Isolation and characterization of actinoramides A–C, highly modified peptides from a marine *Streptomyces* sp.

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ABSTRACT

Reported herein is the isolation and structure elucidation of three highly modified peptides, actinoramides A–C (**1–3**), which are produced by a marine bacterium closely related to the genus *Streptomyces*. The planar structures of the actinoramides, which are composed of the unusual amino acids 2-amino-4-ureidobutanoic acid and 4-amino-3-hydroxy-2-methyl-5-phenylpentanoic acid, were assigned by chemical transformations and by interpretation of spectroscopic data, while the absolute configuration of these new peptides were defined by application of the advanced Marfey's and Mosher's methods.

1. Introduction

Natural products from terrestrial actinomycetes have been used for developing and discovering new medicines for the last 50 years.[1] However, a decline in the discovery of new chemical structures from terrestrial actinomycetes has led to a reduced pipeline of new drugs for pharmaceutical research.[2] We have been investigating marine-derived actinomycetes as a source of novel secondary metabolites. Studies of marine sediments collected off the coast of southern California have revealed considerable actinomycete diversity including many new groups that, to date, have only been reported from marine samples.[3] Included in one of these groups is the seawater-requiring strain CNQ-027. This strain shares only 97.6% 16S rRNA gene sequence identity with a marine *Streptomyces* sp., *S. marinus*, suggesting it may represent a new *Streptomyces* sp.. The genus *Streptomyces* is mainly a terrestrial genus and is abundantly distributed in soil with literally hundreds of species reported. In our studies of the marine environment, we have repeatedly isolated seawater-requiring *Streptomyces* strains indicating that a subdivision of this genus is likely adapted to life in marine systems.[3] In culture, *Streptomyces* strain CNQ-027 produced a series of unprecedented peptides that we have named actinoramides A–C. Herein, we describe the isolation and structural elucidation of these highly modified peptides.
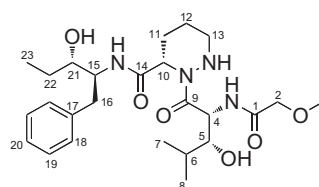
2. Results and discussion

Actinoramide A (**1**) was isolated as a yellow oil, which analyzed for the molecular formula $C_{31}H_{47}N_7O_9$ based on a pseudomolecular ion peak at *m/z* 662.3510 $[M+H]^+$ in the HRESIMS and on interpretation of ^{13}C NMR data. The ^{13}C NMR spectrum of **1** in
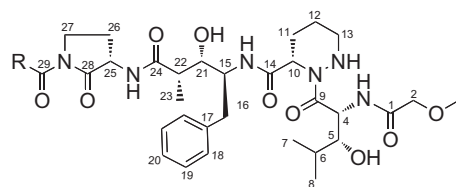
actinoramide A (**1**): R = NH₂

actinoramide B (**2**): R = CH₃

actinoramide C (**3**)

Chemical structures of Actinoramides A-C (**1-3**)

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combination with data from 135° DEPT spectra revealed four methyl, seven methylene, 13 methine, and seven fully substituted carbons. The ^{1}H and gradient (g)COSY NMR spectra from **1** displayed the typical features for a peptide with three amide proton signals [δ_H 8.10, 7.59, 7.43], and four α-amino protons [δ_H 5.50, 4.95, 4.38, 4.13]. The ^{1}H NMR spectrum also revealed mono-substituted benzene protons [δ_H 7.23 (2H), 7.21, 7.14 (2H)], two oxygenated methine protons [δ_H 3.44, and 3.43], three methyl doublets [δ_H 1.05, 0.87 and 0.83], and an *O*-methyl group [δ_H 3.37].

