Published in final edited form as:

J Nat Prod. 2009 March 27; 72(3): 396–402. doi:10.1021/np800617a.

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

Ratnakar N. Asolkar[†], Kelle C. Freer[†], Paul R. Jensen[†], William Fenical^{*,†}, Tamara P. Kondratyuk[§], Eun-Jung Park[§], and John M. Pezzuto[§]

Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92093-0204 and College of Pharmacy, University of Hawaii at Hilo, Hilo, Hawaii 96720

Abstract

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's 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 IC₅₀ values of 3.7 and 1.7 μM, respectively. In addition, the compounds inhibited nitric oxide (NO) and prostaglandin E₂ (PGE₂) 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.

[‡]Dedicated to Dr. David G. I. Kingston, Virginia Polytechnic Institute and State University, for his pioneering work in bioactive natural products.

^{*}To whom correspondence should be addressed. Phone: 858-534-2133. Fax: 858-558-3702. E-mail: wfenical@ucsd.edu.

[†]Scripps Institution of Oceanography

[§]University of Hawaii at Hilo

NFκB regulates the expression of a number of genes, the products of which are involved in tumorigenesis.^{4,5} These include the anti-apoptosis genes *bcl-2* and *bcl-xl*, and COX-2, 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 C₃₆H₅₇N₅O₇ (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 ¹³C 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 ¹³C NMR spectrum, six amide or ester resonances [δ_C 171.9, 171.8, 171.7, 171.0, 168.9, 168.8] and one oxygenated sp³ 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 lined 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.

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 $[\text{M} + \text{Na}]^+ m/z$ 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**.

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 data¹⁰ 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 ¹H NMR, and COSY spectra allowed the assignment of the proton chemical shifts for both esters in proximity of the esterified carbon. Calculation of $\Delta\delta_{\text{S-R}}$ 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, C₃₄H₅₃N₅O₇, 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 ¹H 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 1H , ^{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 support 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 1H 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 1H 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 phenylalanine (Phe) moiety of **1** was replaced by a methionine (Met) unit. Analysis utilizing a combination of 1H , ^{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** based upon the similar optical rotations of all these metabolites.

The discovery of the arenamides from the marine actinomycete *Salinispora 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 vinylamycin11 from *Streptomyces* sp. M1982-63F1, iturin¹² from *Bacillus subtilis*, majusculamide D¹³ and microcolin A¹⁴ from *Lyngbya majuscula*, and beauverolide II from *Beauveria tenella*.¹⁵

The main activated form of NF κ B is a heterodimer composed of p65 and p50 subunits. NF κ B 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-1 β , -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 mediator¹⁸ and PGE₂ levels. At a concentration of 1 μ g/mL, LPS stimulated NO synthesis in RAW cells 10–12-fold, up to 30 μ M, in cell culture media. In the concentration range of 3–10 μ M, arenamides A and B inhibited NO production by approximately 35% in a dose-dependent manner. Similarly, PGE₂ production was reduced by about 50%. In contrast, neither compound affected cell viability at the concentrations found to inhibit NO and PGE₂ production.

NF κ B is known to play a key role in the regulation of cell survival genes and coordinate the synthesis of enzymes like iNOS and COX-2.¹⁹ Our results indicate inhibition of the NF κ B

signal pathway may have functional relevance, such as inhibition of NO and PGE₂ 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 IC₅₀ 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. ¹H, ¹³C, and 2D NMR spectroscopic data were obtained on Varian Inova 500 MHz and Varian Inova 300 MHz NMR spectrometers. High-resolution EIMS were recorded on Thermo MAT900XL double-focusing high resolution mass spectrometer, HRESIMS were recorded on Thermo LTQ Orbitrap XL mass spectrometer, and ESIMS/MS were recorded on Thermo LCODECA mass spectrometer. Low-resolution LC-MS spectra were obtained on a Hewlett-Packard HP1100 integrated LC-MS system with a reversed-phase C₁₈ column (Agilent, 4.6 mm × 100 mm, 5 µm) at the flow rate of 0.7 mL/min. Reversed-phase HPLC separations were performed using a semi-preparative C₁₈ Phenomenex Luna (2) 5 µm, (10 mm × 250 mm) column with a CH₃CN/H₂O gradient solvent system. Preparative HPLC was performed on a Waters 4000 system with a UV variable wavelength detector, monitoring at 210 nm, using a C₁₈ 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 Bankit 1158085).

