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Nocardiamides A and B, Two Cyclohexapeptides from the Marine-Derived Actinomycete *Nocardiopsis* sp. CNX037

Zheng-Chao Wu,[†,‡,§] Sumei Li,[†] Sang-Jip Nam,[⊥] Zhong Liu,[∥] and Changsheng Zhang*[,†]

[†]Key Laboratory of Marine Bio-resources Sustainable Utilization, RNAM Center for Marine Microbiology, Guangdong Key Laboratory of Marine Materia Medica, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, People's Republic of China

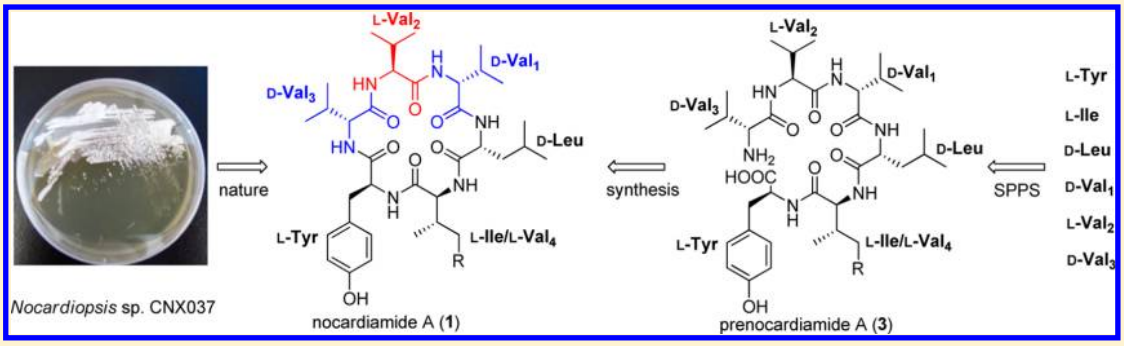
[‡]Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California San Diego, La Jolla, California 92093-0204, United States

[§]University of Chinese Academy of Sciences, Beijing 100049, People's Republic of China

[⊥]Department of Chemistry and Nano Science, Ewha Womans University, Seoul, 120-750, Republic of Korea

[∥]Guangzhoujinan Biomedicine Research and Development Center, Guangdong Key Laboratory of Bioengineering Medicine, Jinan University, 601 West Huangpu Road, Guangzhou 510632, People's Republic of China

Supporting Information



ABSTRACT: Two new cyclic hexapeptides, nocardiamides A (**1**) and B (**2**), were isolated from the culture broth of marine-derived actinomycete CNX037 strain that was identified as a *Nocardiopsis* species. The planar structures of nocardiamides A (**1**) and B (**2**) were assigned on the basis of 1D and 2D NMR and HRESIMS spectroscopic analyses. Their absolute configurations were deduced by the advanced Marfey's method and chiral-phase HPLC analysis. The challenge of locating two D- and one L-valine residue in **1** and **2** was accomplished by total synthesis using solid-phase peptide synthetic methods. Both **1** and **2** showed negligible antimicrobial activities against seven indicator strains and exhibited no cytotoxicity against HCT-116.

Marine-derived actinomycetes are currently being explored as a valuable source for novel drug discovery.[1] A number of biologically active natural products with unique structures have been discovered from marine-derived or obligate marine microorganisms.[2] Peptides or peptide-derived compounds account for more than half (56%) of all bacterial metabolites[3] and have also been shown to be a large group of secondary metabolites from marine-derived actinomycetes.[4] In the last two decades, 35 new peptides or peptide-derived natural products with various bioactivities have been reported from marine-derived actinomycetes.[4,5] These are exemplified by the antibacterial salinamides,[6] by the anti-inflamatory cyclomarins,[5f] and by the antitumor proximicins,[7] piperazimycins,[8] and marthiapeptide A.[9] In the course of searching for new natural products from marine-derived actinomycetes, a bacterium of the genus *Nocardiopsis* sp., strain CNX037, was isolated from a marine sediment sample and was observed to produce new secondary metabolites with molecular ions at m/z 673.4292 and 687.4450, using HRLCMS dereplication analysis against the AntiMarin database.[10] Herein we report the isolation, structure elucidation, synthesis, and biological evaluation of these two new cyclohexapeptides, designated nocardiamides A (**1**) and B (**2**).

RESULTS AND DISCUSSION

The strain CNX037 was isolated from a sediment sample collected at a depth of 18−30 m from the La Jolla Canyon, San Diego, CA. It was identified as a *Nocardiopsis* species on the basis of the phylogenetic tree constructed by a neighbor-joining method (Figure S1). Purification of the EtOAc extracts of the fermentation broth of strain CNX037 led to the isolation of two new cyclohexapeptides, designated nocardiamides A (**1**) and B (**2**).

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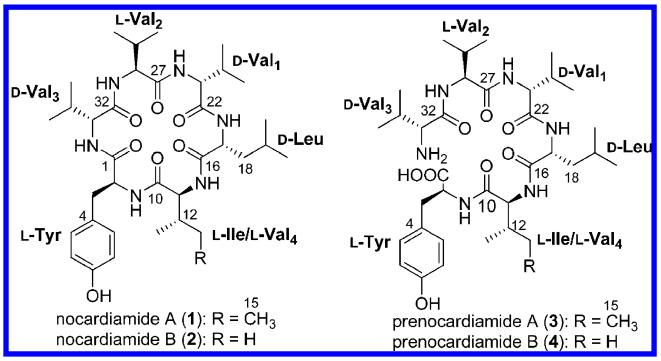