Analysis of gCOSY, gHSQC, and gHMBC NMR spectroscopic data allowed five distinct fragments to be discerned, including two rarely reported amino acids: 3-hydroxyleucine (Hle) and piperazic acid (Pip) and two unusual amino acids, 4-amino-3-hydroxy-2-methyl-5-phenylpentanoic acid (Ahmppa), and 2-amino-4-ureidobutanoic acid (Auba). The Hle and Pip residues were identified by interpretation of gCOSY and gHMBC NMR data. For Hle, gCOSY NMR crosspeaks permitted construction of the spin system from H-4 through H-8 [H-4 (δ_H 5.50), H-5 (δ_H 3.43), H-6 (δ_H 1.64), H-7 (δ_H 0.87), and H-8 (δ_H 0.83)]. The gCOSY NMR correlations observed from H-5 to 5-OH (δ_H 4.79) indicated that the alcohol should be attached to C-5. The Hle residue was also confirmed by gHMBC NMR correlations from H-5 (δ_H 3.43) to C-9 (δ_C 173.0), C-4 (δ_C 50.4), C-6 (δ_C 29.8), C-7 (δ_C 20.8), and C-8 (δ_C 16.1). The connectivity from H-10 through H-13 in the Pip residue was established by analysis of the gCOSY crosspeaks [H-10 (δ_H 4.95), H-11 (δ_H 2.09, 1.63), H-12 (δ_H 1.34), H-13 (δ_H 2.79)]. The NH (δ_H 4.50) proton coupled to H-13 (δ_H 2.79) displayed a gHMBC correlation to C-9 (δ_C 173.0). gHMBC correlations were also observed from the α proton H-10 (δ_H 4.95) to two carbonyl carbons C-14 (δ_C 171.0) and C-9 (δ_C 173.0). However, the α proton, H-10 (δ_H 4.95), was not coupled to the NH (δ_H 4.50), as revealed by interpretation of gCOSY spectral data. On this basis, this residue was assigned as a piperazic acid moiety. The Ahmppa residue was also constructed by analysis of gCOSY NMR correlations from methylene protons H-16 (δ_H 2.79) and an oxygenated proton H-21 (δ_H 3.44) to the α proton H-15 (δ_H 4.13), and from the oxygenated proton H-21 (δ_H 3.44) and the methyl doublet H-23 (δ_H 1.05) to the methine proton H-22 (δ_H 2.23), and by long-range gHMBC correlations from H-16 (δ_H 2.79) to carbons C-15 (δ_C 53.3), C-17 (δ_C 139.9), C-18 (δ_C 129.8), and C-21 (δ_C 72.4) and from the methyl doublet H-23 (δ_H 1.05) to carbons C-21 (δ_C 72.4), C-22 (δ_C 43.7), and C-24 (δ_C 174.8). The cyclic 2-amino-4-ureidobutanoic acid (Auba) residue was assigned by interpretation of gCOSY crosspeaks from the α proton H-25 (δ_H 4.38) and H-27 (δ_H 3.71, 3.43) to H-26 (δ_H 2.15, 1.93), along with gHMBC correlations from the α proton H-25 (δ_H 4.38) to a carbon C-28 (δ_C 175.2) and from proton H-27 (δ_H 3.71) to carbons C-25 (δ_C 52.8), C-26 (δ_C 23.9), C-28 (δ_C 175.2), and C-29 (δ_C 153.4). Lastly, the 2-methoxyacetamide moiety was constructed by analysis of gHMBC correlations from the downfield methylene protons [H-2 (δ_H 3.81)] to C-1 (δ_C 169.0) and C-3 (δ_C 59.2).

The sequence of amino acids in actinoramide A was determined by interpretation of key long-range gHMBC correlations. The linkage of Pip to Hle was achieved on the basis of gHMBC correlations from the α-amino proton of Hle [H-4 (δ_H 5.50)] and the α-amino proton of Pip [H-10 (δ_H 4.95)] to the Hle amide carbonyl [C-9 (δ_C 173.0)]. The gHMBC correlation observed from the α-amino proton of Hle [H-4 (δ_H 5.50)] to the acetamide carbon [C-1 (δ_C 169.0)] established the connectivity of those two residues. The gHMBC correlations from both the α-amino proton of Ahmppa [H-15 (δ_H 4.13)] and the α-amino proton of Pip [H-10 (δ_H 4.95)] to the Pip amide carbonyl [C-14 (δ_C 171.0)] revealed Ahmppa to be adjacent to Pip. Lastly, gHMBC correlation of the α-amino proton of Auba [H-25 (δ_H 4.38)] to the Ahmppa amide carbonyl [C-24 (δ_C 174.8)] allowed the connection between Auba and Ahmppa to be established as shown below (Fig. 1).
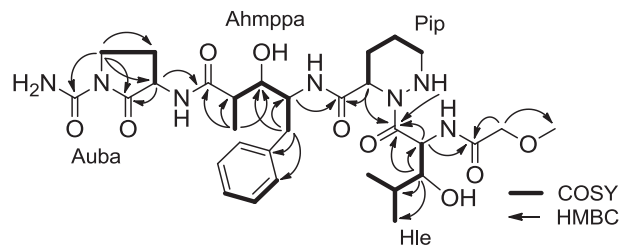

Fig. 1. Structure of actinoramide A (**1**) with COSY (bold lines) and key HMBC (solid arrows) correlations.