Fermentation and Extraction

S. arenicola, strain CNT-088 was cultured at 27 °C for 6 days while shaking at 215 rpm in 5 × 1 liter volumes of the liquid medium (A1Bfe+C) [composed of 10 g starch, 4 g yeast extract, 2 g peptone, 1 g CaCO₃, 40 mg Fe₂(SO₄)₃·4H₂O, 100 mg KBr, per 1 liter 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 C₈ (2), 10 mm × 250 mm, 5 μm) with a gradient solvent system (0–10 min; 25 – 45 % aqueous CH₃CN, 10–35 min; 55 – 65 % aqueous CH₃CN, 35–50 min; 65 – 85 % aqueous CH₃CN, 50–60 min, 100 % CH₃CN) 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; $[\alpha]_D^{25} - 76.3$ (*c* 0.08, CH₃OH); IR (neat) $\nu_{\max}$ 3325, 3240, 2957, 1746, 1672, 1635, 1648, 1446, 1380, 1173, 739, 620 cm⁻¹; ¹H NMR (500 MHz, DMSO-*d*₆) and ¹³C 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 C₃₆H₅₇N₅O₇ - 671.4253).

Arenamide B (2): white crystals; mp 232 °C; $[\alpha]_D^{25} - 96.3$ (*c* 0.27, CH₃OH); IR (neat) $\nu_{\max}$ 3327, 2957, 1748, 1672, 1638, 1548, 1446, 1380, 1273, 698, 621 cm⁻¹; ¹H NMR (500 MHz, DMSO-*d*₆) and ¹³C 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 C₃₄H₅₃N₅O₇ - 643.3940).

Arenamide C (3): white powder; $[\alpha]_D^{25} - 45$ (*c* 0.09, CH₃OH); IR (neat) $\nu_{\max}$ 3379, 2930, 1667, 1554, 1364, 1091, 801, 702 cm⁻¹; ¹H NMR (500 MHz, CD₃OD-*d*₆) and ¹³C NMR (125 MHz, CD₃OD-*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 C₃₂H₅₇N₅O₇S - 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 N₂ to remove traces of HCl. The resultant hydrolysate was dissolved in a small amount of 10 % CH₃CN/H₂O and loaded on a C₁₈ SEPAK (0.5 cm × 1 cm) and the column was eluted with 10 % CH₃CN/H₂O (2 mL). The solvent was evaporated and 1N NaHCO₃ (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 2N HCl (50 μL), and diluted with 50% aqueous CH₃CN (300 μL). About 10 μL of the FDAA derivative was analyzed by HPLC using a C₁₈ column (Luna, 4.6 mm × 100 mm) by LC-MS. Aqueous CH₃CN 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** were added 1.5 mL of 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 saturated NH₄Cl. The resulting mixture was extracted with EtOAc, with the EtOAc phase dried in vacuo, and the methanolysis product obtained purified by HPLC (semi-prep Phenomenex Luna C₁₈ (2), 10 mm × 250 mm, 5 μm) with a gradient solvent system (0–10 min; 50% aqueous CH₃CN, 10–40 min; 55% aqueous CH₃CN, 40–50 min; 85% aqueous CH₃CN, 50–55 min; 100% CH₃CN) at 2.5 mL/min flow rate and UV detection of 210 nm, to give 4.4 mg of the ring open methyl ester derivative of arenamide A (**4**).

