Arenamides A−C, Cytotoxic NFκB Inhibitors from the Marine Actinomycete *Salinispora arenicola*[⊥]

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Three new cyclohexadepsipeptides, arenamides A−C (**1**−**3**), were isolated from the fermentation broth of a marine bacterial strain identified as *Salinispora arenicola*. The planar structures of these compounds were assigned by detailed interpretation of NMR and MS/MS spectroscopic data. The absolute configurations of the amino acids, and those of the chiral centers on the side chain, were established by application of the Marfey and modified Mosher methods. The effect of arenamides A and B on NFκB activity was studied with stably transfected 293/NFκB-Luc human embryonic kidney cells induced by treatment with tumor necrosis factor (TNF). Arenamides A (**1**) and B (**2**) blocked TNF-induced activation in a dose- and time-dependent manner with IC_{50} values of 3.7 and 1.7 μM, respectively. In addition, the compounds inhibited nitric oxide (NO) and prostaglandin E_2 (PGE_2) production with lipopolysaccharide (LPS)-induced RAW 264.7 macrophages. Moderate cytotoxicity was observed with the human colon carcinoma cell line HCT-116, but no cytotoxic effect was noted with cultured RAW cells. Taken together, these data suggest that the chemoprevention and anti-inflammatory characteristics of arenamides A and B warrant further investigation.

Actinomycetes belonging to the obligate marine genus *Salinispora* have proven to be a rich source of novel, biologically active secondary metabolites.[1] We have recently reported that these compounds are produced in species-specific patterns, indicating that the isolation of new species may be a productive strategy for the discovery of new secondary metabolites.[2] It has also been observed that a small proportion of strains produce compounds outside of the standard chemotype for that species, an example being cyclomarin and cyclomarazine production by *S. arenicola* strain CNS-205.[3] In these cases, the additional compounds have been termed accessory metabolites, and there is mounting evidence that their production may be correlated with the geographic location from which the strain was obtained. As part of an investigation into actinomycete diversity in marine sediments around the island nation of Fiji, the actinomycete strain CNT-088 was isolated and identified as *S. arenicola* by 16S rDNA sequence analysis. LC-MS chemotyping revealed that this strain produces an accessory compound not previously observed from any of the three currently recognized *Salinispora* species. Herein we report the isolation, structure elucidation, and NFκB inhibition activities of three new cyclodepsipeptides, arenamides A−C (**1**−**3**), obtained from culture extracts of *S. arenicola* strain CNT-088.

NFκB regulates the expression of a number of genes, the products of which are involved in tumorigenesis.[4,5] These include the antiapoptosis genes *bcl-2* and *bcl-xl*, and COX-2 and MMP-9, genes encoding adhesion molecules, chemokines, inflammatory cytokines, iNOS, and cell cycle-regulatory genes.[6,7] Thus, agents that can suppress NFκB activation have the potential of suppressing carcinogenesis.[8]

Results and Discussion

Arenamide A (**1**) was isolated as a white, crystalline solid, mp 225 °C, which analyzed for the molecular formula $C_{36}H_{57}N_5O_7$ (seven degrees of unsaturation), by EI high-resolution mass spectrometry (obsd M^+ *m/z* 671.4261, calcd M^+ *m/z* 671.4253).

This molecular formula was also supported by ^1H and ^{13}C NMR spectroscopic data (Table 1). The IR spectrum of **1** showed intense sharp absorption bands at 1745 and 1672 cm^{-1}. The ^1H NMR spectrum displayed characteristics of a typical peptide, illustrating five amide NH signals [δ_H 8.63, 8.03, 7.93, 7.88, 7.83], six α-amino protons [δ_H 4.34, 4.19, 4.10, 4.05, 4.03, 3.41], and one ester carbinol proton [δ_H 4.90]. In the ^{13}C NMR spectrum, six amide or ester resonances [δ_C 171.9, 171.8, 171.7, 171.0, 168.9, 168.8] and one oxygenated sp^3 carbon resonance [δ_C 75.8] were observed. Since six carbonyl carbons accounted for six of the seven unsaturations, arenamide A was concluded to be monocyclic. A characteristic IR ester absorption at 1745 cm^{-1} indicated arenamide A is a depsipeptide.

Further analysis of DEPT and 2D NMR spectroscopic data (COSY, HSQC, and HMBC) allowed six subunits to be established, a phenylalanine (Phe), an alanine, (Ala), a valine (Val), a glycine (Gly), a leucine (Leu), and a 3-hydroxy-4-methyldecanoic acid (HMDA). Sequencing the amino acid residues and HMDA in **1** was accomplished by HMBC NMR analysis using correlations between the α-amino proton and/or the secondary amide proton and the carbonyl carbon resonances. Specifically, cross-peaks between the secondary amide proton of Phe [δ_H 8.03] and the carbonyl carbon (C-10) of Ala linked these two amino acids. The linkage of Ala to Leu was established by HMBC correlations from the secondary amide proton of Ala [δ_H 7.83] and the α-amino proton of Leu [δ_H 4.03] to the Leu amide carbonyl [δ_C 170.8]. This tripeptide fragment was in turn linked to the Val unit through interpretation of HMBC data, which demonstrated correlations from the α-amino proton of Leu [H-14, δ_H 4.03] and the α-amino proton of Val [H-20, δ_H 4.10] to C-19 [δ_C 171]. The subsequent connection of Val to Gly was achieved on the basis of HMBC correlations from the α-amino proton of Val [H-20, δ_H 4.10] and the α-amino proton of Gly [H-25a, b, δ_H 4.05, 3.41] to the Gly carbonyl carbon C-24 [δ_C 171.7]. Further, HMBC correlations from H-27 [δ_H 2.46] and the secondary amide proton of Gly [δ_H 7.93] to C-26 [δ_C 171.8] led to the assignment of the Gly to HMDA linkage. Last, the ring closure linkage was secured by a three-bond HMBC correlation from H-28 [δ_H 4.90] to C-1 [δ_C 169.8], which allowed the planar structure of arenamide A (**1**) to be assigned.

