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New Cyclic Depsipeptide Antibiotics, Clavariopsins A and B, Produced by an Aquatic Hyphomycetes, *Clavariopsis aquatica*

2. Structure Analysis

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The structures of new cyclic decadepsipeptides, clavariopsins A and B, were determined to be *cyclo*[-(*R*)-2-hydroxyisovaleryl-L-pipecolyl-L-MeVal-L-Val-L-MeAsp-L-MeIle-L-MeIle-Gly-L-MeVal-L-Tyr(OMe)-] and *cyclo*[-(*R*)-2-hydroxyisovaleryl-L-pipecolyl-L-Val-L-Val-L-MeAsp-L-MeIle-L-MeIle-Gly-L-MeVal-L-Tyr(OMe)-], respectively, by spectroscopic analyses, especially using 2D NMR techniques. The absolute stereochemistry was elucidated by the advanced Marfey's method and chiral HPLC analysis.

Clavariopsins A and B were found from a culture broth of the aquatic hyphomycetes *Clavariopsis aquatica* as antifungal metabolites. The production and biological activity of these compounds have been described in the preceding paper[1)].

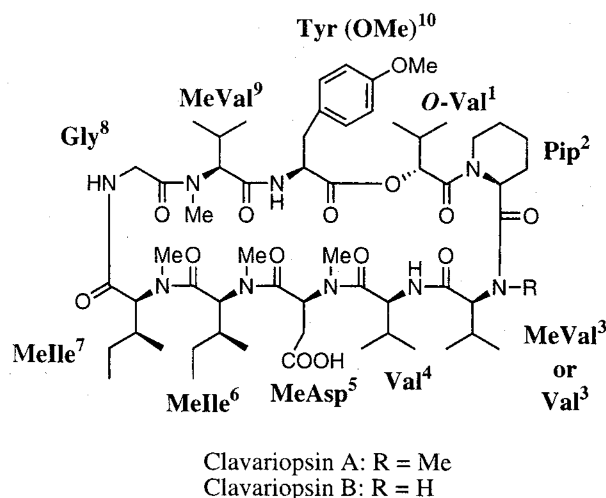
In the present paper we describe the structural elucidation of these compounds.

Results

Spectroscopic Analysis

The molecular formula of clavariopsin A was determined to be $C_{59}H_{95}N_9O_{14}$ by high-resolution FAB-MS. The absorption bands at 1739, 1683 and 1648 cm^{-1} in the IR spectrum of clavariopsin A suggest the presence of ester, carboxyl (hydrogen-bonded) and amide groups, respectively[1)]. The ^1H and ^{13}C NMR data for clavariopsin A are summarized in Table 1. The direct connectivity between protons and carbons was established by a heteronuclear multiple quantum coherence (HMQC) spectrum. In the ^1H NMR spectrum, the signals around 4~7 ppm due to the

Fig. 1. Structures of clavariopsins A and B.



Clavariopsin A: R = Me
Clavariopsin B: R = H

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Table 1. NMR data for clavariopsin A in C_6D_6.