Arenamide A Methanolysis Product (**4**)

White powder; $[\alpha]_D^{25} - 36.8$ (c 0.07, CH₃OH); IR (neat) $\nu_{\max}$ 3326, 2960, 1743, 1642, 1535, 1454, 1409, 1200, 1091, 700 cm⁻¹; ¹H NMR (500 MHz, DMSO-*d*₆) and ¹³C 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 [M – C₁₀H₁₂NO₂ + H]⁺, 454 [M – C₁₀H₁₂NO₂ – Ala + H]⁺, 356 [M – C₁₀H₁₂NO₂ – Ala – Leu + H]⁺, 257 [M – C₁₀H₁₂NO₂ – Ala – Leu – Val + H]⁺; HRESITOFMS *m/z* 726.4420 [M + Na]⁺ (calcd for C₃₇H₆₁N₅O₈Na 726.4412).

Preparation of MTPA Derivatives of (**4**)

Approximately 1 mg of dimethylaminopyridine (DMAP) was added to 0.8 mg of compound **4**. One mL 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 were added. The resultant reaction mixture was stirred at RT for 70 min under N₂. After removal of solvents under vacuum, the residue was purified by RP HPLC (Waters Prep LC 4000 system, Phenomenex Luna C₁₈ (2), 10 mm × 250 mm, 2.5 mL/min, UV detection at 210 nm) using a gradient solvent system (0–10 min; 50% aqueous CH₃CN, 10–40 min; 70% aqueous CH₃CN, 40–50 min; 100% CH₃CN). 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 C₄₇H₆₈F₃N₅O₁₀) 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

PGE₂ 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 PGE₂ 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 $\mu\text{g/mL}$) 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank C. A. Kauffman for assistance with culturing this strain and S. Kelly for performing the antibacterial, antifungal, and cytotoxic bioassays. We also thank A. Mrse (UCSD) for assistance with NMR experiments and Y. Su (UCSD) for HRMS and MS/MS data. This research is a result of financial support from the National Institutes of Health under NCI grants P01 CA48112 and U01-TW007401-01, the latter under the Fogerty Center's International Cooperative Biodiversity Groups program. We thank the Roko Tui Kadavu and Tui Dravuni for their hospitality and for permission to collect in the reefs of Kadavu Province, and W. Aalbersberg (University of the South Pacific) for providing laboratory space and facilitating field collections. We also thank the government of Fiji for permission to export samples.

References and Notes

1. (a) Fenical W, Jensen PR. *Nat Chem Biol* 2006;2:666–673. [PubMed: 17108984] (b) Blunt JW, Copp BR, Hu WP, Munro MHG, Northcote PT, Prinsep MR. *Nat Prod Rep* 2008;25:35–94. [PubMed: 18250897]
2. Jensen PR, Williams PG, Oh DC, Zeigler L, Fenical W. *Appl Environ Microbiol* 2007;73:1146–1152. [PubMed: 17158611]
3. Schultz AW, Oh DC, Carney JR, Williamson RT, Udway DW, Jensen PR, Gould SJ, Fenical W, Moore BS. *J Am Chem Soc* 2008;130:4508–4516.
4. Aggarwal BB, Sethi G, Nair A, Ichikawa H. *Curr Sign Transd Ther* 2006;1:25–52.
5. Melisi D, Chiao PJ. *Expert Opin Ther Targets* 2007;11:133–144. [PubMed: 17227230]
6. Kim HJ, Hawke N, Baldwin AS. *Cell Death Differ* 2006;13:738–747. [PubMed: 16485028]
7. Pikarsky E, Ben-Neriah Y. *Eur J Cancer* 2006;42:779–784. [PubMed: 16530406]
8. Inoue J, Gohda J, Akiyama T, Semba K. *Cancer Sci* 2007;98:268–274. [PubMed: 17270016]
9. Marfey P. *Carlsberg Res Commun* 1984;49:591–596.
10. (a) Ohtani I, Kusumi T, Kakisawa Y. *J Am Chem Soc* 1991;113:4092–4096. (b) Seco JM, Quinoa E, Riguera R. *Tetrahedron: Asymmetry* 2001;12:2915–2925.
11. Igarashi M, Shida T, Sasaki Y, Kinoshita N, Naganawa H, Hanada M, Takeuchi T. *J Antibiot* 1999;52:873–879. [PubMed: 10604756]
12. Hiradate S, Yoshida S, Sugie H, Yada H, Fujii Y. *Phytochemistry* 2002;61:693–698. [PubMed: 12423891]
13. Moore RE, Entzeroth M. *Phytochemistry* 1988;27:3101–3103.