Actinoramide B (**2**) was isolated as a yellow oil, which analyzed for the molecular formula $C_{32}H_{48}N_6O_9$ based on HRESIMS data (a pseudomolecular ion peak at m/z 661.3550 [M+H]$^+$). The ^{1}H NMR spectrum of **2** was almost identical to that of **1** except that **2** displayed an additional methyl singlet. The observation of a gHMBC correlation from the methyl singlet to the C-29 carbon indicated that **2** had a methyl group at C-29 instead of an NH$_2$ group. Thus, **2** was composed of a cyclic 4-acetamido-2-aminobutanoic acid (Aaba) unit. Analysis of 2D NMR spectroscopic data allowed the planar structure of **2** to be assigned as shown.

The molecular formula of actinoramide C (**3**) was assigned as $C_{25}H_{40}N_4O_6$ based on a pseudomolecular ion peak at m/z 493.2999 [M+H]$^+$ observed in the HRESIMS data. The ^{1}H NMR spectrum of **3** had similar features to that of **1**, however differences included the absence of the α-amino proton and two methylene protons for Auba and the presence of a methyl triplet. Further interpretation of gCOSY, gHSQC, and gHMBC NMR spectroscopic data indicated that **3** contained a 2-amino-1-phenylpentan-3-ol moiety (App) in place of the Ahmppa adjacent to the Pip in **1**. Interpretation of gCOSY NMR crosspeaks allowed the construction of two spin systems consisting of a five carbon unit (H-23–H-22–H-21–H15–H-16) and a benzene ring [H-18 (H-18′)–H-19 (H-19′)–H-20]. The connection between C-16 and C-17 was established on the basis of gHMBC correlations from the methylene protons H-16 to carbons C-15, C-17, C-18, and C-21. These data permitted the assignment of the App residue.

The absolute configurations of the actinoramides were assigned by a combination of spectral and chemical methods. We proposed that actinoramides A–C possessed the same absolute configurations based on their simultaneous isolation and similar NMR and CD spectroscopic data (Fig. S1, Supplementary data).

The absolute configuration of C-4 in Hle was determined as *R* by acid hydrolysis followed by the application of the advanced Marfey's method. The 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) derivative of Hle was detected by LC–MS analysis at 46.3 min, while the L- and D-FDLA derivatives of Hle were detected by LC–MS analysis at 46.3 and 39.3 min, respectively. Due to the structural similarity of Hle and threonine (Thr), the elution behavior of FDLA derivatives of Hle was expected to be similar to Thr. The L-FDLA derivative of *R*-Thr eluted later than D-FDLA derivative of *R*-Thr.[4] This result allowed the configuration at C-4 of Hle to be assigned as 4*R*. To determine the absolute configuration of C-5 for Hle, the advanced Mosher's method was applied.[5] Treatment of **1** with (*R*)- and (*S*)-MTPA-Cl [α-methoxy-α-(trifluoromethyl) phenylacetyl chloride] yielded the (*S*)- and (*R*)-MTPA ester derivatives, respectively. Calculation of ^{1}H $\Delta\delta_{S-R}$ values of bis-MTPA esters of **1** from proton NMR data established the absolute configuration of C-21 of Ahmppa as *S*. However, determination of the absolute configuration at C-5 of Hle proved to be more difficult. The ^{1}H $\Delta\delta_{S-R}$ values of the bis-MTPA esters (**4a/b**) were inconsistent (Fig. 2a), thus we prepared mono-MTPA esters using (*R*)- and (*S*)-MTPA-Cl (**5a/b** and **6a/b**). Unfortunately, ^{1}H $\Delta\delta_{S-R}$ values for C-5 of Hle in the mono-MTPA esters were also inconsistent (The ^{1}H $\Delta\delta_{S-R}$ values for

