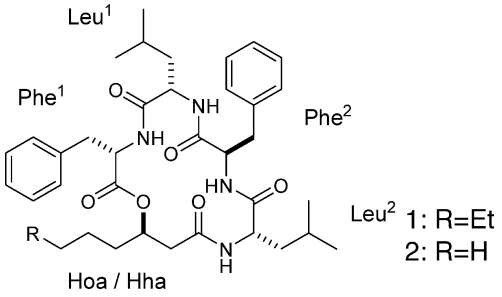

Fig. 1 Structures of unnarmicins C (**1**) and A (**2**).

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Results and Discussion

Taxonomy of Strain MBIC06485

Cells were facultatively anaerobic, motile, polar-flagellated, Gram-negative bacilli with a size of 2.8～3.1 μm in length and 0.6～0.7 μm in width. Spores were not formed. Colonies grown on Marine Agar 2216 were circular, flattened with a wavy surface, white in the center and translucent at the growing edge. The optimal temperature range for growth is 25～30°C. The pH range for growth was 5～10, optimally 6～9. NaCl was required for growth in the range of 1.0～7.0%. Tests for enzymatic activities were positive for urease, oxidase, and catalase, but negative for β-galactosidase. Nitrate was reduced. DNA and starch were hydrolyzed but not esculine. Utilization of citric acid was positive, but hydrogen sulfide and indole production were negative. Acid was produced from D-glucose, D-fructose, maltose, lactose, glycerin, but not from L-arabinose, D-xylose, D-mannose, D-galactose, sucrose, trehalose, D-sorbitol, D-mannitol, inositol, or starch. The major isoprenoid quinone was ubiquinone-8. The G+C content of DNA was 51.7%. A homology search of the 16S rRNA gene sequence of this strain by the BLAST program resulted in the closest type strain being *Photobacterium leiognathi* ATCC25521^T (GenBank/EMBL/DDBJ accession No. X74686, 95% identity). These morphological and physiological characteristics enabled the strain to be identified as a member of the genus *Photobacterium*.

Isolation

The EtOAc extract (780 mg) of the fermentation broth (10 liters) was chromatographed on silica gel eluted with $CHCl_3$/MeOH (20 : 1) to give a crude anti-*Pseudovibrio* fraction (50 mg). Purification of this fraction by reversed phase HPLC eluted with 60% aq MeCN yielded **1** and **2** as white amorphous solids.

Structure Elucidation

The molecular formula of **1** was established as $C_{38}H_{54}N_4O_6$ (*m/z* 663.4138 $[M+H]^+$, calcd for $C_{38}H_{55}N_4O_6$, 663.4122, Δ1.6 mmu) by an HRFAB-MS analysis. The ^{1}H- and ^{13}C-NMR and DEPT spectra in DMSO-d_6 were typical of those for peptides, as showing four each of amide linkages (Table 1: δ_H 7.35～8.94; δ_C 167.73～173.55) and methines in the mid-magnetic regions (δ_H 4.03～4.52; δ_C 50.09～55.88). Interpretation of the COSY and TOCSY spectra gave 5 spin systems, of which four were accounted for by a pair of Leu (Leu^1: δ_H 8.20, 4.03, 1.35, 1.14, 0.97, 0.67, 0.60; Leu^2: δ_H 7.35, 4.52, 1.43, 1.33, 1.47, 0.87, 0.83) and a pair of Phe (Phe^1: δ_H 7.82, 7.29, 7.26, 7.20, 4.36, 3.21, 3.08; Phe^2: δ_H 8.94, 7.27, 7.26, 7.20, 4.31, 2.91) residues and the remaining one by a 3-hydroxyoctanoyl moiety (=Hoa, δ 2.56, 2.14, 4.99, 1.51, 1.43, 1.06, 1.16, 1.21, 0.83). HMBC correlations (Fig. 2) from the amide protons to adjacent amide carbons, δ_H/δ_C 7.82 (Phe^1)/171.95 (Leu^1), 8.20 (Leu^1)/170.99 (Phe^2), 8.94 (Phe^2)/173.55 (Leu^2), revealed an alternating alignment of Phe and Leu, whereas HMBC correlations δ_H/δ_C 7.35/167.73, 4.99/167.73, and 4.99/169.89 were respective evidence for *N*- and *C*-terminal attachment of Hoa to Leu^2 and Phe^1, thereby completing the peptide lactone structure for **1**.