[⊥] Dedicated to Dr. David G. I. Kingston of Virginia Polytechnic Institute and State University for his pioneering work on bioactive natural products.

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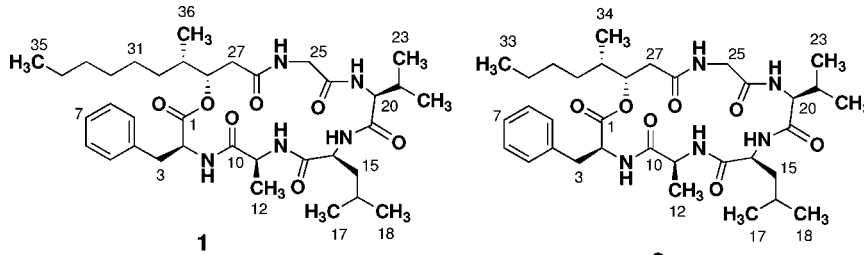

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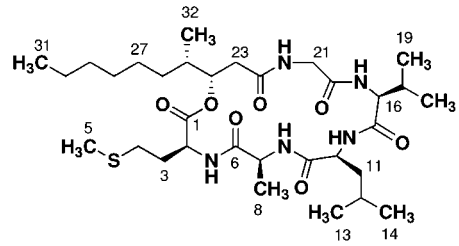

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The amino acid sequence of arenamide A, assigned on the basis of gCOSY and gHMBC correlations, was fully supported by the ESIMS/MS fragmentation pattern of **1**. Key fragmentation ions include those at *m/z* 547, 476, 363, 264, and 241, which indicated cleavage of amide bonds between Phe/Ala, Ala/Leu, Leu/Val, Val/Gly, and Gly/HMDA, respectively. Finally, the ester linkage in **1** was confirmed by methanolysis to yield the methyl ester **4**, Figure 1 (ESIMS $[M + Na]^+$ *m/z* 726). Subsequent analysis of 1D and 2D NMR spectra (Table 2) showed the presence of a new methoxyl substituent [δ_H 3.62 (s); δ_C 52.7] in the NMR spectrum of **4**.

Table 1. NMR Spectroscopic Data for Arenamide A (**1**) in DMSO-d_6[a]

	position	δ_H (*J* Hz)	δ_C		COSY	HMBC
L-Phe	1		169.8	C_q		
	2	4.34, q (14.1, 7.2)	55.6	CH	H-3, 2-NH	C-1, C-3
	3	2.97, dd (14.1,7.2)	38.1	CH_2	H-2	C-2, C-4
		2.95, dd (14.1, 8.2)				
	4		137.2	C_q		
	5/9	7.26, m	129.1	CH	H-6/8	C-4, C-6/8
	6/8	7.26, m	128.2	CH	H-5/9, H7	C-5/9, C-7
	7	7.20, m	126.5	CH	H-6/8	C-6/8
	2-NH	8.03, d (6.4)			H-2	C-1, C-2, C-10
L-Ala	10		168.9	C_q		
	11	4.19, p (14.6, 7.6)	49.0	CH	H-12, 11-NH	C-10, C-12
	12	1.15, d (7.2)	17.8	CH_3	H-11	C-11
	11-NH	7.83, d (7.6)			H-11	C-10, C-11
L-Leu	13		170.8	C_q		
	14	4.03, m	52.6	CH	H-15, 14-NH	C-13, C-15, C-16
	15	1.50, m	39.7	CH	H-14, H-16	C-14, C-17, C-18
	16	1.63, m	25.1	CH_2	H-14, H-17, H-18	C-17, C-18
	17	0.89, d (6.3)	22.9	CH_3	H-16	C-16, C-18
	18	0.84, d (6.3)	21.4	CH_3	H-16	C-16, C-17
	14-NH	8.63, d (6.1)			H-14	C-13, C-14
L-Val	19		171.0	C_q		
	20	4.10, dd (8.5, 7.9)	59.2	CH	H-21,20-NH	C-1, C-21, C-23
	21	1.87, m	30.7	CH	H-20, H-22, H-23	C-20, C-23, C-24
	22	0.88, d (6.8)	18.9	CH_3	H-21	C-21, C-23
	23	0.86, d (6.8)	18.9	CH_3	H-21	C-21, C-22
	20-NH	7.88, d (8.1)			H-20	C-19, C-20
Gly	24		171.7	C_q		
	25	4.05, dd (16.4, 5.4)	43.5	CH_2	H-25b, 25-NH	C-24
		3.41, dd (16.4, 4.3)			H-25a, 25-NH	C-24
	25-NH	7.93, t (5.4, 4.3)			H-25a, H-25b	C-24, C-25
HMDA	26		171.8	C_q		
	27	2.46, d (9.3)	38.4	CH_2	H-28	C-26, C-28, C-24
		2.24, d (14.4)			H-28	C-26, C-28
	28	4.90, dd (9.3,2.1)	75.8	CH	H-27a, H-27b, H-29	C-1, C-26, C-27, C-29
	29	1.51, m	37.1	CH	H-28, H-36	C-28, C-36, C-30
	30	1.16, m	32.5	CH_2		
	31	1.18, m 1.08, m	26.9	CH_2		
	32	1.14, m	30.8	CH_2		C-31
	33	1.16, m	32.1	CH_2		
	34	1.26, m	23.1	CH_2	H-35	C-35
	35	0.83, t (6.7)	13.9	CH_3	H-34	C-34
	36	0.67, d (6.8)	14.5	CH_3	H-29	C-28, C-29