Position	$^1H^a$	$^{13}C^b$	Position	$^1H^a$	$^{13}C^b$
O-Val[1c]			MeIle[6]		
α	5.27 d (3.5)	75.1 d	α	5.43 d (11.1)	56.7 d
β	1.75 m	30.6 d	β	2.30 m	32.8 d
γ CH_3	0.95 d (6.8)	19.0 q	γ CH_2	1.27 m	26.1 t
γ CH_3	0.92 d (6.8)	16.3 q		1.01 m	
CO		172.0 s	δ CH_3	0.83 d (6.7)	15.2 q
Pip[2d]			δ CH_3	0.77 t (7.4)	11.0 q
α	5.74 d (6.5)	46.8 d	CO		171.1 s
β	1.64 br. d (13.6)	28.2 t	NCH_3	3.18 s	30.2 q
	1.20 m		MeIle[7]		
γ	2.20 m	18.9 t	α	2.95 d (10.1)	74.9 d
	1.16 m		β	3.02 m	34.1 d
δ	1.27 m	25.3 t	γ CH_2	1.49 m	24.7 t
	0.89 m			0.88 m	
ε	4.25 ddd (13.5, 13.5, 2.7)	43.4 t	γ CH_3	1.16 d (6.4)	17.8 q
	3.09 br. d (13.5)		δ CH_3	0.80 m	9.7 q
CO		171.6 s	CO		171.1 s
MeVal[3]			NCH_3	3.11 s	41.1 q
α	4.54 d (10.7)	67.4 d	Gly[8]		
β	2.52 m	26.4 d	α	4.12 dd (17.2, 6.0)	41.6 t
γ CH_3	1,27 d (6.5)	19.8 q		3.60 dd (17.2, 2.8)	
γ CH_3	0.73 d (6.8)	19.1 q	CO		169.1 s
CO		168.2 s	NH	7.50 dd (6.0, 2.8)	
NCH_3	2.90 s	28.8 s	MeVal[9]		
Val[4]			α	5.00 d (10.9)	62.4 d
α	4.80 dd (10.4, 10.4)	55.2 d	β	2.24 m	26.9 d
β	2.41 m	29.5 d	γ CH_3	1.08 d (6.6)	19.9 q
γ CH_3	0.90 d (6.9)	18.1 q	γ CH_3	0.66 d (6.7)	18.6 q
γ CH_3	0.88 d (6.4)	20.3 q	CO		169.1 s
CO		171.5 s	NCH_3	2.24 s	28.4 s
NH	7.27 d (10.4)		Tyr(OMe)[10]		
MeAsp[5]			α	5.60 ddd (12.2, 10.0, 5.0)	51.3 d
α	6.58 dd (11.5, 5.4)	52.7 d	β	3.66 dd (13.7, 12.2)	35.0 t
β	3.54 dd (13.0, 11.5)	35.6 t		3.51 dd (13.7, 5.0)	
	3.17 m		1'		130.4 s
γ COOH		172.0 s	2', 6'	7.10 d (8.5)	130.9 d
CO		169.8 s	3', 5'	6.79 d (8.5)	113.8 d
NCH_3	3.17 s	30.9 s	4'		158.8 q
			4'-OMe	3.56 s	55.1 q
			CO		171.8 s
			NH	7.68 d (10.0)	

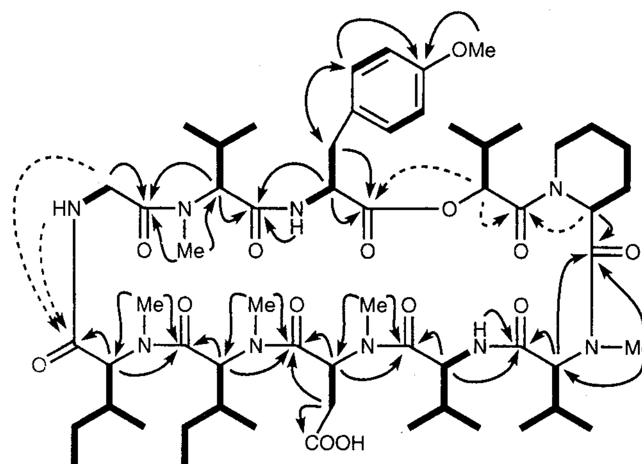
[a] Recorded at 600 MHz. Coupling constants in Hz are in parenthesis.
[b] Recorded at 150 MHz.
[c] *O*-Val = 2-hydroxyisovaleric acid
[d] Pip = pipecolic acid

α-protons of several amino acid residues and the exchangeable signals around 7~8 ppm could be due to amide NH protons. The five singlet methyl groups observed at δ_H 2.24~3.18 and one singlet methyl group at δ_H 3.56 are thought to connect to a nitrogen atom and an oxygen atom because of their carbon chemical shifts of δ_C 28.4~41.1 and δ_C 55.1, respectively. The eleven carbonyl carbons are observed at δ_C 168.2~172.0. These data suggest that clavariopsin A is a highly methylated peptide or peptide like compound. Further analyses by double quantum filter