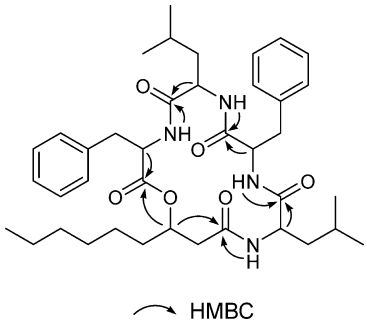


Fig. 2 Key HMBC correlations for **1**.

A chiral analysis of the amino acid components in **1** after derivatizing its acid-hydrolysate with L-FDAA (=(5-fluoro-2,4-dinitrophenyl)-L-alaninamide, Marfey's reagent) revealed the presence of L-Leu and an enantiomeric pair of Phe (Scheme 1). To specify the stereochemistry of each Phe residue, **1** was reduced by $LiBH_4$ and resulting linear product **3** was subjected to the same analytical protocol. Since a new peak (Rt 25.1 minutes, attributable to L-phenylalaninol : L-Phl) appeared in exchange for the disappearance of the L-Phe peak (Rt 23.2 minutes), L- and D-stereochemistry were assigned for Phe^1 and Phe^2, respectively.

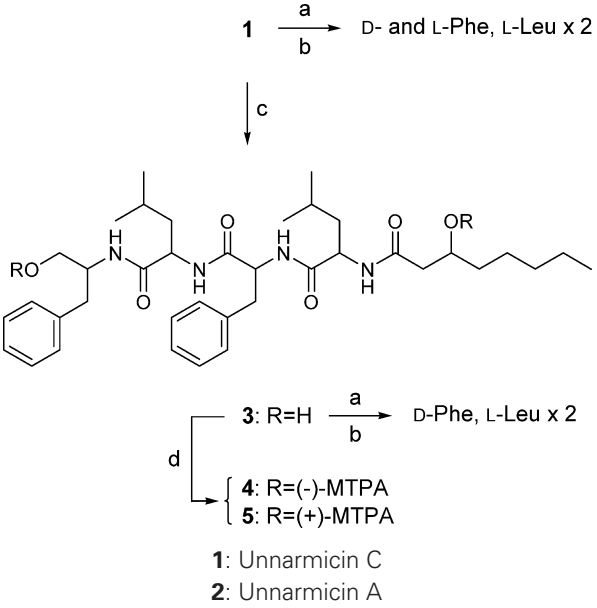
The absolute configuration of C3 in Hoa was determined by applying the modified Mosher method to **3**. The plus and minus $\Delta\delta$ values consistently resided towards the ω- and carboxyl termini, respectively (Fig. 3), enabling the *R*-configuration to be concluded for this stereocenter.

The HRFAB-MS spectrum for **2** gave a molecular ion at *m/z* 635.3812 ($[M+H]^+$, calcd. for $C_{36}H_{51}N_4O_6$, 635.3809, +Δ0.3 mmu), revealing the molecular formula of **2** to be two methylene units smaller than that of **1**. An analysis of the NMR data for **2** immediately disclosed that this difference was due to replacement of the Hoa unit in **1** with a 3-hydroxyhexanoyl (Hha) group. The stereochemistry of **2** was concluded to be the same as that of **1** based on a CD spectral comparison, in which **1** and **2** showed almost identical spectra (Fig. 4).