[a] ^{1}H NMR data were recorded at 500 MHz; ^{13}C NMR spectra were obtained at 125 MHz.

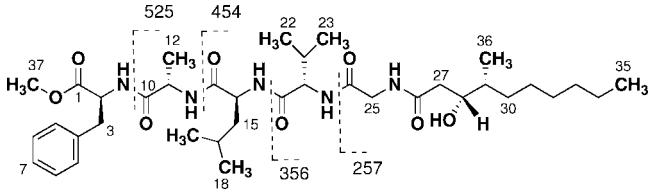

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Figure 1. Structure of methanolysis product **4** and mass spectrometric cleavage ions (*m*/*z* values) observed in the ESIMS/MS spectrum.

The relative configuration of the chiral center at C-29 was determined by analysis of coupling constant data and NOE correlations. The small coupling constant (2.1 Hz) between H-28 and H-29 suggested the *syn*-configuration, which was also supported by observed NOE correlations. The absolute configurations of the amino acid units were determined by complete acid hydrolysis of **1** and HPLC-MSD analysis of Marfey amino acid derivatives.[9] Treatment of the hydrolysate of **1** (6 N HCl, 110 °C, 18 h) with *N*-(3-fluoro-4,6-dinitrophenyl)-L-alaninamide (FDAA) yielded Marfey derivatives that were analyzed by LC-MS under positive-mode electrospray ionization (ESI). All the derivatives were identified by their retention times, molecular weights, UV spectra, and comparison of the appropriate FDAA derivatized D- and L-amino acid standards. In the hydrolysate, the retention times of the phenylalanine FDAA, alanine FDAA, leucine FDAA, and valine FDAA were 30.2, 16.3, 30.1, and 23.9 min, respectively, which correspond to L-Phe, L-Ala, L-Leu, and L-Val FDAA derivatives. Thus, all the amino acids in **1** possessed L-configurations.

The absolute configuration of the HMDA unit in arenamide A (**1**) was determined by analysis of the Mosher ester data[10] derived from the methanolysis product **4** (Figure 2). Methanolysis product **4** was acylated with *R*-(−)- and *S*-(+)-α-methoxy-α-(trifluoromethyl)phenyl acetyl chloride (MTPA-Cl) to furnish the corresponding *S*- and *R*-MTPA esters, respectively. Analysis of ^{1}H NMR and COSY spectra allowed the assignment of the proton chemical shifts for both esters in proximity of the esterified carbon. Calculation of $\Delta\delta_{S-R}$ values clearly established the absolute configuration of C-28 as *R*.

Arenamide B (**2**) was obtained as a white, crystalline solid, mp 232 °C, which analyzed for the molecular formula $C_{34}H_{53}N_5O_7$ by HREIMS (obsd M^+ at *m*/*z* 643.3937, calcd M^+ 643.3940). The molecular composition of **2** indicated the loss of 28 amu as compared to the formula of **1**. Using the same approach as in the assignment of **1**, the overall structure of compound **2** was assigned by interpretation of ESIMS/MS and 1D and 2D NMR spectroscopic data. The ^{1}H NMR spectrum of arenamide B displayed a high degree of similarity to that of **1**, with five amide protons [δ_H 8.63, 8.03, 7.93, 7.88, 7.83], six α-amino protons [δ_H 4.34, 4.19, 4.10, 4.05, 4.03, 3.42], and one ester carbinol proton [δ_H 4.90] being readily observed. The overall NMR data, including analysis of information from HSQC, COSY, and HSQC experiments, revealed the same amino acids and sequence as found in **1**. Analysis of ^{1}H, ^{13}C NMR, COSY, and HMBC data (Table 3) allowed the side chain to be assigned as 3-hydroxy-4-methyloctanoic acid (HMOA).

The relative stereochemistry of the chiral center at C-29 in **2** was determined as *R*, by the same method applied to **1** (coupling constant and NOE correlations), and the absolute configurations of the amino acid units in **2** were similarly defined by Marfey derivatization and LC-MS analysis. The identical sign of optical rotation values observed for **1** and **2** also supports this proposal.