COSY (DQF-COSY) and homonuclear Hartmann-Harn (HOHAHA) experiments revealed the partial structures of nine amino acid residues and an α-hydroxy acid (2-hydroxyisovaleric acid: *O*-Val) residue. These partial structures and the rest of the molecule consisting of eleven carbonyl, six singlet methyl (five NMe and one OMe), and heteroatoms (six nitrogens and two oxygens) were connected by a heteronuclear multiple-bond connectivity (HMBC) spectrum to give the gross structure of clavariopsin A (Fig. 2). Although some important HMBC correlations were not observed when measured in C_6D_6, those were detected using $CDCl_3$ as a solvent. Although the reason for the unusual low-field shifts of the α-carbons of MeVal[3] (δ 67.4) and MeIle[7] (δ 74.9) is unclear, we estimate the presence of some conformational effects; these residues could be located at turn structures in the peptide backbone (unpublished results). The HMBC correlations between *N*-methyl proton and α-carbon elucidate the presence of these residues (Fig. 2).

The gross structure of the minor metabolite clavariopsin B was determined in the same way as that for clavariopsin A. The ^{1}H NMR data (experimental section) were quite similar to those for clavariopsin A except that the signal due to an *N*-methyl group (δ 2.90, s) disappeared and the signal due to an NH proton (δ 6.71, d) newly appeared. These data were supported by the molecular formula of clavariopsin B, $C_{58}H_{93}N_9O_{14}$, which is less than that of clavariopsin A by CH_2. The HMBC correlation between NH of Val[3] and C=O of pipecolic acid (Pip[2]) showed that the *N*-methyl group of MeVal[3] of clavariopsin A was replaced with the new NH group in clavariopsin B.

Fig. 2. Gross structure of clavariopsin A.



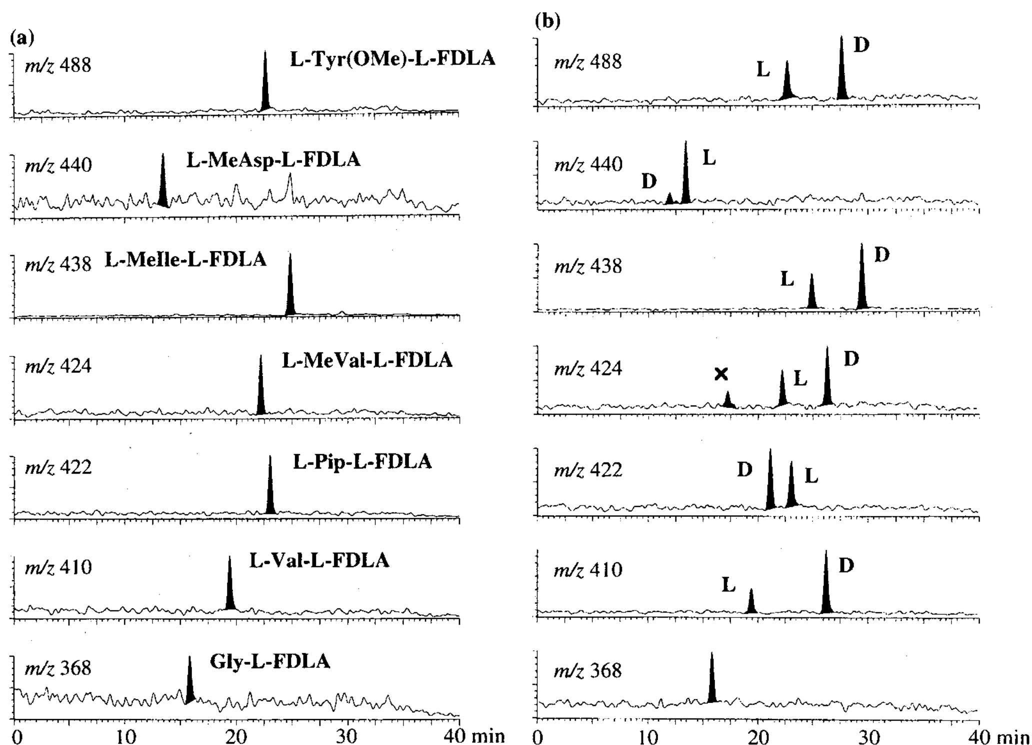
Bold lines show DQF-COSY and/or HOHAHA correlations. Solid and dotted arrows show HMBC correlations in C_6D_6 and $CDCl_3$, respectively.