Table 1 NMR data for unnarmicins C (**1**) and A (**2**)

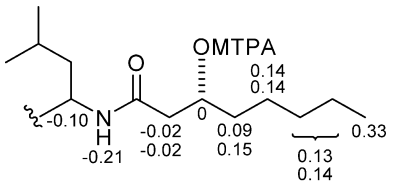
	1			**2**			
Position	δ_C (m)	δ_H	J (Hz)	Position	δ_C (m)	δ_H	J (Hz)
Phe[1]				Phe[1]			
N*H*		7.82	1H, d, 8.8	N*H*		7.80	1H, d, 8.8
α	54.67 (d)	4.36	1H, dt, 5.6, 8.9	α	54.65 (d)	4.36	1H, dt, 5.6, 8.9
β	37.10 (t)	3.21	1H, dd, 9.1, 14.2	β	37.13 (t)	3.19	1H, dd, 9.5, 14.2
		3.08	1H, dd, 5.6, 14.2			3.08	1H, dd, 5.6, 14.2
1	137.57 (s)			1	137.52 (s)		
2/6	128.95 (d)	7.29	2H, m	2/6	128.95 (d)	7.28	2H, m
3/5	127.82 (d)	7.26	1H, m	3/5	127.83 (d)	7.25	1H, m
4	126.18 (d)	7.20	1H, m	4	126.20 (d)	7.19	1H, m
*C*O	169.89 (s)			*C*O	169.85 (s)		
Leu[1]				Leu[1]			
N*H*		8.20	1H, d, 9.0	N*H*		8.20	1H, d, 9.0
α	50.79 (d)	4.03	1H, ddd, 3.3, 9.0,12.4	α	50.85 (d)	3.98	1H, m
β	39.34 (t)	1.35	1H, m	β	39.37 (t)	1.33	1H, m
		1.14	1H, m			1.12	1H, m
γ	23.44 (d)	0.97	1H, m	γ	23.44 (d)	0.97	1H, m
δ	23.13 (q)	0.67	3H, d, 6.7	δ	23.13 (q)	0.67	3H, d, 6.7
δ'	20.58 (q)	0.60	3H, d, 6.4	δ'	20.62 (q)	0.59	3H, d, 6.7
*C*O	171.95 (s)			*C*O	171.97 (s)		
Phe[2]				Phe[2]			
N*H*		8.94	1H, d, 5.8	N*H*		8.92	1H, d, 5.8
α	55.88 (d)	4.31	1H, dt, 5.8, 7.7	α	55.87 (d)	4.30	1H, dt, 6.0, 7.8
β	35.94 (t)	2.91	2H, d, 7.7	β	35.94 (t)	2.91	2H, d, 7.7
1	136.92 (s)			1	136.89 (s)		
2/6	129.06 (d)	7.27	2H, m	2/6	129.06 (d)	7.27	2H, m
3/5	128.05 (d)	7.26	1H, m	3/5	128.06 (d)	7.25	1H, m
4	126.07 (d)	7.20	1H, m	4	126.08 (d)	7.19	1H, m
*C*O	170.99 (s)			*C*O	171.00 (s)		
Leu[2]				Leu[2]			
N*H*		7.35	1H, d, 8.6	N*H*		7.35	1H, d, 8.6
α	50.09 (d)	4.52	1H, dt, 6.3, 9.0	α	50.10 (d)	4.52	1H, dt, 6.3, 9.0
β	40.78 (t)	1.43	1H, m	β	40.77 (t)	1.43	1H, m
		1.33	1H, m			1.33	1H, m
γ	24.18 (d)	1.47	1H, m	γ	24.18 (d)	1.47	1H, m
δ	22.66 (q)	0.87	3H, d, 6.5	δ	22.67 (q)	0.88	3H, d, 6.3
δ'	21.86 (q)	0.83	3H, t, 7.5	δ'	21.85 (q)	0.84	3H, d, 6.7
*C*O	173.55 (s)			*C*O	173.52 (s)		
Hoa				Hha			
1	167.73 (s)			1	167.73 (s)		
2	39.50 (t)	2.56	1H, dd, 4.2, 13.5	2	39.45 (t)	2.57	1H, dd, 4.2, 13.5
		2.14	1H, dd, 4.2, 13.5			2.14	1H, dd, 4.2, 13.5
3	71.55 (d)	4.99	1H, m	3	71.23 (d)	5.01	1H, m
4	32.00 (t)	1.51	1H, m	4	34.12 (t)	1.51	1H, m
		1.43	1H, m			1.43	1H, m
5	24.66 (t)	1.06	2H, m	5	18.19 (t)	1.08	2H, m
6	30.84 (t)	1.16	2H, m	6	13.59 (q)	0.79	3H, d, 6.7
7	21.91 (t)	1.21	2H, m				
8	13.80 (q)	0.83	3H, t, 7.5				