Arenamide C (**3**), a minor metabolite, was isolated as a white powder that analyzed for the molecular formula $C_{32}H_{57}N_5O_7S$ by HREIMS [M^+ *m*/*z* 655.3971 (calcd for $C_{32}H_{57}N_5O_7S$, 655.3973)] and comprehensive analysis of its NMR data. The ^{1}H and ^{13}C NMR spectra of **3** (Table 4) were highly consistent with a cyclic hexadepsipeptide; however, major differences were observed in the aromatic region. The ^{1}H and COSY NMR spectra displayed a spin system consisting of a two-proton multiplet at δ 2.58, a methyl singlet (δ 2.05), and a two-proton multiplet at δ 2.08, corresponding to a $CH_2CH_2SCH_3$ moiety of methionine, which suggested that the phenyalanine (Phe) moiety of **1** was replaced by a methionine (Met) unit. Analysis utilizing a combination of ^{1}H, ^{13}C NMR, HSQC, COSY, and HMBC experiments, coupled with examination of the molecular formula, allowed arenamide C to be assigned structure **3**. This structure was confirmed by the ESIMS/MS fragmentation pattern. Key fragmentation ions included *m*/*z* 547, 476, 363, 264, and 241, which indicated cleavage of amide bonds between Met/Ala, Ala/Leu, Leu/Val, Val/Gly, and Gly/HMDA, respectively. The absolute configuration at C-29 and also those for the amino acids were not determined for **3**, but are expected to be identical to those in **1** on the basis of the similar optical rotations of all these metabolites.

The discovery of the arenamides from the marine actinomycete *S. arenicola* expands our understanding of the biosynthetic capabilities of this species. Other *S. arenicola* strains (CNS-276 and CNS-205) also produce cyclic heptapeptides of the cyclomarin class.[3] Examination of the literature revealed a number of depsipeptides containing 4-methyloctanoic, 4-methyldecanoic acid, and derivatives isolated from bacteria, cyanobacteria, and fungi. Examples include vinylamycin[11] from *Streptomyces* sp. M1982-63F1, iturin[12] from *Bacillus subtilis*, majusculamide D[13] and microcolin A[14] from *Lyngbya majuscula*, and beauverolide II from *Beauveria tenella*.[15]

The main activated form of NFκB is a heterodimer composed of p65 and p50 subunits. NFκB induction in response to carcinogenic or proinflammatory stimuli results in translocation subunits from cytoplasm to nucleus, where it induces the transcription of proinflammatory mediators, such as iNOS, COX-2, and IL-1β, -6 and, -8.[16,17] Using LPS-induced macrophage RAW 264.7 cells as a model, we tested arenamides A and B on production of iNOS, which catalyzes the oxidative deamination of L-arginine, to produce NO, an important pro-inflammatory mediator[18] and PGE_2 levels. At a concentration of 1 μg/mL, LPS stimulated NO synthesis in RAW cells 10−12-fold, up to 30 μM, in cell culture media. In the concentration range 3−10 μM, arenamides A and B inhibited NO production by approximately 35% in a dose-dependent manner. Similarly, PGE_2 production was reduced by about 50%. In contrast, neither compound affected cell viability at the concentrations found to inhibit NO and PGE_2 production.

NFκB is known to play a key role in the regulation of cell survival genes and coordinate the synthesis of enzymes such as iNOS and COX-2.[19] Our results indicate inhibition of the NFκB signal pathway may have functional relevance, such as inhibition of NO and PGE_2 generation. Further studies are required to understand the molecular mechanisms of this inhibition, but lack of strong cytotoxicity implies the response may be of relevance for cancer chemoprevention or anti-inflammatory responses.

Arenamides A−C (**1**−**3**) and the methanolysis product of arenamide A (**4**) were also evaluated in other biological assays. Arenamides A and B exhibited weak in vitro cytotoxicity against HCT-116 human colon carcinoma with IC_{50} values of 13.2 and 19.2 μg/mL, respectively. The arenamides had no significant activity in antimicrobial assays using methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VREF), and amphotericin-resistant *Candida albicans*.

Experimental Section

General Experimental Procedures. Melting points were determined on a Mel-Temp apparatus and are uncorrected. Optical rotations were measured using a JASCO P-2000 polarimeter with a 10 cm cell. IR spectra were acquired on a Perkin-Elmer 1600 series FTIR spectrometer. ^{1}H, ^{13}C, and 2D NMR spectroscopic data were obtained on Varian Inova

Table 2. NMR Spectroscopic Data for Methanolysis Product **4** in DMSO-d_6[a]