4a : R = S-MTPA, 4b : R = R-MTPA

5a : R = S-MTPA, 5b : R = R-MTPA

6a : R = S-MTPA, 6b : R = R-MTPA
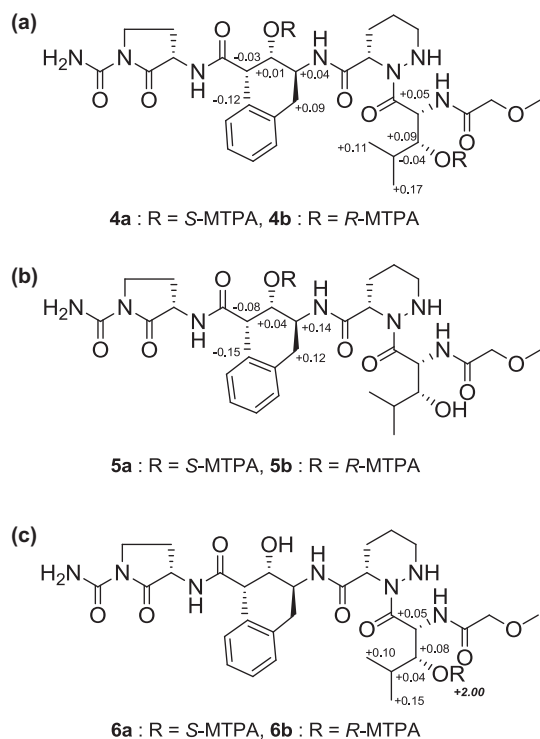
Fig. 2. $\Delta\delta_{S-R}$ of ^1H for S- and R-bis-MTPA esters of actinoramide A (a). $\Delta\delta_{S-R}$ of ^1H for S- and R-mono-MTPA esters of Ahmppa (b), and $\Delta\delta_{S-R}$ of ^1H (plain) and ^{19}F (bold italic) for S- and R-mono-MTPA esters of Hle (c).

C-21 of Ahmppa in the mono-MTPA esters further confirmed the assignment of S revealed by the analysis of the bis-MTPA esters, as shown in Fig. 2b). Finally, calculation of ^{19}F $\Delta\delta_{S-R}$ values for the mono-MTPA esters allowed the absolute configuration at C-5 of Hle to be assigned as S (Fig. 2c).[6] Unfortunately, it became subsequently clear that many configurations have been incorrect when assigned on the basis of ^{19}F NMR data alone.[5,7] Therefore, we prepared the L-FDLA derivative of (2R, 3S)-Hle from lobocyclamide B, a cyanobacterial metabolite isolated from a complex cyanobacterial mat containing *Lyngbya confervoides*.[8a] The retention time of the L-FDLA derivative of (2R, 3S)-Hle of lobocyclamide B was 50.8 min. This result was not consistent for the assigned absolute configuration of C-5 of actinoramide A from the analysis of ^{19}F NMR data.

Next, we prepared L-FDLA derivatives of authentic (2S, 3R)- and (2S, 3S)-Hle from dentigerumycin a metabolite produced by

Pseudonocardia sp.[8b] The retention times of the L-FDLA derivatives of (2S, 3R)- and (2S, 3S)-Hle were 36.8 and 39.3 min, respectively. The elution order of L-FDLA derivatives of four stereoisomers of Hle were consistent with those reported by MacMillan et al., who demonstrated the elution behaviors of 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (L-FDAA) derivatives of Hle.[8a] Furthermore, while Hle from actinoramide A has $2R$ and $3R$ configurations, the D-FDLA derivative of (2S, 3S)- and L-FDLA derivative of (2R, 3R)-Hle, because they are enantiomers, are expected to have identical retention times. The D-FDLA derivative of (2S, 3S) was observed at 46.3 min, which was the elution time of L-FDLA derivative of Hle from actinoramide A. These data suggested that the absolute configurations of Hle from actinoramide would be $4R$, and $5R$, respectively.

The configuration of Pip was determined as S by acid hydrolysis and application of Marfey's method using standards of known configuration. The L-FDLA derivative of Pip in actinoramide A (**1**) was detected at 38.5 min by LC−MS analysis. L-FDLA derivatives of authentic R-Pip and S-Pip were eluted by LC−MS analysis at 35.3 and 38.5 min, respectively.

The absolute configuration of Auba was assigned as S by acid hydrolysis and comparison of the advanced Marfey's products by LC−MS. The L-FDLA derivative of Auba with $m/z=456$ [M+H]$^+$ was detected by LC−MS analysis at 31.5 min, while the D- and L-FDLA derivatives of Auba were detected at 29.0 and 31.5 min, respectively. The structure of the L-FDLA derivative of Auba (**7**) was further characterized by NMR (Scheme 1). Although, there is no standard available for Auba, we identified two amino acids (ureido-alanine, and citrulline) with similar structures to Auba. Ureido-alanine has a side chain that is one carbon shorter than Auba, while citrulline (Cit) has a side chain that is one carbon larger than Auba. It is reasonable to expect the elution behavior of FLDA derivatives of these two amino acids to be similar to those of Auba. L-FDLA derivatives of commercial S-Cit eluted later than that of R-Cit. Furthermore, the amino L-FDLA derivatives of S-ureido-ala-nine and L-FDLA derivatives of S-Cit were detected by LC−MS analysis at 30.5 and 32.7 min, respectively, while the L-FDLA derivative of Auba was detected at 31.5 min. These results allowed the absolute configuration of Auba to be assigned as S.