Stereochemistry

To determine the absolute configuration of the amino acid constituents in clavariopsin A, we applied the "advanced MARFEY's method"[2~4]. An acid hydrolysate (6 N HCl, 110°C, 2 hours) of clavariopsin A was derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) and D-FDLA, and then analyzed by LC/MS using negative-mode electrospray ionization (ESI). Fig. 3 shows the mass chromatograms obtained by monitoring at *m/z* values for the deprotonated pseudomolecular ion $[M-H]^-$ of the amino acid constituents derivatized with FDLA. The desired peaks of L-FDLA derivatives of seven kinds of amino acids were clearly detected in the mass chromatograms monitored at the respective *m/z* values (Fig. 3a). In the mass chromatograms of the hydrolysate derivatized with DL-FDLA (Fig. 3b), the corresponding diastereomers (D-FDLA derivatives) newly appeared. It is reported that L-amino acid-L-FDLA derivatives are usually eluted prior to the D-amino acid-L-FDLA (and also L-amino acid-D-FDLA) under reversed-phase conditions in spite of a few exceptions[2,3]. However, since the L-FDLA derivatives of DL-Pip and *N*-methyl-DL-aspartic acid (DL-MeAsp) gave the opposite elution order (D-amino acid-L-FDLA→L-L) in an experiment using standard amino acids, both Pip[2] and MeAsp[5] have the L-configuration as well as the other amino acid constituents. The mass chromatogram of MeIle-L-FDLA derivatized from the hydrolysate was compared with that of the four standard stereoisomers [*i.e.* L-MeIle-L-FDLA (L-L), *allo*-D-L, L-D (=D-L), and *allo*-D-D (=*allo*-L-L)], and the retention time was identical with L-MeIle-L-FDLA (data not shown). From these results, we concluded that all the nine amino acid constituents of clavariopsin A had the L-configuration.

Since hydroxyl group is known to be much less reactive with FDLA, we could not determine the absolute configuration of 2-hydroxyisovaleric acid (*O*-Val) moiety of clavariopsin A by the above method. Hence, we carried out the HPLC analysis of *O*-Val using a chiral stationary phase. After thorough hydrolysis (6 N HCl, 110°C, 65 hours) of clavariopsin A, the resulting hydrolysate was separated by reversed-phase HPLC to give *O*-Val, which was analyzed by chiral HPLC (Fig. 4). The analysis of the standard samples showed that (*S*)-*O*-Val was eluted prior to (*R*)-isomer (Fig. 4b), which is the opposite elution order to usual amino acids under the same HPLC conditions. On the

Fig. 3. Mass chromatograms of the L-FDLA derivatives (a) and the DL-FDLA derivatives (b) of a clavariopsin A hydrolysate using negative-mode ESI LC/MS.