	position	δ_H (J Hz)	δ_C		COSY	HMBC
L-Phe	1		172.7	C_q		
	2	4.45, m	54.5	CH	H-3, 2-NH	C-1, C-3
	3	3.02, m	37.4	CH_2	H-2	C-1, C-2, C-4, C-5/9
	4		137.9	C_q		
	5/9	7.20, m	129.9	CH	H-6/8	C-3, C-7
	6/8	7.26, m	128.9	CH	H-5/9, H7	C-5/9, C-7
	7	7.22, m	127.3	CH	H-6/8	
	2-NH	8.27, d (7.5)			H-2	C-1, C-2
L-Ala	10		172.1	C_q		
	11	4.26, m	48.8	CH	H-12, 11-NH	C-10, C-12
	12	1.15, d (7.1)	19.0	CH_3	H-11	C-11, C-10
	11-NH	7.94, d (7.5)			H-11	C-10
L-Leu	13		170.8	C_q		
	14	4.27, m	51.7	CH	H-15, 14-NH	
	15	1.56, m	41.2	CH	H-14, H-16	C-17, C-18
	16	1.58, m	24.9	CH_2	H-14, H-17, H-18	C-17, C-18
	17	0.88, d (6.9)	23.8	CH_3	H-16	C-15, C-18
	18	0.81, d (6.9)	21.2	CH_3	H-16	C-15, C-17
	14-NH	7.94, d (7.4)			H-14	C-13, C-14
L-Val	19		171.0	C_q		
	20	4.17, m	58.7	CH	H-21,20-NH	C-19, C-21, C-22
	21	1.95, m	31.4	CH	H-20, H-22, H-23	C-19, C-20, C-23
	22	0.99, d (7.1)	18.8	CH_3	H-21	C-21, C-23
	23	0.83, d (7.1)	20.1	CH_3	H-21	C-21, C-22
	20-NH	7.78, d (8.1)			H-20	C-19, C-20
Gly	24		171.7	C_q		
	25	3.74, m	42.8	CH_2	H-25, 25-NH	C-24
	25-NH	8.04, t (8,4)			H-25	C-24, C-25
HMDA	26		172.9	C_q		
	27	2.19, m	41.2	CH_2	H-28	C-26, C-28
	28	3.74, m	71.2	CH	H-27a, H-27b, H-29	C-26
	29	1.41, m	38.8	CH	H-28, H-36	C-27, C-31
	30	1.24,, m	31.2	CH_2		
	31	1.18, m	26.8	CH_2		
	32	1.14, m	29.0	CH_2		
	33	1.15, m	32.3	CH_2		
	34	1.26, m	22.9	CH_2	H-35	C-35, C-33
	35	0.85, t (6.3)	14.8	CH_3	H-34	C-33
	36	0.78, d (6.9)	14.9	CH_3	H-29	C-28, C-29
	37	3.62, s	52.7	CH_3		C-1

[a] ^{1}H NMR data were recorded at 500 MHz; ^{13}C NMR spectra were obtained at 125 MHz.

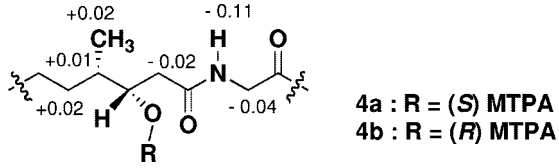

Figure 2. $\Delta\delta_{S-R}$ values for the Mosher esters **4a** and **4b** from the methanolysis product **4**.

500 MHz and Varian Inova 300 MHz NMR spectrometers. High-resolution EIMS were recorded on a Thermo MAT900XL double-focusing high-resolution mass spectrometer, HRESIMS were recorded on a Thermo LTQ Orbitrap XL mass spectrometer, and ESIMS/MS were recorded on a Thermo LCOdeca mass spectrometer. Low-resolution LC-MS spectra were obtained on a Hewlett-Packard HP1100 integrated LC-MS system with a reversed-phase C_{18} column (Agilent, 4.6 mm × 100 mm, 5 μm) at a flow rate of 0.7 mL/min. Reversed-phase HPLC separations were performed using a semipreparative C_{18} Phenomenex Luna (2) 5 μm (10 mm × 250 mm) column with a CH_3CN/H_2O gradient solvent system. Preparative HPLC was performed on a Waters 4000 system with a UV variable-wavelength detector, monitoring at 210 nm, using a C_{18} Nova-Pak 6 μm, 60 Å (40 mm × 300 mm) column.

Bacterial Isolation and Identification. The actinomycete strain CNT-088 was obtained from a marine sediment sample collected at a depth of ca. 20 m off the Great Astrolab Reef, in the Kandavu Island chain, Fiji, in 2006. The sediment was dried at room temperature under a laminar flow hood and then stamped onto a seawater agar medium as previously described.[20] Strain CNT-088 was obtained in pure culture by repeated, single colony transfer on agar media and identified as *Salinispora arenicola* based on 16S rDNA analysis (sequence deposited in GenBank under accession number FJ487867).

Fermentation and Extraction. *S. arenicola* strain CNT-088 was cultured at 27 °C for 6 days while shaking at 215 rpm in 5 × 1 L volumes of the liquid medium (A1Bfe+C) [composed of 10 g starch, 4 g yeast extract, 2 g peptone, 1 g $CaCO_3$, 40 mg $Fe_2(SO_4)_3 \cdot 4H_2O$, 100 mg KBr, per 1 L seawater]. Amberlite XAD-7 resin (20 g/L) was added at the end of the fermentation period to adsorb extracellular secondary metabolites. The culture and resin were shaken at low speed for two additional hours. The resin and cell mass were collected by filtration through cheesecloth and washed with DI water to remove salts. The resin, cell mass, and cheesecloth were then soaked for 2 h in acetone, after which the acetone extract was filtered and the solvent removed under vacuum to give 1.2 g of solid material from a 5 L culture.

Isolation of Arenamides A−C (1−3). The crude extract was fractionated by HPLC (Altima Si gel 10 × 250 mm, flow rate 5 mL/min; UV detection at 210 nm, 10% ethyl acetate in isooctane for 10 min, a linear gradient up to 100% over 40 min and then 15% methanol in ethyl acetate for 15 min) to give nine fractions. Fraction nine, which contained the peptides, was further purified by RP HPLC (Phenomenex Luna C_8 (2), 10 mm × 250 mm, 5 μm) with a gradient solvent system (0−10 min; 25−45% aqueous CH_3CN, 10−35 min; 55−65% aqueous CH_3CN, 35−50 min; 65−85% aqueous CH_3CN, 50−60 min, 100% CH_3CN) at 2.5 mL/min flow rate and UV detection of 210 nm. Arenamides A (**1**, 35 mg), B (**2**, 55 mg), and C (**3**, 1.2 mg) were eluted at 51.6, 41.5, and 44.2 min, respectively.