The antibacterial activity of **1** and **2** against two Gram-positive bacteria (*Firmicutes*) and seven halophilic Gram negative bacteria belonging to *Bacteroidetes*, *Alphaproteobacteria*, and *Gammaproteobacteria* were evaluated by a plate diffusion assay. As summarized in Table 2, the peptides specifically inhibited the growth of two *Pseudovibrio* strains (*Alphaproteobacteria*). Such unique action suggests potential application of the peptides, in combination with korormicin [10] which is a selective antibiotic against halophilic *Gammaproteobacteria*, as an ideal selection additive to media to suppress fast-growing bacteria that often dominate on solidified media and hamper the isolation/purification of slow-growing colonies. The few precedents of secondary metabolites identified from *Photobacterium* are all pteridines [18, 19], thus the



Scheme 1

Reagents and conditions: (a) 6 N HCl, 110°C, 18 hours; (b) L-FDAA, 0.1 N $NaHCO_3$/Me_2CO (2 : 1), 70°C; (c) $LiBH_4$, THF, 0°C to rt, 2 hours; (d) (−)- or (+)-MTPACl, pyridine, rt, 2 hours.



$\Delta\delta=\delta_H(\mathbf{4})-\delta_H(\mathbf{5})$

Fig. 3 Distribution of $\delta\Delta$ values calculated for the Hoa unit.

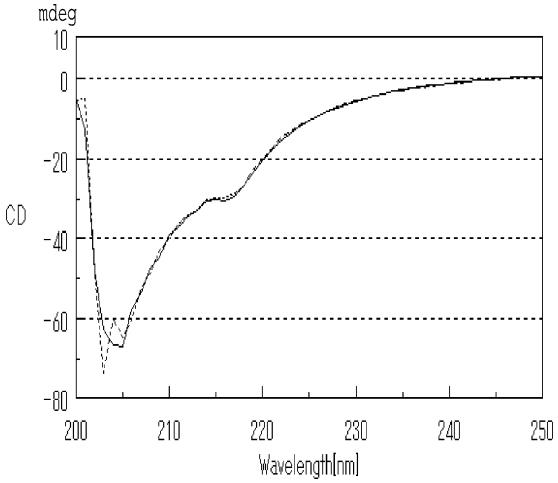


Fig. 4 Superimposed CD spectra for **1** (solid line) and **2** (dotted line) in MeOH. Concn.: 80 μM

Table 2 Antibacterial activities of **1** and **2** in the plate diffusion assay

Tested strains	**1**		**2**		PG[a]	PB[b]	CP[c]	Koro[d]
	13[e]	2.6	13	2.6	2.6	2.6	2.6	2.6
Staphylococcus aureus IFO12732					13[f]	24	14	
Bacillus subtilis IFO3134					9	24	19	
Cytophaga marinoflava IFO14170								
Pseudovibrio denitrificans JCM12308	7	7	18	10	15			
Pseudovibrio sp. MBIC3368	8	7	12	9	31			
Agrobacterium kieliense IAM12618					14		16	
Caulobacter halobacteroides NCIMB2022					13			
Salinivibrio costicola ATCC33508						9		25
Vibrio harveyi IFO15634							13	20