Nocardiamide A (**1**) was obtained as a white powder. The molecular formula was determined as $C_{36}H_{58}N_6O_7$ by HRESIMS (*m/z* 687.4450 $[M + H]^+$, calcd 687.4440, Figure S2), indicating 11 degrees of unsaturation. The peptide nature of **1** was deduced from the ^1H and ^{13}C NMR and IR spectra (Table 1). The IR spectrum of **1** showed an absorption band at 1635 cm^{-1}, characteristic for the amide functionality. The ^1H NMR spectrum of **1** displayed six NH resonances (δ_H 7.0−8.5), six α-amino acid hydrogen resonances (δ_H 3.5−5.0), and 10 methyl doublets or triplets (δ_H 0.5−1.0). The ^{13}C NMR spectrum of **1** exhibited six amide carbonyl signals resonating between δ_C 169 and 172 ppm and six α-amino acid carbon signals between δ_C 53 and 60 ppm. Detailed analysis of the 1D

Table 1. ^1H (500 MHz) and ^{13}C (75 MHz) NMR Data for Nocardiamides A (1) and B (2) in DMSO-d_6

		nocardiamide A (**1**)			nocardiamide B (**2**)	
amino acids	pos.	δ_C, mult	δ_H, mult (*J* in Hz)	HMBC	δ_C, mult	δ_H, mult (*J* in Hz)
Tyr	1	170.0, C			170.0, C	
	2	53.9, CH	4.44, m	1, 3a, 3b	53.9, CH	4.44, m
	3a	35.4, CH_2	2.73, dd (14.0, 10.0)	2, 4	35.4, CH_2	2.73, dd (13.5, 9.5)
	3b		3.08, dd (14.0, 10.0)	2, 4		3.08, dd (13.5, 9.5)
	4	128.0, C			128.0, C	
	5 or 9	129.9, CH	6.95, d (8.5)		129.9, CH	6.95, d (8.5)
	6 or 8	114.7, CH	6.59, d (8.5)		114.7, CH	6.59, d (8.5)
	7	155.7, C			155.7, C	
	NH		7.56, d (8.0)	10		7.56, d (8.0)
	OH		9.11, s			9.11, s
Ile/Val_4	10	170.5, C			170.5, C	
	11	59.0, CH	3.80, t (7.0)	10	59.7, CH	3.76, t (7.0)
	12	35.3, CH	1.61, m	13, 14	29.1, CH	1.85, m
	13	15.3, CH_3	0.56, d (7.0)	11, 12, 14	18.0, CH_3	0.69, d (6.5)
	14	23.9, CH_2	1.03, m	12, 13, 15	19.2, CH_3	0.62, d (6.5)
			1.03, m			
	15	10.7, CH_3	0.69, t (7.5)	14		
	NH		8.10, d (7.0)	16		8.10, d (7.5)
Leu	16	171.8, C			171.8, C	
	17	51.9, CH	4.46, m	16	51.9, CH	4.46, m
	18	41.4, CH_2	1.55, m	17	41.4, CH_2	1.55, m
			1.48, m	17		1.48, m
	19	24.0, CH	1.50, m		24.0, CH	1.50, m
	20	22.3, CH_3	0.88, d (6.0)	18, 19	22.3, CH_3	0.88, d (5.5)
	21	23.1, CH_3	0.83, d (5.0)	18, 19	23.1, CH_3	0.83, d (8.0)
	NH		7.62, d (7.0)	22		7.62, d (6.5)
Val_1	22	170.6, C			170.6, C	
	23	58.1, CH	4.12, dd (9.0, 5.5)	22, 24, 25	58.1, CH	4.12, dd (9.5, 5.5)
	24	29.6, CH	2.20, m	23, 25, 26	29.6, CH	2.20, m
	25	17.2, CH_3	0.84, d (5.0)		17.2, CH_3	0.84, d (5.0)
	26	19.4, CH_3	0.82, overlapped	23, 24, 25	19.4, CH_3	0.82, d (7.0)
	NH		8.22, d (9.0)	27		8.22, d (9.0)
Val_2	27	171.4, C			171.4, C	
	28	59.7, CH	3.98, dd (8.5, 7.0)	27, 29	59.7, CH	3.98, t (7.5)
	29	28.5, CH	1.91, m	28, 30, 31	28.5, CH	1.91, m
	30	19.1, CH_3	0.86, d (6.5)	29	19.1, CH_3	0.86, d (7.0)
	31	19.1, CH_3	0.92, d (7.0)	29	19.1, CH_3	0.92, d (7.0)
	NH		8.28, d (6.5)	32		8.28, d (6.5)
Val_3	32	170.7, C			170.7, C	
	33	57.3, CH	4.20, t (8.0)	32, 34	57.3, CH	4.20, t (8.0)
	34	30.4, CH	1.88, m	33, 35, 36	30.4, CH	1.88, m
	35	18.6, CH_3	0.76, d (7.0)	33, 34	18.6, CH_3	0.76, d (7.0)
	36	18.6, CH_3	0.81, d (7.0)		18.6, CH_3	0.81, d (7.0)
	NH		7.39, d (3.5)	1		7.39, d (8.0)

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(^{1}H, ^{13}C, DEPT) and 2D (HSQC, TOCSY, and HMBC) NMR spectroscopic data (Figure S2) revealed that **1** was a hexapeptide containing Tyr, Ile, Leu, and three Val residues (Table 1). The hexapeptide **1** was supposed to have the sequence Tyr-Ile-Leu-Val$_1$-Val$_2$-Val$_3$ on the basis of the following HMBC correlations: NH-Tyr (δ_H 7.56)/Ile C═O (δ_C 170.5), NH-Ile (δ_H 8.10)/Leu C═O (δ_C 171.8), Leu-NH (δ_H 7.62)/Val$_1$ C═O (δ_C 170.6), Val$_1$-NH (δ_H 8.22)/Val$_2$ C═O (δ_C 171.4), and Val$_2$-NH (δ_H 8.3)/Val$_3$ C═O (δ_C 170.7) (Figure 1). These six amino acids accounted for 10 degrees of