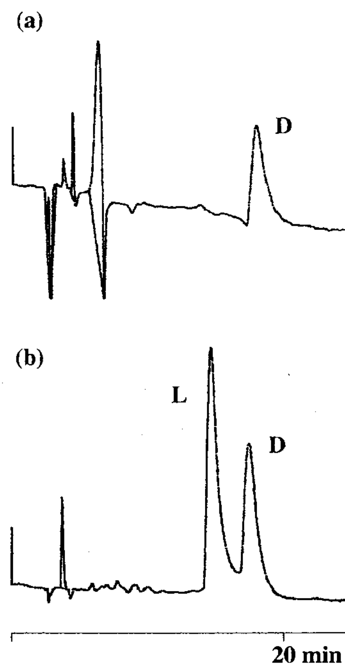


other hand, *O*-Val derived from clavariopsin A was eluted at the retention time identical with (*R*)-isomer (Fig. 4a). We thus concluded that the *O*-Val moiety of clavariopsin A had the D-configuration, and that clavariopsin A was *cyclo*[-(*R*)-2-hydroxyisovaleryl-L-Pip-L-MeVal-L-Val-L-MeAsp-L-MeIle-L-MeIle-Gly-L-MeVal-L-Tyr(OMe)-]. Clavariopsin B is thought to possess the same configurations because of similarity in the NMR spectra and optical rotations.

Discussion

Clavariopsins A and B are cyclic depsipeptides containing uncommon *N*-methylated or *O*-methylated amino acids and an α-hydroxy acid. All the nine amino acid residues of clavariopsins have the L-configuration but the 2-hydroxyisovaleric acid (*O*-Val^1) residue has the D-(*S*-) configuration. These characteristic peptides are structurally related to antifungal depsipeptides, aureobasidins[5] and much similar to a cyclopeptolide[6]. Cyclopeptolide exhibits antifungal activity at acidic pH against *Candida* species[6] but its mode of action is still unknown. On the other hand,

Fig. 4. Chiral-phase HPLC chromatograms of *O*-Val derived from a clavariopsin A hydrolysate (a) and *O*-Val standard (D : L = 1 : 3) (b).



several derivatives of cyclopeptolide such as SDZ 280-446 have the ability to chemosensitize P-glycoprotein-mediated multidrug resistance of tumor cells *in vitro* and *in vivo*[7]. Since clavariopsins and cyclopeptolide are closely resemble, clavariopsins are thought to have the same activities as cyclopeptolide.

Experimental

General

Organic extracts were dried over anhydrous Na_2SO_4, and solvents were evaporated with a rotary evaporator under reduced pressure. HPLC was performed on a JASCO high-pressure gradient system with PU-980 pumps and a MD-915 multi-wavelength detector. Optical rotation was measured with a JASCO DIP-370 digital polarimeter. IR spectra were recorded on a JASCO FT/IR-7000S. UV spectra were recorded on a JASCO Ubest-50 UV/VIS spectrophotometer. NMR spectra were recorded on a Bruker-AMX600 (600 MHz) or a Bruker-ARX400 (400 MHz). NMR chemical shifts were referenced to the solvent peak of δ_H 7.26 (residual $CHCl_3$), 7.15 (residual benzene), 3.75 (additional dioxane in D_2O), δ_C 77.0 ppm for $CDCl_3$, or 128.0 for C_6D_6 as an internal standard. Mass spectra were recorded on a JEOL Mstation JMS-700 mass spectrometer in the FAB mode using *m*-nitrobenzyl alcohol as a matrix.

Clavariopsin A

$[\alpha]_D^{24}$ −210 (*c* 0.37, $CHCl_3$), TLC: Rf=0.82 (silica gel, $CHCl_3$-MeOH=9:1), UV (CH_3CN) 205 (end, 58800), 222 (sh, 25700), 277 (1900), 288 (1700) nm, IR (KBr) 3385, 1739, 1683, 1648, 1614, 1515, 1472, 1249 cm^{-1}. The other physico-chemical data are reported in ref. 1, and the NMR data are in Table 1.