Arenamide A (1): white crystals; mp 225 °C; $[\alpha]^{25}_D$ −76.3 (*c* 0.08, CH_3OH); IR (neat) ν_{max} 3325, 3240, 2957, 1746, 1672, 1635, 1648, 1446, 1380, 1173, 739, 620 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6)

Table 3. NMR Spectroscopic Data for Arenamide B (**2**) in DMSO-d_6[a]

	position	δ_H (J Hz)	δ_C		COSY	HMBC
L-Phe	1		169.8	C_q		
	2	4.34, q (14.1, 7.1)	55.6	CH	H-3, 2-NH	C-1, C-3
	3	2.97, dd (14.1,7.1)2.95, dd (14.1, 8.2)	38.1	CH_2	H-2	C-2, C-4
	4		137.2	C_q		
	5/9	7.26, m	129.1	CH	H-6/8	C-4, C-6/8
	6/8	7.26, m	128.2	CH	H-5/9, H7	C-5/9, C-7
	7	7.20, m	126.5	CH	H-6/8	C-6/8
	2-NH	8.03, d (6.7)			H-2	C-1, C-2
L-Ala	10		168.9	C_q		
	11	4.19, p (14.6, 7.2)	49.0	CH	H-12, 11-NH	C-10, C-12
	12	1.15, d (7.2)	19.0	CH_3	H-11	C-11
	11-NH	7.83, d (8.0)			H-11	C-10, C-11
L-Leu	13		170.8	C_q		
	14	4.03, m	52.6	CH	H-15, 14-NH	C-13, C-15, C-16
	15	1.50, m	39.7	CH	H-14, H-16	C-14, C-17, C-18
	16	1.63, m	25.1	CH_2	H-14, H-17, H-18	C-17, C-18
	17	0.89, d (6.3)	22.9	CH_3	H-16	C-16, C-18
	18	0.84, d (6.3)	21.4	CH_3	H-16	C-16, C-17
	14-NH	8.63, d (7.2)			H-14	C-13, C-14
L-Val	19		171.0	C_q		
	20	4.10, t (8.5)	59.2	CH	H-21,20-NH	C-1, C-21, C-23
	21	1.87, m	30.7	CH	H-20, H-22, H-23	C-20, C-23, C-24
	22	0.88, d (6.8)	18.9	CH_3	H-21	C-21, C-23
	23	0.86, d (6.8)	18.9	CH_3	H-21	C-21, C-22
	20-NH	7.88, d (8.1)			H-20	C-19, C-20
Gly	24		171.7	C_q		
	25	4.05, dd (16.4,5.3)	43.5	CH_2	H-25b, 25-NH	C-24
		3.42, dd (16.4, 2.4)			H-25a, 25-NH	C-24
	25-NH	7.93, t (5.3, 2.4)			H-25a, H-25b	C-24, C-25
HMOA	26		171.8	C_q		
	27	2.46, d (9.3)	38.4	CH_2	H-28	C-26, C-28
		2.25, d (14.5)			H-28	C-26, C-28
	28	4.90, dd (9.3, 2.0)	75.8	CH	H-27a, H-27b, H-29	C-1, C-26, C-27, C-29
	29	1.51, m	37.1	CH	H-28, H-34	C-28, C-34, C-30
	30	1.15, m	32.5	CH_2		
	31	1.14, m	30.8	CH_2		
	32	1.26, m	23.1	CH_2	H-33	C-33
	33	0.83, t (6.7)	13.9	CH_3	H-32	C-32
	34	0.67, d (6.8)	14.5	CH_3	H-29	C-28, C-29

[a] ^{1}H NMR data were recorded at 500 MHz; ^{13}C NMR spectra were obtained at 125 MHz.

and ^{13}C NMR (125 MHz, DMSO-d_6), see Table 1; ESIMS m/z 672 [M + H]$^+$, 694 [M + Na]$^+$; ESIMS/MS (fragmentation of m/z 694) 547 [M − Phe + Na]$^+$, 476 [M − Phe − Ala + Na]$^+$, 363 [M − Phe − Ala − Leu + Na]$^+$, 264 [M − Phe − Ala − Leu − Val + Na]$^+$, 207 [M − Phe − Ala − Leu − Gly + Na]$^+$; HREIMS m/z 671.4261 M$^+$ (calcd for $C_{36}H_{57}N_5O_7$ 671.4253).

Arenamide B (2): white crystals; mp 232 °C; $[\alpha]^{25}_D$ −96.3 (*c* 0.27, CH_3OH); IR (neat) ν_{max} 3327, 2957, 1748, 1672, 1638, 1548, 1446, 1380, 1273, 698, 621 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6), see Table 3; ESIMS m/z 664 [M + H]$^+$, 666 [M + Na]$^+$; ESIMS/MS (fragmentation of m/z 666) 519 [M − Phe + Na]$^+$, 448 [M − Phe − Ala + Na]$^+$, 335 [M − Phe − Ala − Leu + Na]$^+$, 236 [M − Phe − Ala − Leu − Val + Na]$^+$, 179 [M − Phe − Ala − Leu − Gly + Na]$^+$; HREIMS m/z 643.3937 M$^+$ (calcd for $C_{34}H_{53}N_5O_7$ 643.3940).