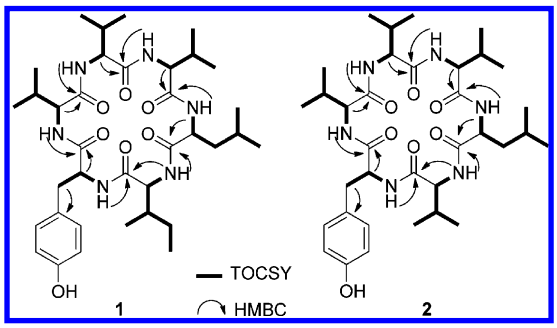

Figure 1. Selected key TCOSY and key HMBC correlations for **1** and **2**.

unsaturation, indicating that **1** was a monocyclic peptide. In support of the cyclic nature of **1**, the NH of the Val$_3$ (δ_H 7.39) also showed an HMBC correlation to the carbonyl of Tyr (δ_C 170.0). Therefore, the planar structure of **1** was deduced as cyclo-(Tyr-Ile-Leu-Val$_1$-Val$_2$-Val$_3$). In order to precisely define the connectivity of alpha protons and amide NH protons to specific carbonyls, especially for three carbonyls with very close chemical shifts (δ_C 170.5, 170.6, and 170.7), we performed additional HRESIMS/MS analysis (Figure 2, Figure S3). The

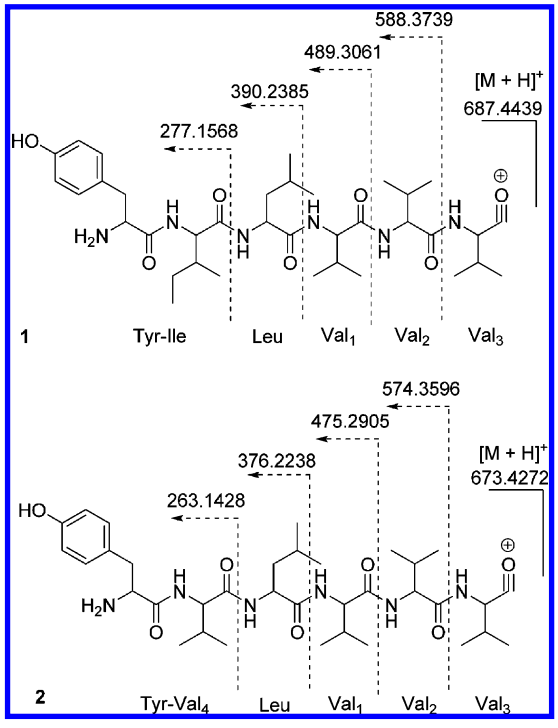

Figure 2. HRESIMS/MS of fragmentation patterns of nocardiamides A (**1**) and B (**2**).

fragmentation of the isolated molecular ion *m/z* 687.4439 [M + H]$^+$ for **1** yielded MS^2 ions *m/z* 277.1568 [M + H]$^+$ for Tyr-Ile, *m/z* 390.2385 [M + H]$^+$ for Tyr-Ile-Leu, *m/z* 489.3061 [M + H]$^+$ for Tyr-Ile-Leu-Val, and *m/z* 588.3739 [M + H]$^+$ for Tyr-Ile-Leu-Val-Val. This fragmentation pattern was in good agreement with the proposed structure cyclo-(Tyr-Ile-Leu-Val$_1$-Val$_2$-Val$_3$). Therefore, the planar structure of **1** was unambiguously established as cyclo-(Tyr-Ile-Leu-Val$_1$-Val$_2$-Val$_3$).

The absolute configurations of the amino acid residues in **1** were determined by application of the advanced Marfey's method and by chiral-phase HPLC analysis. Compound **1** was subjected to acid hydrolysis and derivatizated with the advanced Marfey's reagent D-FDLA or D-FDAA.[11] Comparison of the retention times and *m/z* values between the chiral derivatives of the amino acid residues of **1** and derivatives of authentic amino acids indicated the presence of L-Tyr, D-Leu, L-Ile or L-allo-Ile or L-Leu, D- and L-Val (Table S1, Figure S4). The D-FDLA derivatives of L-Ile, L-*allo*-Ile, and L-Leu (t_R 55.7 min for all three standards) were indistinguishable by reversed-phase HPLC analysis. We then applied a chiral-phase HPLC method to differentiate the isomers of L-Ile, L-*allo*-Ile, and L-Leu. The chiral-phase analysis established the presence of an L-Ile (t_R 15.8 min), but not an L-*allo*-Ile (t_R 11.1 min) or an L-Leu (t_R 13.3 min) in compound **1**, by comparing with authentic standards (Figure S5).

Due to an overlap between the D-Val derivative and an unidentified *m/z* 314 compound from the hydrolysate reaction mixture (Figure S4), the ratio of D- and L-Val in compound **1** could not be determined. Therefore, compound **1** was alternatively subjected to Marfey's reaction with the reagent D-FDAA. Both D-FDAA derivatives of D-Val (t_R 34.3 min, *m/z* 370 [M + H]$^+$; t_R 33.9 min for standard) and L-Val (t_R 39.2 min, *m/z* 370 [M + H]$^+$; t_R 39.1 min for standard) were obtained and were estimated in a 2:1 (D:L) ratio (Figure S4).