[a] Penicillin G. [b] Polymyxin B. [c] Chloramphenicol. [d] Korormicin. [e] Amount of compound (mg) applied to the paper disk. [f] Diameter of inhibition zone (mm).

discovery of the unnarmicins demonstrates a rather richer biosynthetic potential for this genus. Details of the biological properties of **1** and **2** will be published elsewhere.

Experimental

General Experimental Procedures

Optical rotations were measured with a Horiba SEPA-300 high sensitivity polarimeter. UV and IR spectra were obtained with Beckman DU640 and Jasco FT-IR 7000 spectrophotometers, respectively, whereas CD spectra with Jasco J-725 spectropolarimeter in MeOH. NMR spectra were recorded with Varian UNITY INOVA 750 or 500 spectrometers, referencing chemical shifts to the residual solvent signals at δ_H 2.50 and δ_C 39.5 ppm in DMSO-d_6 and at δ_H 7.25 and δ_C 77.0 ppm in $CDCl_3$. Mass spectra were measured with a Jeol JMS-SX102A instrument for HRFAB-MS and with a ThermoFinigan LCQ Advantage instrument for ESI-MS.

Producing Microorganisms and Fermentation

Photobacterium sp. strain MBIC06485 was isolated from coastal seawater collected off Onna Beach, Okinawa, Japan. A subculture of this isolate has been deposited at National Institute of Technology and Evaluation (Kisarazu, Chiba, Japan) with accession number NITE P-62.

To produce the unnarmicins, a 2-ml seed culture of strain MBIC06485 grown in Marine Broth 2216 (Difco) was transferred to 300 ml of the same medium in a 1-liter baffled Erlenmeyer flask. The flask was shaken at 100 rpm and 30°C for 4 days before harvesting the fermentation products.

Isolation of 1 and 2

The fermentation broth was centrifuged at 6000 rpm for 20 minutes to remove the bacterial cells. The resulting supernatant (10 liters) was extracted with EtOAc and the extract was dried over anhydrous Na_2SO_4, after which the solvent was removed *in vacuo* to give an oily solid (780 mg). This material was fractionated by SiO_2 chromatography using isocratic elution with $CHCl_3$: MeOH (20 : 1), and the combined antibacterial fraction (50 mg) against *Pseudovibrio* sp. MBIC3368 was purified by reversed-phase HPLC (Shiseido CAPCELL PAK C18 UG120 column, 20×250 mm, 60% aq MeCN as a solvent, detection at 210 nm) to yield **1** (6.0 mg) and **2** (3.7 mg).

Unnarmicin C (**1**)

White amorphous solid; $[\alpha]_D^{30}$ 67° (*c* 0.084, MeOH); UV (MeOH) λ_{max} 258.5 nm (ε 400); CD (MeOH) λ_{ext} 216 nm ($\Delta\varepsilon$ −12, sh), 205 (−65); IR (KBr) ν_{max} 3297, 2956, 2927, 2854, 1747, 1717, 1670, 1654, 2359, 1691, 1631, 1557, 1542, 1523, 1508 cm^{-1}; HRFAB-MS *m/z* 663.4138 $[M+H]^+$, calcd. for $C_{38}H_{55}N_4O_6$, 663.4122. ^{1}H- and ^{13}C-NMR data, see Table 1.