The relative configurations of the remaining stereocenters in Ahmppa were determined by spectroscopic data analysis. 5-Benzyl-4-hydroxy-3-methylpyrrolidin-2-one (**8**) was isolated from the acid hydrolysis products of actinoramide A after multiple modifications of the hydrolysis conditions (Scheme 2). The relative configuration of **8** was assigned by interpretation of NOESY correlation data. The methyl protons H-3 (δ_H 0.99) showed strong correlations
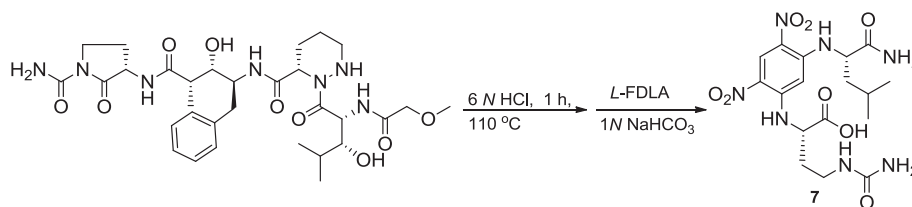
Scheme 1. Reaction sequence for determination of the absolute configuration of 2-amino-4-ureidobutanoic acid.
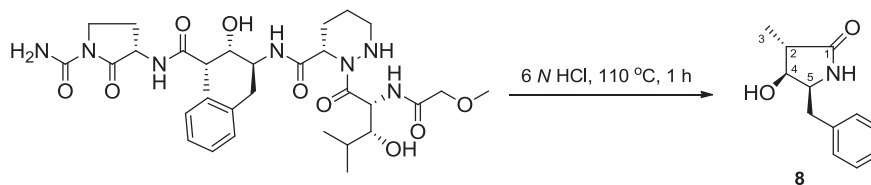
Scheme 2. Reaction for determination of the absolute configuration of 4-amino-3-hydroxy-2-methyl-5-phenylpentanoic acid.

with H-4 (δ_H 3.84), and H-5 (δ_H 3.65) in the NOESY spectrum, which allowed the relative configuration to be designated as (2R*, 4R*, 5R*). On the basis of their relative configurations and the previously described application of advanced Mosher's method, the absolute configuration of Ahmppa in actinoramide A was assigned as 15S, 21S, 22S.

Actinoramide A is unique in its incorporation of the uncommon amino acids 2-amino-4-ureidobutanoic acid (Auba), and 4-amino-3-hydroxy-2-methyl-5-phenylpentanoic acid (Ahmppa), and rare known amino acids, piperazic acid (Pip) and 3β-hydroxyleucine (Hle). The Auba unit was previously reported in the synthetic literature, as a reagent for preparing peptidomimetics[9] and the Ahmppa unit was also reported in the synthesis of bleomycin analogs.[10] This is the first report of Auba and Ahmppa components in a natural product.

The crude culture extract of *Streptomyces* strain CNQ-027 showed NF-κB inhibitory activity, but unfortunately this activity was lost and actinoramides were found not to be active. The actinoramides were evaluated in limited bioassays. They displayed no significant activity against HCT-116 human colon carcinoma, methicillin-resistant *Staphylococcus aureus*, or the breast cancer-related enzyme aromatase.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured using a Rudolph Research Autopol III polarimeter with a 10-cm cell. UV data were recorded using a Varian Cary UV-visible spectrophotometer with a path length of 1 cm. The IR spectrum was recorded on a Perkin–Elmer 1600 FT-IR spectrometer. Proton and 2D NMR spectra were recorded in DMSO-d_6 or methanol-d_4 containing Me$_4$Si as internal standard on Varian Inova spectrometers at 500 or 600 MHz. C-13 NMR spectra were acquired on Varian Inova spectrometers at 75 or 150 MHz. High resolution mass TOF spectra were acquired at The Scripps Research Institute, La Jolla, CA. Low resolution LC–MS data were obtained 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.

3.2. Collection and phylogenetic analysis of strain CNQ-027

The marine *Streptomyces* strain CNQ-027 was isolated from a marine sediment sample collected at a depth of 50 m off the coast of San Diego, CA. NCBI blastn analysis of the partial (1480 base pairs) 16S rDNA gene sequence of CNQ-027 (GenBank accession number EU214912) indicates that this strain shares only 97.6% sequence identity to *Streptomyces marinus*, thus is likely to be a new marine species.