Given that both D- and L-Val enantiomers (D:L = 2:1) were encountered, the configurations of the individual Val units in the triple-Val subunit in **1** could not be easily established by these analyses. Theoretically, there should be three possible configurations for the triple-Val subunit: D-Val$_1$-D-Val$_2$-L-Val$_3$, L-Val$_1$-D-Val$_2$-D-Val$_3$, or D-Val$_1$-L-Val$_2$-D-Val$_3$. Subsequently, careful analysis of NMR-derived distance restraints based on the key ROESY correlations and the comparison with the three predicted molecular models (Figure 3)[12] revealed D-Val$_1$-L-Val$_2$-D-Val$_3$ as the most favorable configuration of the triple-Val subunit. The ROESY correlations were observed between NH (Tyr) and NH (Leu) and between NH (Tyr) and NH (Ile) in **1** (Figure 3). Three energy-minimized structure models were constructed for the three possible configurations: D-Val$_1$-L-Val$_2$-D-Val$_3$ (Figure 3A), D-Val$_1$-D-Val$_2$-L-Val$_3$ (Figure 3B), and L-Val$_1$-D-Val$_2$-D-Val$_3$ (Figure 3C). Based on these models, the interproton distances for NH (Tyr) and NH (Leu) were found to be 4.12 Å (model A), 5.77 Å (model B), and 6.18 Å (model C), while the interproton distances for NH (Tyr) and NH (Ile) were predicted to be 2.93 Å (model A), 4.43 Å (model B), and 5.23 Å (model C), respectively (Figure 3, Table S3). The calculated interproton distances for NH (Tyr) and NH (Leu) and for NH (Tyr) and NH (Ile) in models B and C (Figure 3) were significantly different from the observed ROSEY correlations. In contrast, the calculated interproton distances in model A (Figure 3) matched well with NMR-derived distance restraints (Figure 3, Table S3). Therefore, we assumed

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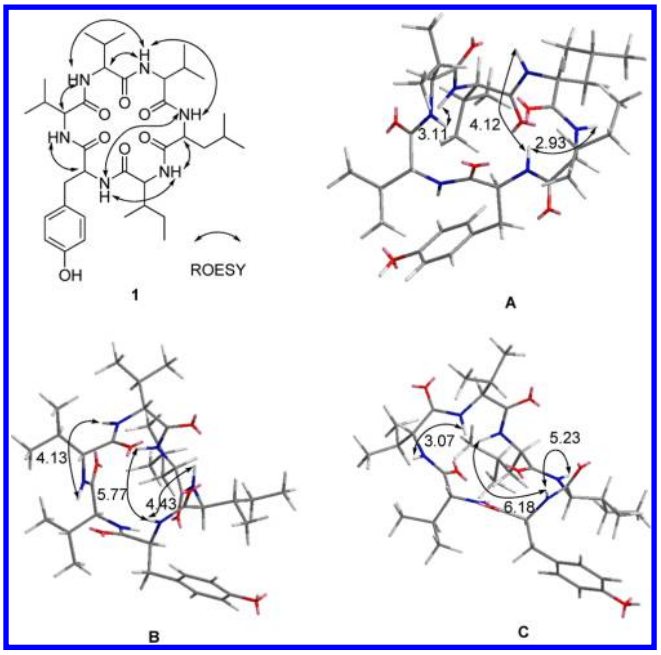


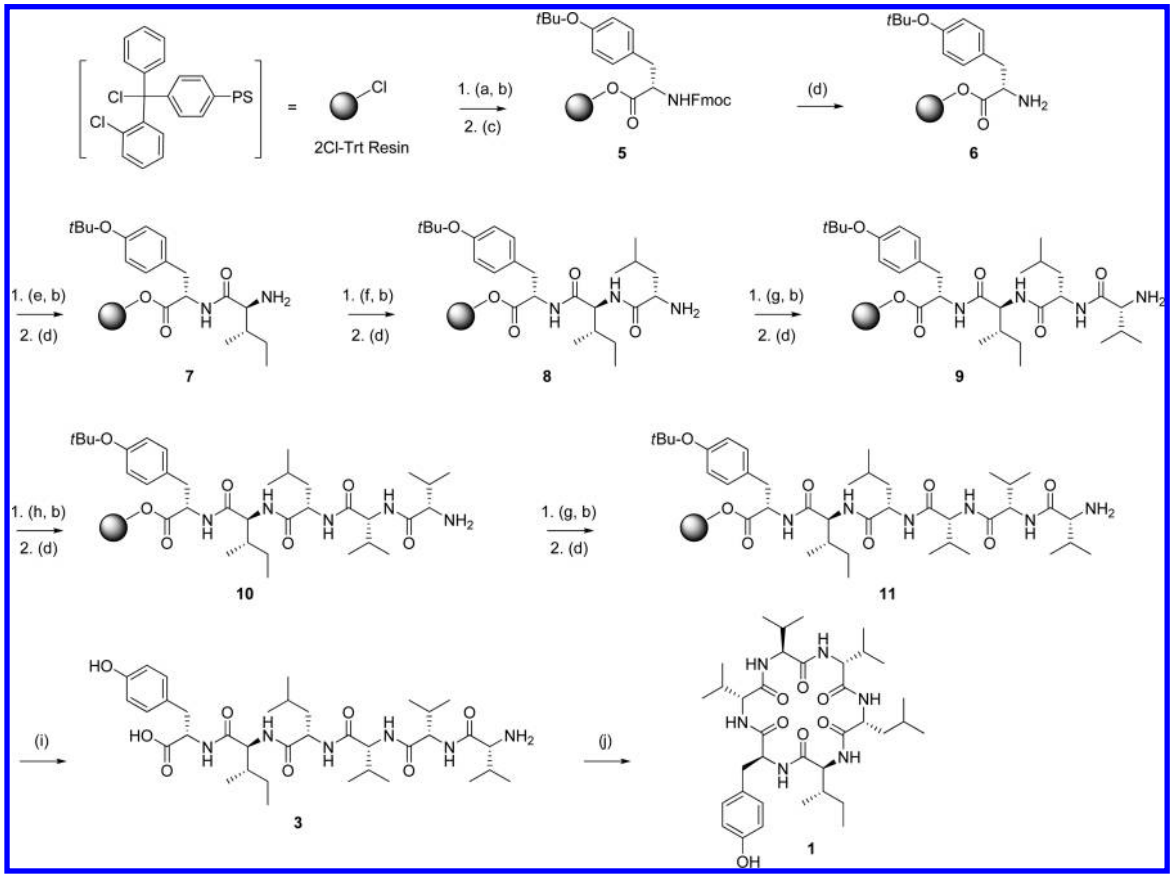
Figure 3. Key ROSEY correlations and energy-minimized models of three possible configurations, D-Val_1-L-Val_2-D-Val_3 (A), D-Val_1-D-Val_2-L-val_3 (B), and L-Val_1-D-Val_2-D-Val_3 (C), of nocardiamide A (**1**).