Clavariopsin B

$[\alpha]_D^{24}$ −200 (*c* 0.078, $CHCl_3$), TLC: Rf=0.64 (silica gel, $CHCl_3$-MeOH=9:1), UV (CH_3CN) 222 (sh, 20700), 277 (1200), 283 (970) nm, IR (KBr) 3389, 1731, 1681, 1645, 1514, 1468, 1247 cm^{-1}, ^1H NMR (C_6D_6) δ 0.75 (t, *J*=7.0 Hz, 3H), 0.76 (m, 1H), 0.77 (d, *J*=6.8 Hz, 3H), 0.77 (d, *J*=6.8 Hz, 3H), 0.83 (t, *J*=6.8 Hz, 3H), 0.84 (d, *J*=6.7 Hz, 3H), 0.87 (m, 1H), 0.88 (m, 1H), 0.97 (d, *J*=8.0 Hz, 3H), 0.97 (m, 1H), 0.99 (d, *J*=6.7 Hz, 3H), 1.06 (d, *J*=6.8 Hz, 3H), 1.06 (m, 1H), 1.10 (d, *J*=6.6 Hz, 3H), 1.15 (d, *J*=6.7 Hz, 3H), 1.17 (d, *J*=6.6 Hz, 3H), 1.20 (m, 1H), 1.21 (d, *J*=6.6 Hz, 3H), 1.24 (m, 1H), 1.46 (m, 1H), 1.50 (m, 1H), 1.81 (m, 1H), 2.27 (m, 1H), 2.32 (m, 1H), 2.37 (s, 3H), 2.42 (m, 1H), 2.47 (m, 1H), 2.60 (br. d, *J*=13.0 Hz, 1H), 2.80 (s, 3H), 2.89 (d, *J*=10.1 Hz, 1H), 2.93 (m, 1H), 3.04 (m, 1H), 3.06 (s, 3H), 3.08 (m, 1H), 3.08 (m, 1H), 3.10 (m, 1H), 3.12 (m, 1H), 3.13 (s, 3H), 3.39 (s, 3H), 3.50 (dd, *J*=17.3 Hz, 3.2 Hz, 1H), 3.62 (m, 1H), 4.13 (dd, *J*=17.3 Hz, 5.9 Hz, 1H), 4.49 (d, *J*=5.3 Hz, 1H), 5.04 (m, 1H), 5.06 (m, 1H), 5.27 (d, *J*=10.4 Hz, 1H), 5.39 (d, *J*=11.0 Hz, 1H), 5.58 (ddd, *J*=10.3 Hz, 10.3 Hz, 6.1 Hz, 1H), 5.75 (br. d, *J*=4.6 Hz, 1H), 6.43 (d, *J*=7.6 Hz, 1H), 6.48 (dd, *J*=11.9 Hz, 3.4 Hz, 1H), 6.71 (d, *J*=6.8 Hz, 1H), 6.73 (d, *J*=8.5 Hz, 2H), 7.15 (d, *J*=8.5 Hz, 2H), 7.32 (br. d, *J*=5.8 Hz, 1H), 7.40 (d, *J*=9.7 Hz, 1H), ^{13}C NMR (100 MHz, C_6D_6) δ 10.2 (q), 11.0 (q), 15.4 (q), 17.5 (q), 18.0 (q), 18.7 (q), 18.7 (q), 18.8 (q), 19.2 (q), 19.4 (q), 19.6 (q), 20.2 (q), 20.7 (t), 24.2 (t), 25.2 (t), 25.6 (t), 26.1 (t), 27.8 (d), 28.6 (q), 30.1 (q), 30.2 (d), 31.0 (q), 31.3 (d), 32.5 (d), 32.8 (d), 34.0 (d), 34.1 (t), 34.3 (t), 40.7 (q), 41.7 (t), 44.1 (t), 51.4 (d), 52.6 (d), 54.0 (d), 54.6 (d), 55.0 (q), 57.1 (d), 61.1 (d), 61.8 (d), 74.8 (d), 76.1 (d), 113.7 (d, 2C), 129.6 (s), 131.0 (d, 2C), 159.0 (s), 168.0 (s), 168.6 (s), 169.1 (s), 169.5 (s), 169.6 (s), 170.2 (s), 170.5 (s), 170.8 (s), 172.3 (s), 173.4 (s), 173.4 (s). The other physico-chemical properties are reported in ref. 1.