Unnarmicin A (**2**)

White amorphous solid; $[\alpha]_D^{30}$ 70° (*c* 0.19, MeOH); UV (MeOH) λ_{max} 258.5 nm (ε 410); CD (MeOH) λ_{ext} 216 nm ($\Delta\varepsilon$ −12, sh), 205 (−67); IR (KBr) ν_{max} 3297, 2956, 2927, 2854, 1747, 1717, 1670, 1654, 2359, 1691, 1631, 1557, 1542, 1523, 1508 cm^{-1}; HRFAB-MS *m/z* 635.3812 $[M+H]^+$, calcd. for $C_{36}H_{51}N_4O_6$ for 635.3809. ^{1}H- and ^{13}C-NMR data, see Table 1.

Antibacterial Assay

The antibacterial activities of **1** and **2** was evaluated by the paper disk method. A liquid culture of each test strain (4.0 ml, cultured for 24 hours) was smeared with a sterile cotton-tipped swab onto a Marine Agar 2216 (Difco) or Nutrient Agar (Difco) medium using a sterile cotton-tipped swab. Methanolic solutions of the peptides were prepared at concentrations of 1.0 and 0.2 mg/ml, and a 13 μl-aliquot from each solution was applied to a 6 mm-paper disk. The disks were air-dried for 10∼20 minutes and then transferred to the test plates, which were incubated at 30°C for 24 hours. The antibacterial potency of each peptide was evaluated by measuring the diameter of the generated inhibitory zones.

Chiral Analysis of the Component Amino Acids in 1

A 50-μg portion of **1** was hydrolyzed in constant-boiling hydrochloric acid (6 N, 200 μl) at 105°C for 19 hours in a N_2 atmosphere. The dried acid hydrolysate, which was obtained after removing hydrochloric acid under a stream of N_2, was dissolved in 0.1 N aq $NaHCO_3$ (100 μl) and reacted with L-FDAA (0.1% solution in Me_2CO, 50 μl) at 50°C for 30 minutes. The reaction was quenched with 0.2 N hydrochloric acid (50 μl) and the mixture was diluted with 50% aq MeCN containing 50 mM NH_4OAc (100 μl) to give an analyte solution. A 10-μl aliquot of this solution was analyzed by reversed-phase HPLC on a Cosmosil AR-II column (4.6×150 mm) with the elution program of 15% MeCN - 50 mM NH_4OAc for 5 minutes (1.0 ml/minute flow rate), 0.67%/minute concentration gradient of MeCN to 45%, and then an 11%/minute gradient to 100%, and the same concentration held for 5 minutes. Analytical standards were prepared from 0.1 mg each of authentic amino acids with the same derivatization procedure as that already described. Retention times (minute): L-Leu, 21.3:

L-Phe, 23.2; D-Leu, 28.5; D-Phe, 29.5; acid hydrolysate of **1**, 21.3, 23.2, and 29.5.