that D-Val_1-L-Val_2-D-Val_3 was the mostly likely configuration in **1**.

In order to confirm the structure of **1** and to definitively assign the locations of the D- and L-amino acids, the linear precursor of **1**, L-Tyr-L-Ile-D-Leu-D-Val-L-Val-D-Val (**3**), designated prenocardiamide A, was synthesized using a solid-phase peptide synthetic (SPPS) approach (Scheme 1). The structure of **3** was confirmed by interpretation of MS and 1D and 2D NMR spectra (Table S2, Figure S6). Cyclization of compound **3** yielded a synthetic product, whose spectroscopic properties were identical to those of the natural product **1** (Figure S7). Therefore, the structure of nocardiamide A (**1**) was established as cyclo-(L-Tyr-L-Ile-D-Leu-D-Val-L-Val-D-Val).

Nocardiamide B (**2**) was obtained as a white powder. The molecular formula was determined as $C_{35}H_{56}N_6O_7$ by HRESIMS, indicating 11 degrees of unsaturation. The ^1H and ^{13}C NMR spectra of **2** are similar to those of **1** (Table 1). Detailed comparison of the 1D and 2D NMR spectroscopic data of **1** and **2** (Figure S8) revealed that the Ile residue in **1** was replaced by a Val residue in **2** (Figure 1). Comparing NMR data of **1** and **2**, signals for the ethyl group of Ile (δ_C 23.9, δ_H 1.03 and δ_C 10.7, δ_H 0.69) in **1** were absent in **2**, which was replaced by a methyl doublet (δ_C 19.2, δ_H 0.62) (Table 1). Furthermore, the HMBC correlations (Figure 2) supported the assignment of the planar structure of **2** as cyclo-(Tyr-Val_4-Leu-Val_1-Val_2-Val_3), which was also confirmed by the HRESIMS/MS analysis (Figure 2, Figure S9). Subsequently, the absolute

Scheme 1. Total Synthesis of Nocardiamide A (1)[a]



[a]Reagents: (a) Fmoc-L-Tyr(tBu)-OH; (b) HBTU, DIEA; (c) MeOH; (d) 20% piperdine, DMF; (e) Fmoc-L-Ile-OH; (f) Fmoc-D-Leu-OH; (g) Fmoc-D-Val-OH; (h) Fmoc-L-Val-OH; (i) TFA/thioanisole/PhOH/EDT/DI H_2O; (j) HBTU, DIEA, DMF (yield 7.2%). The yield for off resin step (i) to get compound **3** was 31.1%, and the yield of the cyclization step (j) to convert **3** to **1** was 7.2%.

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configuration of **2** was determined by the advanced Marfey's method, in the same manner as described for **1**. HPLC-MS analysis of the resulting hydrolysates revealed the presence of L-Tyr, D-Leu, and D- and L-Val (in a 1:1 ratio) (Table S1, Figure S4). Given that the valine isomers in the triple-Val subunit of **2** exhibited the same chemical shifts as those in **1** (Table 1), the absolute configurations of the valine residues in **2** were also assigned as D–L–D. The remaining valine residue was thus deduced to have an L configuration, to satisfy the equal ratio of D- and L-Val in **2** (Table S1, Figure S4). Therefore, the structure of compound **2** was deduced as cyclo-(L-Tyr-L-Val_4-D-Leu-D-Val_1-L-Val_2-D-Val_3).

Compound **2** was also prepared by synthesis in a similar fashion to the synthesis of **1** (Scheme 1). The linear intermediate prenocardiamide B (**4**) was first synthesized using SPPS methods (Table S2, Figure S10), and the cyclization of **4** produced the target molecule **2**. Synthetic **2** displayed an indistinguishable HPLC profile and identical NMR data to natural **2** (Figure S11). Consequently, the structure of **2** was confirmed as cyclo-(L-Tyr-L-Val_4-D-Leu-D-Val_1-L-Val_2-D-Val_3).

Compounds **1**–**4** showed negligible antimicrobial activities against the seven indicator strains, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Bacillus thuringensis* SCSIO BT01, *B. subtilis* SCSIO BS01, *Micrococcus luteus* SCSIO ML01, and *Candida albicans* ATCC 10231. Compounds **1** and **2** failed to exhibit cytotoxicity toward the human colon carcinoma cell line HCT-116.