Arenamide C (3): white powder; $[\alpha]^{25}_D$ −45 (*c* 0.09, CH_3OH); IR (neat) ν_{max} 3379, 2930, 1667, 1554, 1364, 1091, 801, 702 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD-d_6) and ^{13}C NMR (125 MHz, CD_3OD-d_6), see Table 4; ESIMS m/z 656 [M + H]$^+$, 678 [M + Na]$^+$; ESIMS/MS (fragmentation of m/z 678) 547 [M − Phe + Na]$^+$, 476 [M − Phe − Ala + Na]$^+$, 363 [M − Phe − Ala − Leu + Na]$^+$, 264 [M − Phe − Ala − Leu − Val + Na]$^+$, 207 [M − Phe − Ala − Leu − Gly + Na]$^+$; HREIMS m/z 655.3971 M$^+$ (calcd for $C_{32}H_{57}N_5O_7S$ 655.3973).

Acid Hydrolysis and Marfey Analysis. Hydrolysis of arenamides A (**1**) and B (**2**) (0.6 mg each) was achieved, in separate experiments, by the addition of 1 mL of 6 N HCl at 115 °C for 18 h. The hydrolysate was evaporated to dryness and resuspended in water (1 mL) and again evaporated to dryness under a stream of N_2 to remove traces of HCl. The resultant hydrolysate was dissolved in a small amount of 10% CH_3CN/H_2O and loaded on a C_{18} SEPAK (0.5 cm × 1 cm), and the column was eluted with 10% CH_3CN/H_2O (2 mL). The solvent was evaporated and 1 N $NaHCO_3$ (100 μL) and *N*-(3-fluoro-4,6-dinitrophenyl)-L-alaninamide (FDAA, 50 μL [10 mg/mL solution in acetone]) were added, and the mixture was heated at 80 °C for 3 min. The reaction mixture was cooled, neutralized with 2 N HCl (50 μL), and diluted with 50% aqueous CH_3CN (300 μL). About 10 μL of the FDAA derivative was analyzed by HPLC using a C_{18} column (Luna, 4.6 mm × 100 mm) by LC-MS. Aqueous CH_3CN containing 1% TFA was used as the mobile phase with linear gradient elution (10−60% for 50 min) at a flow rate of 0.7 mL/min. FDAA-derivatized amino acids were detected by absorption at 340 nm. A Hewlett-Packard Series 1100 MSD mass spectrometer was used for detection in API-ES (positive, mass range 100−1200 Da) mode. The retention times (min) of the derivatives were compared with those of authentic derivatized standards L-Phe (30.3), L-Ala (16.3), L-Val (23.8), L-Leu (30.1), D-Phe (34.2), D-Ala (19.6), D-Val (29.2), and D-Leu (35.3), which showed that the phenylalanine, alanine, valine, and leucine amino acids of arenamides A (**1**) and B (**2**) possess L configurations.

Methanolysis of Arenamide A (1). To 7.2 mg of **1** was added 1.5 mL of a 0.5 N NaOMe solution, and the reaction was stirred at room temperature for 2 h under an argon atmosphere. LC-MS analysis indicated the complete conversion of the starting material to the desired product. The mixture was neutralized by adding 1 mL of saturated NH_4Cl. The resulting mixture was extracted with EtOAc, with the EtOAc phase dried in vacuo, and the methanolysis product obtained was purified by HPLC (semipreparative Phenomenex Luna C_{18} (2), 10 mm × 250 mm, 5 μm) with a gradient solvent system (0−10 min; 50% aqueous CH_3CN, 10−40 min; 55% aqueous CH_3CN, 40−50 min; 85% aqueous CH_3CN, 50−55 min; 100% CH_3CN) at 2.5 mL/min flow rate and UV detection of 210 nm, to give 4.4 mg of the ring-opened methyl ester derivative of arenamide A (**4**).

Arenamide A Methanolysis Product (4): white powder; $[\alpha]^{25}_D$ −36.8 (*c* 0.07, CH_3OH); IR (neat) ν_{max} 3326, 2960, 1743, 1642, 1535, 1454, 1409, 1200, 1091, 700 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6), see Table 2; ESIMS m/z 704 [M + H]$^+$, 726 [M + Na]$^+$; ESIMS/MS (fragmentation of m/z 704) 525

Table 4. NMR Spectroscopic Data for Arenamide C (**3**) in CD_3OD-d_4[a]