Stereochemical Analysis of the Phe Residues in 1

To the peptide (1.5 mg) in a sealed pear-shaped flask was added a solution of $LiBH_4$ in THF (2.0 M, 200 μl) and the reaction mixture was stirred first at 0°C for 5 minutes and then at rt for 2 hours. The mixture was diluted with Et_2O (0.5 ml) and the reaction was quenched by stirring with a saturated aqueous NH_4Cl solution (0.2 ml) until the generation of bubbles had ceased. The mixture was again diluted with Et_2O (4.0 ml) and dried over anhydrous $MgSO_4$ for 10 minutes. The Et_2O solution and the washings were passed through a plug of glasswool layered on cotton in a Pasteur pipette. The residue in the flask was dissolved in H_2O and loaded onto ODS (2 ml), which was successively eluted with H_2O and MeOH. The MeOH eluate was combined with the ethereal filtrate and purified by HPLC on a Cosmosil AR-II column (1×25 cm) with a gradient elution with aq MeCN (30～60%) to give linear reduction product **3** (0.2 mg): ESI-MS *m*/*z* 667.4 $[M+H]^+$; ^{1}H-NMR (750 MHz, $CDCl_3$) δ 3.76 (brs, 1H, CH_2OH, Phl), 3.74 (d, J=12.2 Hz, 1H, -CH_2OH, Phl), 3.42 (m, 1H, -CH_2OH, Phl), 4.10 (m, 1H, αH, Phl), 2.87 (m, 1H, βHa, Phl), 2.97 (m, 1H, βHb, Phl), 7.25 (H2/6, Phl), 7.27 (H3/5, Phl), 7.19 (H4, Phl), 6.84 (d, J=12.2 Hz, N*H*, Phl), 4.08 (αH, Leu^1), 1.57 (βHa, Leu^1), 1.16 (βHb, Leu^1), 0.84 (m, 1H, γH, Leu^1), 0.66 (d, J=6.4 Hz, 3H, δH, Leu^1), 0.71 (d, J=6.7 Hz, 3H, δ'H, Leu^1), 5.79 (d, J=7.3 Hz, N*H*, Leu^1), 4.20 (m, 1H, αH, Phe), 3.00～3.04 (m, 1H, βH, Phe), 7.17 (H2/6, Phe), 7.30 (H3/5, Phe), 7.26 (H4, Phe), 7.27 (N*H*, Phe), 4.47 (αH, Leu^2), 1.55 (βHa, Leu^2), 1.75 (βHb, Leu^2), 1.63 (γH, Leu^2), 0.92 (δH, Leu^2), 0.93 (δ'H, Leu^2), 6.76 (d, J=8.7 Hz, N*H*, Leu^2), 2.31 (dd, J=14.2, 6.2 Hz, H2a, Hoc), 2.50 (dd, J=3.9, 14.3 Hz, H2b, Hoc), 3.94 (H3, Hoc), 3.86 (brs, O*H*, Hoc), 1.61 (H4a, Hoc), 1.70 (m, H4b, Hoc), 1.40 (H5, Hoc), 1.49 (H5, Hoc), 1.35 (H6, Hoc), 1.32～1.35 (H7, Hoc), 0.90 (H8, Hoc); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 62.8 (-*C*H_2OH, Phl), 52.9 (αC, Phl), 36.8 (βC, Phl), 138.5 (C1, Phl), 129.4 (C2/6, Phl), 128.5 (C3/5, Phl), 126.5 (C4, Phl), 171.2 (*C*O, Leu^1), 53.2 (αC, Leu^1), 40.3 (βC, Leu^1), 24.5 (γC, Leu^1), 21.5 (δC, Leu^1), 23.3 (δ'C, Leu^1), 172.2 (*C*O, Phe), 57.5 (αC, Phl), 37.3 (βC, Phl), 135.9 (C1, Phl), 129.1 (C2/6, Phl), 128.9 (C3/5, Phl), 127.3 (C4, Phl), 173.6 (*C*O, Leu^2), 50.9 (αC, Leu^2), 38.8 (βC, Leu^2), 25.0 (γC, Leu^2), 22.5 (δC, Leu^1), 23.0 (δ'C, Leu^1), 173.2 (C1, Hoa), 43.1 (C2, Hoa), 69.3 (C3, Hoa), 36.6 (C4, Hoa), 25.8 (C5, Hoa), 22.8 (C6, Hoa), 31.9 (C7, Hoa), 14.2 (C8, Hoa). A 10 μg-portion of **3** was hydrolyzed and subjected to the same analytical protocol as described in the former section, resulting in a loss of the L-Phe peak. Retention time (minute): acid-hydrolysate of **3**, 21.2 and 29.3.

Preparation of *Bis*-MTPA Esters from Linear Derivative 3

To each 0.1 mg-aliquot of derivative **3** in a V-bottom vial was added 10 drops of dry pyridine and (−)- or (+)-MTPACl (9 μl). The reaction vial was vigorously stirred at rt for 30 minutes and then centrifuged at 6000 rpm for 1.5 hours. After removing pyridine and the excess reagent by lyophilization, the residue was purified by reversed-phase HPLC (Cosmosil AR-II column, 1×25 cm, 50% aq MeCN to MeCN as the solvent) to give *bis*-MTPA esters, respectively.