Cyclic hexapeptides are a large group of natural products that are widely discovered in nature, from plants to microorganisms. Representative plant-derived cyclic hexapeptides include bouvardins,[13] rubiaceae-type compounds,[14] and rubiyunnanins.[15] Ascidians of the genera *Lissoclinum* and *Didemnum* are prolific producers of cyclic hexapeptides,[12a,16] including bistratamides, cycloxazoline, comoramides, didmolamides, and mollamides. Also, many cyclic hexapeptides have been reported from cyanobacteria,[17] which are exemplified by westiellamide, nostocyclamides, raocyclamides, dendroamides, tenuecyclamides, and microcyclamides. Various cyclic hexapeptides have been reported from terrestrial *Streptomyces* species (such as chloptosin, phepropeptins, mannopeptimycins, and NW-G05),[18] the terrestrial- and marine-derived fungus *Aspergillus sclerotiorum* (scleramide and sclerotides),[19] and the marine-derived bacteria *Halobacillus litoralis* YS3106 (halolitoralin A).[20] To our surprise, nocardiamides A (**1**) and B (**2**) represent the first examples of cyclic hexapeptides isolated from marine-derived actinomycetes. Unlike many cyclic hexapeptides from ascidians and cyanobacteria with modified amino acid residues that form thiazole, oxazole, thiazoline, or oxazoline rings,[12a,16,17] nocardiamides contain only unmodified amino acids.

Nocardiamides A (**1**) and B (**2**) feature a triple-Val subunit in their structures. The same triple-Val subunit is also found in some other natural peptides, such as malaysiatin from a marine sponge[21] and tolybyssidin B from a cyanobacterium.[22] A similar subunit, triple-*N*-Me-Val, is naturally occurring in apramide G and dragonamide from marine cyanobacteria,[23] dictyonamide A from a marine-derived fungus,[24] and kendarimide A from a marine sponge.[25] The L-Val configurations of all Val (or *N*-Me-Val) residues in these peptides have been easily identified by using Marfey's method.[21−25] However, in the case where multiple Val (or *N*-Me-Val) residues of both L- and D-configurations are found in a natural peptide, such as malevamide B[26] and kahalalide F (an anticancer drug in clinical trial) and its analogues,[27] it becomes challenging to determine the absolute configuration for a single Val (or *N*-Me-Val) residue. It was shown that four *N*-Me-Val residues in malevamide B existed in a ratio of 3:1 (L:D); however, the precise location of the single *N*-Me-D-Val turned out to be not yet determined.[26] In the case of the marine cyanobacterium-derived cyclic depsipeptide ulongapeptin, the location of both enantiomers of *N*-Me-Val residues was differentiated by molecular modeling coupled with NMR-derived restraints.[28] The challenge of determining the absolute configurations of mixed L- and D-Val residues in kahalalide F was accomplished almost 10 years after its discovery by a series of degradation reactions[29] and eventually by total synthesis.[30] In this study, the D–L–D absolute configurations of the triple-Val subunit in nocardiamides A (**1**) and B (**2**) were deduced by structure modeling and confirmed by total synthesis.

Cyclic hexapeptides exhibited a diverse range of bioactivities, including antibacterial (e.g., chloptosin and mannopeptimycins),[18] antifungal (e.g., halolittoralin A and sclerotides),[19,20] antialgal (nostocyclamide),[17b] cytotoxic against human cancer cell lines (e.g., bouvardins, bistratamides, westiellamide, comoramides, didmolamides, microcyclamides, and mollamides) or against sea urchin embryos (raocyclamides and tenuecyclamides),[16,17] inhibitory to nitric oxide (NO) production (rubiyunnanins),[15] and inhibitory to proteasome (phepropeptins).[18b] Therefore, more bioactivities, not limited by antibacterial and cytotoxic activities, should be screened for nocardiamides **1** and **2** in the future.

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotations were measured using a Rudolph Research Autopol III polarimeter. UV spectra were recorded on a Varian Cary UV–visible spectrophotometer with a path length of 1 cm, and IR spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrometer. ^{1}H and 2D NMR spectroscopic data were recorded at 500 MHz in DMSO-d_6 with TMS as an internal standard on Varian Inova or Bruker AV500 spectrometers. ^{13}C NMR spectroscopic data were acquired at 75 MHz on Varian Inova spectrometers or at 125 MHz on Bruker AV500 spectrometers. HRESIMS were provided by the Agilent 6230 Accurate-Mass time-of-flight mass spectrometry facility at the Department of Chemistry and Biochemistry at the University of California, San Diego, La Jolla, CA. HRESIMS/MS data were acquired using a Bruker Maxis quadrupole-time-of-flight mass spectrometer. Low-resolution LC-MS data were measured using a Hewlett-Packard series 1100 LC/MS system with a reversed-phase C_{18} column (Phenomenex Luna, 4.6 × 100 mm, 5 μm) at a flow rate of 0.7 mL/min. The chirality of the liberated amino acids was analyzed by HPLC with a chiral-phase column (MCIGEL CRS10W, 4.6 × 50 mm, 0.5 mL/min, 2 mM $CuSO_4$(aq), UV detection at 254 nm).

Collection and Phylogenetic Analysis of Strain CNX037. The marine-derived actinomycete, strain CNX037, was isolated from a sediment sample collected at a depth between 18 and 30 m in August 2009 near the La Jolla Canyon, San Diego (sediment sample #CA09-129-C). The strain was identified as a *Nocardiopsis* sp. on the basis of 16S rRNA gene analysis (GenBank accession number KC149962) (Figure S1).