	position	δ_H (J Hz)	δ_C		COSY	HMBC
L-Met	1		173.1	C_q		
	2	4.65, dd (8.4, 5.5)	53.0	CH	H-3	C-1, C-3, C-6
	3	2.08, m	31.3	CH_2	H-2, H-4	C-2
	4	2.58, m	30.7	CH_2	H-3	C-2, C-3, C-5
	5	2.05, s	15.2	CH_3		C-4
L-Ala	6		174.0	C_q		
	7	4.35, q (7.2)	50.1	CH	H-8	C-6, C-8, C-9
	8	1.40, d (7.2)	17.5	CH_3	H-7	C-6, C-7
L-Leu	9		173.9	C_q		
	10	4.14, dd (13.4, 7.1)	54.7	CH	H-11	C-9, C-11, C-12
	11	1.50, m	39.8	CH	H-10, H-12	C-9, C-10, C-14
	12	1.68, m	28.3	CH_2	H-11, H-13, H-14	C-11, C-13, C-14
	13	0.95, d (6.7)	23.1	CH_3	H-12	C-12, C-14
	14	1.00, d (6.7)	22.2	CH_3	H-12	C-12, C-13
L-Val	15		173.3	C_q		
	16	4.23, m	60.3	CH	H-17	C-15, C-20
	17	2.08, m	32.3	CH	H-16, H-18, H-19	C-16, C-18, C-19
	18	0.97, d (7.0)	19.5	CH_3	H-17	C-16, C-19
	19	0.96, d (7.0)	19.2	CH_3	H-17	C-17, C-18
Gly	20		170.6	C_q		
	21a	4.23, d (16.6)	44.1	CH_2	H-21b	C-20
	21b	3.53, d (16.6)			H-21a	C-20, C-22
HMDA	22		172.8	C_q		
	23a	2.55, m	40.4	CH_2	H-23b, H-24	C-22, C-24
	23b	2.45, d (14.4)			H-23a	C-22
	24	5.05, dd (9.1,2.0)	77.7	CH	H-23a, H-25	C-1, C-23
	25	1.41, m	38.8	CH	H-24, H-32	
	26	1.22, m	32.9	CH_2		
	27	1.30, m	31.0	CH_2		
	28	1.29, m	25.9	CH_2		
	29	1.26, m	33.8	CH_2		
	30	1.31, m	23.7	CH_2	H-31	C-31
	31	0.94, t (6.8)	14.8	CH_3	H-30	C-30
	32	0.91, d (6.7)	14.4	CH_3	H-25	C-24, C-25

[a] ^{1}H NMR data were recorded at 500 MHz; ^{13}C NMR spectra were obtained at 125 MHz.

$[M - C_{10}H_{12}NO_2 + H]^+$, 454 $[M - C_{10}H_{12}NO_2 - Ala + H]^+$, 356 $[M - C_{10}H_{12}NO_2 - Ala - Leu + H]^+$, 257 $[M - C_{10}H_{12}NO_2 - Ala - Leu - Val + H]^+$; HRESITOFMS m/z 726.4420 $[M + Na]^+$ (calcd for $C_{37}H_{61}N_5O_8Na$ 726.4412).

Preparation of MTPA Derivatives of 4. Approximately 1 mg of dimethylaminopyridine (DMAP) was added to 0.8 mg of compound **4**. One milliliter of freshly distilled pyridine was added to this mixture, and the mixture was stirred at RT for 30 min. Next, 15 μL of (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride was added. The resultant reaction mixture was stirred at RT for 70 min under N_2. After removal of solvents under vacuum, the residue was purified by RP HPLC (Waters Prep LC 4000 system, Phenomenex Luna C_{18} (2), 10 mm × 250 mm, 2.5 mL/min, UV detection at 210 nm) using a gradient solvent system (0−10 min; 50% aqueous CH_3CN, 10−40 min; 70% aqueous CH_3CN, 40−50 min; 100% CH_3CN). The *S*-MTPA ester obtained (**4a**, 1.1 mg) eluted at 31.40 min. The identical procedure was carried out to obtain the *R*-MTPA ester (**4b**, 0.95 mg) with (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride. The unit masses of *R*- and *S*-MTPA esters ($[M + H]^+$ m/z 920, $[M + Na]^+$ m/z 942, molecular formula $C_{47}H_{68}F_3N_5O_{10}$) were identified by ESI-LC-MS analysis.

NFκB Luciferase Assay. Human embryonic kidney cells 293 were used to monitor alteration of the NFκB pathway. This cell line contains chromosomal integration of a luciferase reporter construct regulated by the NFκB response element. Transcription factors can bind to the response element when stimulated by certain agents, allowing transcription of the luciferase gene. The gene product, luciferase, reacts with substrate, emitting light that was detected using a LUMIstar Galaxy BMG luminometer. After incubating treated cells, they were lysed in Reporter Lysis buffer, and the assay was performed using the Luc assay system from Promega.[21]

PGE_2 Production. RAW 264.7 cells were incubated in 96-well plates for 24 h. The medium was changed, and various concentrations of compounds **1** and **2** in DMSO were added and incubated for 30 min, followed by LPS treatment for 24 h. The medium was collected and diluted in PBS for PGE_2 determination using an ELISA method.[22]

Measurement of Nitric Oxide (NO). NO has a short half-life and is subsequently oxidized to a stable end product, nitrite, the amount of which was determined with Griess reagent. RAW 264.7 cells (1×10^5 cells/well) were incubated in 96-well culture plates for 24 h. The cells were treated with serially diluted compounds **1** and **2** dissolved in phenol red-free DMEM for 30 min followed by treatment with LPS (1 μg/mL) for 24 h. Nitrite in the media of cultured macrophages was reacted with Griess reagent [1:1 mixture (v/v) of 1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine in 2.5% H_3PO_4], and absorbance was measured at 540 nm. A standard curve was created by using known concentrations of sodium nitrite.[23]

RAW Cell Cytotoxicity Assay. To evaluate the cytotoxic effect of the compounds with RAW 264.7 cells using the NCI protocol,[24] briefly, 20% trichloroacetic acid was added to the incubated plate for 1 h, adherent cells were stained with 100 μL of 0.4% sulforhodamine B in 1% acetic acid, and cell viability was quantified by measuring absorbance at 515 nm.

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Supporting Information Available: ^{1}H, ^{13}C, gCOSY, gHSQC, and gHMBC spectra of **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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