Preparation of 2-Hydroxyisovaleric acid[8]

To a solution of DL-Val (1.17 g) in a mixture of water (40 ml), 1 N HCl (10 ml) and AcOH (20 ml) was added a solution of $NaNO_2$ (7 g) in water (12 ml) dropwise at 0°C with vigorous stirring over 15 minutes. The resulting mixture was stirred at 0°C for 1 hour, then at room temperature overnight. The reaction mixture was diluted with conc HCl (10 ml), and extracted with diethyl ether (1×100 ml, 3×50 ml). The organic layers were combined, dried, and concd to give 323 mg of racemic 2-hydroxyisovaleric acid as an oil: ^1H NMR (CD_3OD) δ 3.93 (1H, d, *J*=3.7 Hz), 2.04 (1H, m), 0.99 (3H, d, *J*=6.8 Hz), 0.91 (3H, d, *J*=6.8 Hz); FAB-MS *m/z* 117 $[M-H]^-$.

(*S*)-2-Hydroxyisovaleric acid was prepared from L-Val in the same manner as that for the racemic one. $[\alpha]_D^{23}$ +9 (*c* 1.0, $CHCl_3$), FAB-MS and ^1H NMR data were identical with the racemic sample.

Preparation of *N*-Methyl-L-amino Acids[9]

A typical procedure is as follows. To the solution of 542 mg of *N*-Boc-L-Val in anhydrous THF (7 ml) under a N_2 atmosphere was added 1.25 ml of anhydrous CH_3I at 0°C. To the mixture was added 260 mg of NaH (60% oil suspension) with stirring under a N_2 flow. The resulting mixture was stirred at room temperature for 7.5 hours under a N_2 atmosphere then diluted with saturated aqueous NH_4Cl

(10 ml). The solution was extracted with diethyl ether (10 ml), the organic layer was extracted with saturated aqueous $NaHCO_3$-water (1 : 1) (3×5 ml). The aqueous layers were combined, adjusted to pH 3 with 10% citric acid, and then extracted with EtOAc (2×5 ml). The combined organic layers were dried and concd to give 525 mg of *N*-Boc-*N*-methyl-L-Val as a crude oil. 20.0 mg of the crude oil was dissolved in anhydrous CH_2Cl_2 (1 ml) under a N_2 atmosphere with stirring at 0°C. To the solution was added 1 ml of trifluoroacetic acid, and the mixture was stirred at 0°C for 1.5 hours under a N_2 atmosphere and then concd to give 14.6 mg of *N*-methyl-L-Val (TFA salt) as an oil: ^{1}H NMR (CD_3OD) δ 3.74 (1H, d, J=3.5 Hz), 2.72 (3H, s), 2.29 (1H, m), 1.13 (3H, d, J=7.0 Hz), 1.06 (3H, d, J=7.0 Hz); FAB-MS *m/z* 130 $[M-H]^-$.

N-Methyl-L-Ile (TFA salt) was prepared from *N*-Boc-L-Ile 1/2H_2O: oil; ^{1}H NMR (CD_3OD) δ 3.82 (1H, d, J=3.2 Hz), 2.72 (3H, s), 1.99 (1H, m), 1.63 (1H, ddq, J=13.6, 7.2, 7.2 Hz), 1.43 (1H, ddq, J=13.6, 7.2, 7.2 Hz), 1.02 (3H, d, J=7.2 Hz), 1.01 (3H, t, J=7.2 Hz); FAB-MS *m/z* 144 $[M-H]^-$.

N- Methyl-L-Asp was prepared from *N*-Cbz-L-Asp(OBu^t): oil; ^{1}H NMR (D_2O) δ 4.24 (1H, t, J=5.0 Hz), 3.19 (2H, m), 2.83 (3H, s); FAB-MS *m/z* 146 $[M-H]^-$.