Fermentation, Extraction, and Isolation. The strain CNX037 was fermented at 27 °C by shaking at 250 rpm in 10 × 2.8 L Fernbach flasks each containing 1 L of the seawater-based medium A1BFe [10 g starch, 4 g yeast extract, 2 g peptone, 0.04 g $Fe_2(SO_4)_3{\cdot}4H_2O$, 0.1 g KBr].[31] After cultivation for 6 days, sterilized XAD-16 resin (20 g/L) was added to the fermentation broth to absorb the organic products. After shaking at 215 rpm for 2 h, the resin was filtered through cheesecloth, washed with 2 L of deionized water, and eluted with 5 L

of acetone. The acetone was removed under reduced pressure to provide an aqueous layer, which was further extracted with 2 L of EtOAc to afford a crude residue (220 mg). The crude residue was then dissolved in 10 mL of MeOH, and the insoluble materials were collected by centrifugation to obtain the organic extract (90 mg). The extract (90 mg) was subjected to normal-phase silica gel column chromatography (200−300 mesh, 3 g), eluting with CH_2Cl_2/CH_3OH (100/0, 100/1, 100/2, 100/5, 100/20, 100/100, and 0/100, v/v, each of 20 mL) to obtain seven subfractions (Fr.1 to Fr.7). The two fractions (Fr.5 and Fr.6, 14.0 mg) containing nocardiamide A were further purified by semipreparative HPLC with a reversed-phase C_{18} column (Dynamax column, 3 mL/min, UV 210 nm; solvent A, 0.1% formic acid in H_2O; solvent B, MeCN; eluted with constant 50% solvent B) to obtain nocardiamide A (**1**, 8.0 mg). Fr.7 (15.0 mg) was further purified by semipreparative HPLC with a reversed-phase C_{18} column (Dynamax column, 2.5 mL/min, UV 210 nm; solvent A, 0.1% formic acid in H_2O; solvent B, MeCN; eluted with constant 45% solvent B) to obtain nocardiamide B (**2**, 3.0 mg).

*Nocardiamide A (**1**):* white powder; $[\alpha]_D^{20}$ −3 (*c* 0.25, DMSO); UV (MeOH) λ_{max} (log ε) 224 (3.5), 278 (2.7) nm; IR (KBr) ν_{max} 3668, 3645, 3440, 2962, 2928, 1682, 1635, 1555, 1538, 1435, 1205, 1139, 1026, 1001, 803, 723 cm^{-1}; ^1H and 2D-NMR (500 MHz, DMSO-d_6); ^{13}C NMR (75 MHz, DMSO-d_6), see Table 1; HRESIMS *m/z* 687.4450 $[M + H]^+$ (calcd for $C_{36}H_{59}N_6O_7$, 687.4440).

*Nocardiamide B (**2**):* white powder; $[\alpha]_D^{20}$ −2 (*c* 0.11, DMSO); UV (MeOH) λ_{max} (log ε) 224(4.1), 278 (3.4) nm; IR (KBr) ν_{max} 3423, 3263, 1629, 1539, 1456, 520, 473 cm^{-1}; ^1H and 2D-NMR (500 MHz, DMSO-d_6); ^{13}C NMR (75 MHz, DMSO-d_6), see Table 1; HRESIMS *m/z* 673.4292 $[M + H]^+$ (calcd for $C_{35}H_{57}N_6O_7$, 673.4282).

Determination of the Absolute Configurations of the Amino Acids in 1 and 2 by the Advanced Marfey's Method and Chiral-Phase Analysis. The FDLA- or FDAA-derivatized hydrolysates of compounds **1** (1.0 mg) and **2** (1.0 mg) and the standard amino acids were prepared according to the published method.[11] The following solvent system was used to separate the FDLA or FDAA derivatives of **1** and **2** with UV detection at 340 nm: solvent A, H_2O with 0.1% TFA; solvent B, MeCN with 0.1% TFA. The LCMS program for detecting the derivatives was set as 10−50% solvent B (0−50 min, linear gradient), 50−100% solvent B (50−63 min), 100% solvent B (63−65 min), 100−10% solvent B (65−67 min), and 10% solvent B (67−70 min). The chiral-phase HPLC with an analytical column (MCIGEL CRS10W, 4.6 × 50 mm, Mitsubishi Chemical Corporation) was performed using a constant solvent system (2 mM $CuSO_4$ in H_2O) for 40 min at a flow rate of 1 mL/min, under the UV detection of 254 nm. The retention times were determined as follows: standard L-Ile (15.8 min), L-*allo*-Ile (11.1 min), L-Leu (13.3 min), the Ile residue in **1** (15.5 min).

The retention times and ion peaks for the Marfey's D-FDLA derivatives of the amino acid standards were determined as follows: D-Val (t_R 43.8 min, *m/z* 412 $[M + H]^+$), L-Val (t_R 52.6 min, *m/z* 412 $[M + H]^+$), D-Leu (t_R 48.3 min, *m/z* 426 $[M + H]^+$), L-Leu (t_R 55.8 min, *m/z* 426 $[M + H]^+$), D/L-Ile mixtures (t_R 47.4, 55.6 min, *m/z* 426 $[M + H]^+$), D-*allo*-Ile (t_R 47.4 min, *m/z* 426 $[M + H]^+$), L-Ile (t_R 55.6 min, *m/z* 426 $[M + H]^+$), L-*allo*-Ile (t_R 55.6 min, *m/z* 426 $[M + H]^+$), D-Tyr (t_R 57.6 min, *m/z* 770 $[M + H]^+$), L-Tyr (t_R = 58.7 min, *m/z* 770 $[M + H]^+$ for Tyr bis derivatives resulting from FDLA reactions with both the amino and hydroxyphenyl groups of Tyr).[11a] Comparison of the retention times and ion peaks of the D-FDLA derivatives for the hydrolysate of compound **1**, cyclo-(Tyr-Ile-Leu-Val_1-Val_2-Val_3), with those from the amino acid standards indicated the presence of L-Tyr (t_R 58.8 min, *m/z* 770 $[M + H]^+$), L-Ile or L-*allo*-Ile or L-Leu (t_R 55.7 min, *m/z* 426 $[M + H]^+$), D-Leu (t_R 48.3 min, *m/z* 426 $[M + H]^+$), D-Val (t_R 44.3 min, *m/z* 412 $[M + H]^+$), and L-Val (t_R 52.8 min, *m/z* 412 $[M + H]^+$). Similarly, LCMS analysis of the D-FDLA derivatives of the hydrolysate of compound **2**, cyclo-(Tyr-Val_4-Leu-Val_1-Val_2-Val_3), revealed the presence of L-Tyr (t_R 58.8, *m/z* 770 $[M + H]^+$), D-Leu (t_R 48.3 min, *m/z* 426 $[M + H]^+$), D-Val (t_R 43.8 min, *m/z* 412 $[M + H]^+$), and L-Val (t_R 52.8 min, *m/z* 412 $[M + H]^+$).