3.3. Cultivation and extraction

Marine *Streptomyces* sp. (strain CNQ-027) was cultured in forty 2.8 L Fernbach flasks each containing 1 L of a seawater-based medium (10 g starch, 4 g yeast extract, 2 g peptone, 1 g CaCO$_3$, 40 mg Fe$_2$(SO$_4$)$_3$·4H$_2$O, 100 mg KBr) and shaken at 230 rpm at 27 °C. After seven days of cultivation, sterilized XAD-16 resin (20 g/L) was added to adsorb the organic substances, and the culture and resin were shaken at 215 rpm for 2 h. The resin was filtered through cheesecloth and washed with deionized water, and eluted with acetone. The acetone was removed under reduced pressure, and the resulting aqueous layer was extracted with ethyl acetate (3×500 mL). The ethyl acetate-soluble fraction was dried in vacuo to yield 4.5 g of crude material from a 40 L culture.

3.4. Isolation of actinoramides A–C (1–3)

The crude extract (4.5 g) from strain CNQ-027 was fractionated by open column chromatography on silica gel (25 g) eluting with a step gradient of dichloromethane and methanol. The dichloromethane/methanol 10:1 fraction contained a mixture of actinoramide peptides, which were purified by reversed-phase HPLC (Phenomenex Ultracarb C30, 5 μm, 100 Å, 250×100 mm, 2.0 mL/min, UV=210 nm), eluting with 35% CH$_3$CN in H$_2$O to afford actinoramide A (**1**, 374.0 mg), B (**2**, 5.0 mg), and C (**3**, 24.0 mg), as pale yellow oils.

3.5. Properties of actinoramides A–C (1–3)

*3.5.1. Actinoramide A (**1**).* [α]$^{21}_D$ −18 (c 0.1, methanol); IR (KBr) ν_{max} 3395, 2941, 1725, 1650, 1540, 1361 cm^{-1}; UV (methanol) λ_{max} (log ϵ) 211 (3.2), 252 (1.4) nm; ^{1}H and ^{13}C NMR data, see Table 1; ESI-TOF m/z 662 [M+H]$^+$; HR ESI-TOF m/z 662.3510 (calcd for C$_{31}$H$_{48}$N$_7$O$_9$, 662.3508).

*3.5.2. Actinoramide B (**2**).* [α]$^{21}_D$ −27 (c 0.1, methanol); IR (KBr) ν_{max} 3394, 2940, 1724, 1651, 1542, 1360 cm^{-1}; UV (methanol) λ_{max} (log ϵ) 211 (3.2), 252 (1.4) nm; ^{1}H and ^{13}C NMR data, see Table 2;

Table 1
NMR Data of **1** (DMSO-d_6)[a]

	No.	δ_C, Mult.[b]	δ_H (J in Hz)	COSY	HMBC
	1	169.0, C			
	2	71.8, CH$_2$	3.81, d (2.0)		1, 3
	3	59.2, CH$_3$	3.37, s		2
Hle	4-NH		7.43, d (8.8)	4	1, 9
	4	50.4, CH	5.50, dd (8.8, 8.8)	4-NH, 5	1, 5, 6, 9
	5	75.7, CH	3.43[c]	4, 5-OH, 6	4, 6, 7, 8, 9
	5-OH		4.79, d (7.1)	5	
	6	29.8, CH	1.64, m	5, 7, 8	4, 5, 7, 8
	7	20.8, CH$_3$	0.87, d (7.1)	6	5, 6, 8
	8	16.1, CH$_3$	0.83, d (7.1)	6	5, 6, 7
	9	173.0, C			
Pip	13-NH		4.50, br s	13	9
	10	51.5, CH	4.95, d (4.9)	11	9, 11, 12, 14
	11	26.5, CH$_2$	2.09, m, 1.63, m	10, 12	10, 12, 13
	12	21.4, CH$_2$	1.34, m	11, 13	10, 11, 13
	13	46.7, CH$_2$	2.79[c]	12	11, 12
	14	171.0, C			
Ahmppa	15-NH		7.59, d (8.0)		
	15	53.3, CH	4.13, dd (8.0, 8.0)	15-NH, 16, 21	14, 16, 17, 21, 22
	16	38.3, CH$_2$	2.79[c]	15	15, 17, 18, 21
	17	139.9, C			
	18, 18′	129.8, CH	7.21, dd (7.4, 1.2)	19	16, 17, 19, 20
	19, 19′	128.8, CH	7.23, dd (7.4, 7.4)	18, 20	17, 18, 20
	20	126.6, CH	7.14, dd (7.4, 1.2)	19	18, 19
	21	72.4, CH	3.44[c]	15, 21-OH, 22	15, 22, 23, 24
	21-OH		5.08, d (7.1)	21	
	22	43.7, CH	2.23, q (6.6)	21, 23	15, 21, 23, 24
	23	15.7, CH$_3$	1.05, d (6.6)	22	21, 22, 24
	24	174.8, C			
Auba	25-NH		8.10, q (10.3)	25	24, 28
	25	52.8, CH	4.38, dd (10.3, 8.1)	25-NH, 26	24, 26, 28
	26	23.9, CH$_2$	2.15, q (10.3), 1.93, q (10.3)	25, 27	25, 27, 28
	27	42.1, CH$_2$	3.71 dd (10.3, 10.3), 3.43[c]	26	25, 26, 28, 29
	28	175.2, C			
	29	153.4, C			
	29-NH$_2$		7.40, br s, 7.74, br s		