14. Koehn FE, Longley RE, Reed JK. *J Nat Prod* 1992;55:613–619. [PubMed: 1517734]
15. Jegorov A, Sedmera P, Mat'ha V, Simek P, Zahradnickova H, Landa Z, Eyal J. *Phytochemistry* 1994;37:1301–1303. [PubMed: 7765754]
16. Karin M, Ben-Neriah Y. *Annu Rev Immunol* 2000;18:621–663. [PubMed: 10837071]
17. Lappas M, Permezel M, Georgiou HM, Rice GE. *Biol Reprod* 2002;67:668–673. [PubMed: 12135912]
18. Chung HT, Choi BM, Kwon YG, Kim YM. *Methods Enzymol* 2008;441:329–338. [PubMed: 18554543]
19. Harikumar KB, Aggarwal BB. *Cell Cycle* 2008;7:1020–1035. [PubMed: 18414053]
20. Gontang EA, Fenical W, Jensen PR. *Appl Environ Microbiol* 2007;73:3272–3282. [PubMed: 17400789]
21. Homhual S, Bunyaphrathasara N, Kondratyuk TP, Herunsalee A, Chaukul W, Pezzuto JM, Fong HHS, Zhang HJ. *J Nat Prod* 2006;69:421–424. [PubMed: 16562850]
22. Homhual S, Zhang H-J, Bunyaphrathasara N, Kondratyuk TP, Santarsiero BD, Mesecar AD, Herunsalee A, Chaukul W, Pezzuto JM, Fong HHS. *Planta Med* 2006;72:255–260. [PubMed: 16534731]
23. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. *Anal Biochem* 1982;126:131–138. [PubMed: 7181105]
24. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bodesch H, Kenney S, Boyd MR. *J Natl Cancer Inst* 1990;82:131–138.

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

4a : R = (*S*) MTPA
4b : R = (*R*) MTPA

Figure 2.
 $\Delta\delta_{S-R}$ values for the Mosher esters **4a** and **4b** from the methanolysis product **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)			
L-Ala	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
	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
L-Leu	11-NH	7.83, d (7.6)		H-11	C-10, C-11
	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
Gly	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
	24		171.7 C _q		

Position	δ_H (J/Hz)	δ_C	COSY	HMBC
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	26.9 CH ₂		
	1.08, m			
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

^a¹H NMR data were recorded at 500 MHz; ¹³C NMR spectra were obtained at 125 MHz.

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

	position	δ_H (J Hz)	δ_C	COSY	HMBC
HMDA	25-NH	8.04, t (8.4)		H-25	C-24, C-25
	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

^a¹H NMR data were recorded at 500 MHz; ¹³C NMR spectra were obtained at 125 MHz.

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)	38.1 CH ₂	H-2	C-2, C-4
		2.95, dd (14.1, 8.2)			
	4		137.2 C _q		
L-Ala	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
	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
L-Leu	11-NH	7.83, d (8.0)		H-11	C-10, C-11
	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
L-Val	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
	19		171.0 C _q		
	20	4.10, t (8.5)	59.2 CH	H-21, 20-NH	C-1, C-21, C-23
Gly	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
	24		171.7 C _q		

position	δ_H (J Hz)	δ_C	COSY	HMBC
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

^a₁H NMR data were recorded at 500 MHz; ¹³C NMR spectra were obtained at 125 MHz.

Table 4

NMR Spectroscopic Data for Arenamide C (3) in CD₃OD-*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	

position	δ_H (J Hz)	δ_C	COSY	HMBC
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

^a₁H NMR data were recorded at 500 MHz; ¹³C NMR spectra were obtained at 125 MHz.