Racemization of *N*-Methyl Amino Acids[10]

In a typical procedure, 1~2 mg of *N*-methyl-amino acid was taken into a sealed tube and dissolved in 100 μl of water. To the solution were added 40 μl of triethylamine and 40 μl of acetic anhydride, and the mixture was stirred at 60°C for 1 hour. The reaction mixture was evaporated to dryness by a vacuum pump, which was dissolved in 6 N HCl (200 μl) in the same tube. The tube was evacuated by an aspirator and then sealed. The solution was stirred at 110°C for 12 hours and then dried to give a racemized amino acid, which was subsequently derivatized with FDLA.

Derivatization of Standard Amino Acids with D- or L-FDLA[2~4]

50 μl of a 50 mM amino acid solution in water was taken into a 1.5 ml centrifugal polypropylene tube. After adjusting to approximate pH 9 with 1 M $NaHCO_3$, 100 μl of 1% D- or L-FDLA in acetone was added. After being vortexed, the mixture was incubated at 37°C for 1 hour and quenched with 20 μl of 1 N HCl. The reaction mixture was diluted up to 1000 μl with acetonitrile (HPLC grade), 1 μl of which was analyzed by photodiode array detecting HPLC to check the production of the FDLA derivative and then subjected to LC/MS analysis.

Hydrolysis of Clavariopsin A Followed by D- or L-FDLA Derivatization

420 μg of clavariopsin A (0.36 μmol) was taken into a sealed tube and dissolved in 6 N HCl (200 μl). The reaction tube was evacuated by an aspirator and sealed. The reaction mixture was stirred at 110°C for 2 hours and then concd to dryness, which was dissolved in 100 μl of water to give 3.6 mM solution of each amino acid component theoretically. To each a half portion (50 μl) were added 40 μl of 1 M $NaHCO_3$ and 50 μl of 1% D- or L-FDLA in acetone, respectively. After incubation (see previous section), the reaction mixture was quenched with 20 μl of 1 N HCl and diluted up to 500 μl with acetonitrile. 5 μl of each solution of FDLA derivatives was analyzed by LC/MS.

LC/MS Analysis of FDLA Derivatives

The analysis of the L- and DL-FDLA (mixture of D- and L-FDLA) derivatives was performed on a Develosil ODS-HG-5 column (250×4.6 mm i.d., Nomura Chemical) at 40°C using a JASCO TU-100 temperature control unit. An aqueous acetonitrile containing 0.01 M TFA was used as a mobile phase under a linear gradient elution mode (acetonitrile 30~60%, 30 minutes) at a flow rate of 1.0 ml/minute. A VG Platform II mass spectrometer (Fisons) was used for detection in ESI (negative) mode. The cone voltage and electron multiplier voltage were kept at 30 V and 650 V, respectively, and the ion source at 70°C. Nitrogen gas was used as a sheath gas at 250 liters/hour. A mass range of *m/z* 200~1000 was scanned in 1 scond.

Isolation and HPLC Analysis of (*R*)-2-Hydroxyisovalric Acid from Clavariopsin A Hydrolysate

The hydrolysate from 240 μg of clavariopsin A was separated by reversed-phase HPLC [Develosil ODS-HG-5 (250×4.6 mm i.d.), acetonitrile - water - TFA (5 : 95 : 0.05), 1.0 ml/minute] to give the fraction containing 2-hydroxyisovaleric acid (Rt=16~17 minutes), which was concd and redissolved in 50 μl of water. 7 μl ($\approx$0.03 μmol) of this solution was analyzed on a CHIRALPAK WH column (250×4.6 mm i.d., Daicel Chemical Industries) at 50°C using a JASCO TU-100 temperature control unit with detection at 254 nm. 0.25 mM aq $CuSO_4$ was used as a mobile phase at a flow rate of 1.0 ml/minute.

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