Molecular Modeling. In order to determine the configurations of the triple-Val subunit in nocardiamide A (**1**), the energy minimization of the three possible configurations (D-Val_1-L-Val_2-D-Val_3, D-Val_1-D-Val_2-L-Val_3, and L-Val_1-D-Val_2-D-Val_3) were performed by ChemBio3D (version 12.0.2) using the MM2 molecular mechanics force field with minimum RMS gradient of 0.100.

Total Synthesis of Nocardiamide A. The SPPS method (Scheme 1) was designed to synthesize nocardiamide A (**1**), in a similar manner to that reported for the turnagainolides.[32] Solid-phase 2Cl-Trt resin 0.56 g (loading amount 1 mmol/g) was placed in a clean 20 mL vessel and soaked with dimethylformamide (DMF) for 0.5 h. The first amino acid, Fmoc-L-Tyr(tBu)-OH (0.27 g, 0.56 mmol), was added in DMF; then 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU, 0.21 g, 0.56 mmol) and *N*-ethyldiisopropylamine (DIEA, 2 mL) were added to allow the reaction to proceed for 1.5 h. The reaction was capped by the addition of MeOH to make sure that there were no active resin Cl atoms left. After 30 min, the reaction mixture was washed three times by dichloromethane (DCM) and DMF (15 mL, 1 min each) and then drained. The Fmoc protecting group in L-Tyr was removed by treatment with 20% piperidine in DMF (10 mL) for 15 min under N_2. The solid-phase resin was then washed with DCM and DMF three times (10 mL, 1 min each). The ninhydrin test was checked for blue color to indicate the Fmoc protecting group in L-Tyr had been removed and the NH_2 was available. For coupling of the second amino acid, Fmoc-L-Ile-OH (0.59 g, 1.68 mmol) was added in DMF; then HBTU (0.64 g, 1.68 mmol) and DIEA (2 mL) were added to allow the reaction to proceed for 0.5 h. The reaction mixture was washed three times with DCM and DMF (15 mL, 1 min each) and then drained for the ninhydrin color test. The cycle (deprotection/washing/coupling/washing) was repeated to assemble the other four amino acids in the sequence D-Leu, D-Val, L-Val, and D-Val by using approximately 1.68 mmol of each reagent to yield the resin-bound peptide (6.12 g).

The cleavage reagent mixture TFA/thioanisole/PhOH/ethanedithiol (EDT)/DI H_2O (82.5:5:5:2.5:5) was added to the resin-bound peptide to remove the peptide from the resin to obtain the crude linear hexapeptide (1.81 g). The crude linear peptide was purified by semipreparative RP-HPLC (Biomisil ODS-P-C18, 30 × 250 mm, 5 μm; phase A 0.1% TFA/MeCN, phase B 0.1% TFA/H_2O; gradient 21% A to 31% B, 20 mL/min; UV 220 nm) to yield the pure linear hexapeptide prenocardiamide A (**3**) (124.4 mg, 31.1%). The cyclization of the linear peptide was carried out as follows: prenocardiamide A (**3**) (124.4 mg) was dissolved in 150 mL of DMF to give a ca. 10^{-3}−10^{-4} M solution of **3**. Then HBTU (0.064 g, 0.17 mmol) was added to the solution, adjusting to pH 8−9 by DIEA. The reaction proceeded by stirring at room temperature and was monitored by HPLC. Crude cyclohexapeptide was obtained by concentration *in vacuo*. The synthetic nocardiamide A (**1**) (8.7 mg, 7.2%) was finally purified by semi RP-HPLC (gradient: 23% A to 33% B).

Total Synthesis of Nocardimide B. The nocardiamide B was also subjected to solid-phase synthesis according to the designed linear sequence of L-Tyr-L-Val_4-D-Leu-D-Val_1-L-Val_2-D-Val_3, using the same method as described for the synthesis of prenocardiamide A (**3**), yielding 191 mg of the linear hexapeptide prenocardiamide B (**4**) (47.8%). The subsequent cyclization of **4** afforded 19.9 mg of synthetic nocardiamide B (**2**) (10.7%).

Bioassays. Minimum inhibitory concentration (MIC) values for compounds **1**−**4** were determined against seven strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Bacillus thuringensis* SCSIO BT01, *Bacillus subtilis* SCSIO BS01, *Micrococcus luteus* SCSIO ML01, and *Candida albicans* ATCC 10231) by previously described methods.[33] Cytotoxicities of compounds **1** and **2** were assayed against HCT-116 tumor cell lines by previously described methods.[34]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel/Fax: +86 20 89023038. E-mail: czhang2006@gmail.com.

Notes

The authors declare no competing financial interest.

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