[a] 500 MHz for ^{1}H NMR (500 MHz) and 75 MHz for ^{13}C NMR.
[b] Numbers of attached protons were determined by analysis of DEPT spectra.
[c] The coupling constant was not determined because of overlapping signals.

Table 2
NMR Data of **2** and **3** (DMSO-d_6)[a]

	No.	2 δ_C, mult.[b]	δ_H (J in Hz)	3 δ_C, mult.[b]	δ_H (J in Hz)
	1	168.3, C		169.0, C	
	2	71.1, CH$_2$	3.81, d (2.0)	71.1, CH$_2$	3.81, d (2.0)
	3	58.6, CH$_3$	3.37, s	58.5, CH$_3$	3.32, s
Hle	4-NH		7.44, d (8.8)	Hle	7.48, d (8.4)
	4	49.9, CH	5.50, dd (8.8, 8.8)	50.0, CH	5.48, dd (8.4, 8.4)
	5	75.1, CH	3.43[c]	75.0, CH	3.40[c]
	5-OH		4.93, br s		4.84, br s
	6	29.2, CH	1.64, m	29.1, CH	1.64, m
	7	20.0, CH$_3$	0.87, d (7.1)	20.1, CH$_3$	0.88, d (6.8)
	8	15.5, CH$_3$	0.83, d (7.1)	15.6, CH$_3$	0.83, d (6.8)
	9	172.2, C		172.7, C	
Pip	13-NH		4.50, br s	Pip	4.43, br s
	10	51.0, CH	4.95, d (4.9)	50.6, CH	4.95, d (4.9)
	11	25.9, CH$_2$	2.09, m, 1.63, m	26.0, CH$_2$	2.03, m, 1.63, m
	12	20.7, CH$_2$	1.34, m	20.7, CH$_2$	1.34, m, 1.15, m
	13	46.6, CH$_2$	2.79[c]	46.0, CH$_2$	2.79[c]
	14	170.2, C		170.5, C	
Ahmppa	15-NH		7.66, d (8.0)	App	7.69, d (8.0)
	15	52.7, CH	4.13, dd (8.0, 8.0)	53.0, CH	4.03, dd (8.0, 8.0)
	16	37.9, CH$_2$	2.79[c]	36.4, CH$_2$	2.68, dd (14.0, 6.5), 2.85, dd (14.0, 6.5)
	17	139.9, C		139.9, C	
	18, 18′	129.1, CH	7.21, dd (7.4, 1.2)	129.1, CH	7.21, dd (7.4, 1.2)
	19, 19′	128.8, CH	7.23, dd (7.4, 7.4)	128.1, CH	7.23, dd (7.4, 7.4)
	20	126.6, CH	7.14, dd (7.4, 1.2)	126.6, CH	7.14, dd (7.4, 1.2)
	21	71.8, CH	3.44[c]	71.8, CH	3.21[c]
	21-OH		4.82, br s		
	22	43.0, CH	2.23, q (6.6)	26.4, CH$_2$	1.28, m
	23	14.9, CH$_3$	1.05, d (6.6)	11.3, CH$_3$	0.79, t (6.8)
	24	174.2, C			
Aaba	25-NH		8.20, q (10.3)		
	25	52.1, CH	4.38, dd (10.3, 8.1)		
	26	23.2, CH$_2$	2.15, q (10.3), 1.93, q (10.3)		
	27	41.3, CH$_2$	3.71 dd (10.3, 10.3), 3.43[c]		
	28	173.3, C			
	29	170.3, C			
	30	25.5, CH$_3$	2.37, s		

[a] 500 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR.
[b] Numbers of attached protons were determined by analysis of 2D spectroscopic data.
[c] The coupling constant was not determined because of overlapping signals.

