A New Cyclic Peptide from a Marine-Derived Bacterium of the Genus *Nocardiopsis*

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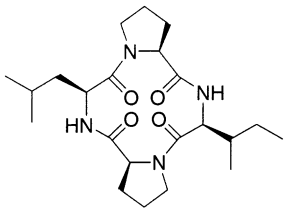
A new cyclic tetrapeptide (**1**) was isolated from the culture broth of an actinomycete of the genus *Nocardiopsis* collected from the Pacific deep-sea sediment. The structure of this compound was determined to be cyclo-(L-isoleucyl-L-prolyl-L-leucyl-L-prolyl) on the basis of combined chemical and spectral methods.

Marine microorganisms are widely recognized as emerging sources of secondary metabolites.[1] These organisms, flourishing in diverse marine environments, have produced a wide variety of structurally unique and biologically active compounds that have attracted considerable attention for biomedical studies.[1−3] During the course of our search for novel natural products from marine bacteria, we collected an actinomycete of the genus *Nocardiopsis* from the Pacific deep-sea sediment. The culture broth of this strain contained several amino acid-derived diketopiperazines and a new cyclic peptide.[4] We describe herein the isolation and structure elucidation of MKN-349A (**1**), a cyclic tetrapeptide.



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The strain M0349 was inoculated in SC-KNO_3 medium and fermented in a 20 L fermenter for 10 days. The culture broth was filtered through a membrane and subjected to HP20 adsorption chromatography. The moderately polar fraction was separated by vacuum flash chromatography on silica gel followed by ODS HPLC to yield compound **1** as a colorless gum.

The molecular formula of compound **1** was assigned as $C_{22}H_{36}N_4O_4$ on the basis of its combined HRFABMS and ^{13}C NMR spectral features. The peptide nature of this compound was evident from the presence of characteristic signals in the ^1H and ^{13}C NMR data as well as a strong absorption band at 1670 cm^{-1} in the IR spectrum. The identification of each amino acid unit was accomplished by detailed interpretation of the ^1H COSY and TOCSY data. Beginning with signals of the methine protons at δ 4.06 and 3.84, proton correlations including the upfield methyl signals readily revealed the presence of Ile and Leu residues (Table 1). Also illustrated were two series of similar proton correlations containing signals of the methine protons at δ 4.25 and 4.18 in the ^1H NMR spectrum, which were interpreted to be two Pro residues. Confirmation of the spectral interpretation as well as the stereochemical assignment of each amino acid residue was established by Marfey analysis.[5] Acid-catalyzed hydrolysis of **1** followed by treatment with L-Marfey's reagent and HPLC analysis showed that all of the amino acids had the L-configuration. The stereochemistry at the side chain of the Ile residue was also determined to be 2*S*,3*S* on the basis of HPLC co-injection with derivatives prepared from both Ile and *allo*-Ile. Thus, compound **1** consisted of all L-forms of Ile, Leu, Pro(I), and Pro(II).

Table 1. ^1H and ^{13}C NMR Assignments for Compound **1**

position		^1H[a]	^{13}C	
Pro(I)	1		172.4	s
	2	4.18, dd (9.9, 6.3)	60.0	d
	3	2.31, m; 1.93, m	29.5	t
	4	2.00, m; 1.95, m	23.2	t[c]
	5	3.52, m; 3.47, m	46.2	t
Ile	1		167.6	s
	2	4.06, dd (2.4, 2.0)	61.3	d
	3	2.15, m	37.1	d
	4	1.43, m; 1.31, m	25.4	t
	5	0.92, t (7.3)	12.6	q
	6	1.06, d (7.3)	15.5	q
	NH	6.51, br s[b]		
Pro(II)	1		171.6	s
	2	4.25, dd (9.5, 6.6)	59.3	d
	3	2.34, m; 1.93, m	29.9	t
	4	2.00, m; 1.95 m	23.1	t[c]
	5	3.55, m; 3.47, m	46.7	t
Leu	1		169.1	s
	2	3.84, ddd (9.3, 5.4)	57.1	d
	3	1.67, ddd (13.7, 9.3, 5.4)	43.7	t
		1.56, ddd (13.7, 8.8, 5.4)		
	4	1.76, m	25.5	d
	5	0.98, d (6.8)[d]	23.3	q[e]
	6	0.95, d (6.8)[d]	21.9	q[e]
	NH	5.98, br s[b]		

[a] Obtained in CD_3OD solutions. [b]Obtained in $CDCl_3$ solutions. [c−e] Interchangeable signals.

Further structural assignment and sequencing of the amino acid residues was accomplished with the aid of gradient-HMBC experiments. Long-range correlations of the carbonyl carbon at δ 169.1 with protons at δ 3.84 (H-2 of Leu), 3.52 (H-5 of Pro(I)), 1.67 and 1.56 (H-3 of Leu) placed a peptide linkage between Leu and Pro(I). Correlations of the carbonyl carbon at δ 171.6 with protons at δ 4.25 (H-2 of Pro(II)), 3.84 (H-2 of Leu), and 2.34 (H-3 of Pro(II)) also denoted a peptide linkage between Leu and