Bis-(−)-MTPA Ester (**4**)

^{1}H-NMR (750 MHz, $CDCl_3$) δ 4.47 (-CH_2OH, Phl), 4.12 (m, 1H, αH, Phl), 2.76 (m, 1H, βHa, Phl), 2.86 (m, 1H, βHb, Phl), 6.97 (br, N*H*, Phl), 4.30 (αH, Leu^1), 1.51 (βH, Leu^1), 1.23 (γH, Leu^1), 0.78 (d, J=6.8 Hz, 3H, δH, Leu^1), 0.79 (d, J=6.8 Hz, 3H, δ'H, Leu^1), 6.30 (br, N*H*, Leu^1), 4.54 (αH, Phe), 2.77 (m, βHa, Phe), 3.21 (dd, J=6.9, 13.6 Hz, βHb, Phe), 6.76 (br, N*H*, Phe), 4.11 (αH, Leu^2), 1.47 (βH, Leu^2), 1.18 (γH, Leu^2), 0.77 (d, J=6.0 Hz, δH, Leu^2), 0.80 (d, J=6.0 Hz, δ'H, Leu^2), 6.07 (d, J=6.0 Hz, N*H*, Leu^2), 2.44 (dd, J=15.0, 4.0 Hz, H2a, Hoc), 2.50 (dd, J=7.8, 15.0 Hz, H2b, Hoc), 5.50 (m, H3, Hoc), 1.69 (H4a, Hoc), 1.75 (H4b, Hoc), 1.28～1.31 (H5, Hoc), 1.27～1.32 (H6/7, Hoc), 0.85 (t, J=6.8 Hz, H8, Hoc), 3.46, (s, CH_3O-, MTPA) 3.50 (s, CH_3O-, MTPA). Signals for phenyl rings were not assigned.

Bis-(+)-MTPA Ester (**5**)

^{1}H-NMR (750 MHz, $CDCl_3$) δ 4.12 (dd, J=3.4, 10.6 Hz, -CH_2OH, Phl), 4.51 (dd, J=7.3, 10.5 Hz, -CH_2OH, Phl), 4.466 (m, 1H, αH, Phl), 2.816 (m, 1H, βHa, Phl), 2.88 (dd, J=7.1, 13.9 Hz, 1H, βHb, Phl), 7.04 (br N*H*, Phl), 4.31 (αH, Leu^1), 1.52 (βH, Leu^1), 1.16 (γH, Leu^1), 0.76 (d, J=6.6 Hz, 3H, δ/δ'H, Leu^1), 6.08 (d, J=7.8 Hz, N*H*, Leu^1), 4.455 (αH, Phe), 2.814 (βHa, Phe), 3.19 (dd, J=7.4, 13.8 Hz, βHb, Phe), 6.87 (br, N*H*, Phe), 4.21 (dt, J=7.1, 7.1 Hz, αH, Leu^2), 1.51 (βH, Leu^2), 1.20 (γH, Leu^2), 0.77 (d, J=5.9 Hz, δH, Leu^2), 0.78 (d, J=5.9 Hz, δ'H, Leu^2), 6.28 (d, J=6.5 Hz, N*H*, Leu^2), 2.46 (dd, J=15.2, 4.3 Hz, H2a, Hoc), 2.52 (dd, J=8.3, 15.2 Hz, H2b, Hoc), 5.50 (m, H3, Hoc), 1.60 (H4a, Hoc), 1.14～1.17 (H5, Hoc), 1.13～1.19 (H6/7, Hoc), 0.82 (t, J=6.9 Hz, H8, Hoc), 3.52 (s, CH_3O-, MTPA) 3.50 (s, CH_3O-, MTPA). Signals for phenyl rings were not assigned.

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