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Pro(II). The placement of a peptide linkage between Ile and Pro(I) was secured by correlations of the carbonyl carbon at δ 172.4 with protons at δ 4.18 (H-2 of Pro(I)), 4.06 (H-2 of Leu), and 2.31 (H-3 of Pro(I)). Although the long-range correlations between the C-1 carbon of Ile at δ 167.6, assigned on the basis of its correlation with a proton at δ 4.06 (H-2 of Ile), and protons of Pro(II) were not observed in the *g*HMBC data, consideration of the molecular formula, as well as the moderate polarity of this compound, allowed construction of a peptide linkage between this carbon and nitrogen of Pro(II). Thus, the structure of MKN-349A (**1**) was determined to be cyclo-(L-isoleucyl-L-prolyl-L-leucyl-L-prolyl). A literature survey revealed that fenestin A, a cyclic tetrapeptide from the marine sponge *Leucophloeus fenestrata*, had the same amino acid composition but differed from **1** in the sequence of amino acid residues.[6] A similar compound consisting of two units each of Leu and Pro was also reported from the marine ascidian *Cystodytes delle chiajei*.[7]

The crude extract and chromatographic fractions containing compound **1** exhibited significant cytotoxicity toward the leukemia cell-line K-562 (LC_{50} < 0.05 μg/mL). However, the same measurement using pure compound revealed that the bioactivity was not attributed to this compound.

Experimental Section

General Experimental Procedures. Optical rotation was measured on a JASCO digital polarimeter using a 5 cm cell. The IR spectrum was recorded on a Mattson Galaxy spectrophotometer. NMR spectra were recorded in $CDCl_3$ and CD_3OD solutions containing Me_4Si as internal standard, on a Varian Unity 500 spectrometer. Proton and carbon NMR spectra were measured at 500 and 125 MHz, respectively. Mass spectral data were provided by the Korea Basic Science Institute, Taejeon, Korea. All solvents used were spectral grade or were distilled from glass prior to use.

Collection and Taxonomic Identification. The bacterial strain M0349 was isolated using a modified Bennett agar plate (0.1% yeast extract, 0.1% beef extract, 0.2% tryptone, 1% dextrose, 1.5% agar) from underwater sediment (depth 3000 m) collected at Clarion-Clipperton Fracture Zone (16.5° N, 126.5° W), Mid-Pacific, during an expedition of the Korea Deep Ocean Study Program in July 1998. The strain was maintained on a yeast extract−malt extract agar containing 70% natural seawater. The colony morphology of the strain M0349 grown on ISP-5 agar at 30 °C for 3 days was round, regular, entire, and matt type with brownish gray vegetative, having a gray mycelia with conidia sporopore.

For taxonomic identification, this strain was analyzed following the 16S rDNA partial sequence method.[8,9] The sequence of 539 base pairs was in good agreement with several type strains of *Nocardiopsis*: 99.07, 98.70, 98.52, and 98.52% similarities to *N. synnemataformans* JCM 10456, *N. dassonvillei* DSM 43111, *N. prasina* DSM 43845, and *N. lucentensis* DSM 44048, respectively. The strain is currently on deposit in the Microbial Collection, KORDI, under the curatorship of J.-R.R.

Fermentation. The slant culture of M0349 was inoculated in a 500 mL Erlenmeyer flask containing 100 mL of SC-KNO_3 medium (starch 1%, K_2HPO_4 0.2%, KNO_3 0.2%, NaCl 2%, casein 0.03%, $MgSO_4 \cdot 7H_2O$ 0.005%, $CaCO_3$ 0.002%, $FeSO_4 \cdot 7H_2O$ 0.001%) in distilled water and incubated at 27 °C for 7 days on a rotary shaker (150 rpm). Fermentation was carried out by transfer of 500 mL of seed culture to a 20 L fermenter containing 10 L of the same medium and incubation for 10 days under similar conditions.

Extraction and Isolation. The combined fermentation broth (70 L) was filtered using a membrane (pore size 0.45 μm) in a tangential filter system. The filtered broth was subjected to Diaion HP20 adsorption chromatography sequentially using H_2O, 50% aqueous MeOH, 50% aqueous acetone, MeOH, acetone, and EtOAc as eluents. The fraction eluted with MeOH was dried in vacuo (1.15 g) and separated by silica vacuum flash chromatography using in the order of elution 10% gradient mixture of *n*-hexane and EtOAc, acetone, and MeOH. The fractions eluted with EtOAc and acetone were combined (113.2 mg), redissolved in MeOH, and filtered through a membrane (pore size 0.45 μm), and the residue (58.5 mg) was separated by reversed-phase HPLC (YMC-ODS-A column, 100% MeOH) to yield 3.9 mg of pure **1** as a colorless gum.

MKN-349A (1): $[\alpha]^{25}_D$ −41.3° (*c* 0.12, MeOH); IR (KBr) ν_{max} 3500−3200 (br), 2960, 1670, 1575, 1450, 1300, 1145 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRFABMS *m/z* 421.1269 [M + H]$^+$ (calcd for $C_{22}H_{37}N_4O_4$, 421.1281).

Amino Acid Analysis of 1. Amino acid composition of compound **1** was accomplished following the general guidelines of Marfey analysis.[5] Compound **1** (200 μg) was hydrolyzed with 6 N HCl (500 μL) containing phenol (50 μg) at 110 °C for 16 h. After drying the solution in a stream of N_2, to the residue were added 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide in acetone (1.5 mg/mL, 50 μL) and 1 N $NaHCO_3$ (100 μL), and the mixture was kept at 80 °C for 3 min. The reaction mixture was neutralized with 2 N HCl (50 μL) and diluted with 50% aqueous MeCN (300 μL). A portion of this solution was analyzed with C_{18} reversed-phase HPLC (YMC-ODS-A column, 4.5 mm × 250 mm, ramp from 0% to 50% aqueous MeCN with 0.1% TFA in 60 min, UV at 340 nm). Peaks were identified by co-injection with standards prepared from authentic D- and L-*allo*-Ile, D- and L-Ile, D- and L-Leu, and D-and L-Pro.

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