ESI-TOF m/z 661 [M+H]$^+$; HR ESI-TOF m/z 661.3550 (calcd for C$_{32}$H$_{49}$N$_6$O$_9$, 661.3555).

3.5.3. Actinoramide C (3). [α]^{21}$_D$ −52 (c 0.2, methanol); IR (KBr) ν_{max} 3393, 2941, 1725, 1653, 1540, 1361 cm^{-1}; UV (methanol) λ_{max} (log ϵ) 211 (3.1), 252 (1.4) nm; ^{1}H and ^{13}C NMR data, see Table 2; ESI-TOF m/z 493 [M+H]$^+$; HR ESI-TOF m/z 493.2999 (calcd for C$_{25}$H$_{41}$N$_4$O$_6$, 493.3020).

3.6. Bis-MTPA esters of actinoramide A

Actinoramide A (**1**, 10.0 mg) was dissolved in freshly distilled dry pyridine (1 mL) with several dry crystals of dimethylaminopyridine. The mixture was stirred for 15 min at room temperature. Treatment with *R*(-)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) at 60 °C yielded the *S*-MTPA ester after 3 days. The reaction was quenched with 1 mL of methanol and extracted with EtOAc. After removal of solvent under vacuum, the residue was purified by reversed-phase HPLC (Phenomenex Luna C18 (2), 5 μm, 250×100 mm, 100 Å, UV=254 nm) using a gradient solvent system (isocratic 70% aqueous CH$_3$CN for 20 min; 70% aqueous CH$_3$CN to 100% aqueous CH$_3$CN over 40 min; 100% CH$_3$CN for 15 min). *S*-MTPA ester was obtained at 42 min. *R*-MTPA ester

was prepared with *S*-MTPA-Cl in the same manner. $\Delta\delta_{S-R}$ values for bis- *S*- and *R*-MTPA esters of actinoramide were recorded in parts per million in methanol-d_4.

3.6.1. Bis-S-MTPA ester (4a). ^{1}H NMR (500 MHz, methanol-d_4): δ 7.59 (d, 2H, J=8.0 Hz), 7.49–7.38 (m, 8H), 7.25 (dd, 2H, J=8.0, 8.0 Hz), 7.18 (m, 3H), 5.90 (d, 1H, J=7.4 Hz), 5.52 (dd, 1H, J=7.4, 3.2 Hz), 5.45 (dd, 1H, J=7.4, 3.2 Hz), 4.78 (dd, 1H, J=11.4, 8.4 Hz), 4.61 (m, 2H), 3.99 (d, 1H, J=4.8 Hz), 3.91 (m, 2H), 3.60 (m, 1H), 3.53 (s, 3H), 3.51 (s, 3H), 3.40 (s, 3H), 2.93 (dd, 1H, J=12.0, 4.3 Hz), 2.82 (m, 2H), 2.72 (d, 1H, J=12.1 Hz), 2.56 (t, 1H, J=12.1 Hz), 2.37 (m, 1H), 2.07 (m, 1H), 1.99 (t, 1H, J=11.0 Hz), 1.88 (d, 1H, J=13.0 Hz), 1.19 (d, 1H, J=13.0 Hz), 1.09 (d, 3H, J=8.0 Hz), 1.05 (d, 3H, J=6.8 Hz), 1.03 (d, 3H, J=6.8 Hz), 0.95 (m, 2H); ESI-MS: m/z 1095 [M+H]$^+$, ESI-MS m/z 1117 [M+Na]$^+$.

3.6.2. Bis-R-MTPA ester (4b). ^{1}H NMR (500 MHz, methanol-d_4): δ 7.63 (m, 2H), 7.55 (m, 2H), 7.46 (m, 3H), 7.42–7.41 (m, 3H), 7.22 (dd, 2H, J=8.0, 8.0 Hz), 7.15 (dd, 1H, J=8.0, 8.0 Hz), 7.09 (d, 2H, J=8.0 Hz), 5.83 (d, 1H, J=7.4 Hz), 5.51 (dd, 1H, J=7.4, 3.2 Hz), 5.36 (dd, 1H, J=7.4, 3.2 Hz), 4.77 (dd, 1H, J=11.4, 8.4 Hz), 4.53 (m, 1H), 3.91 (s, 1H), 3.89 (m, 2H), 3.61 (m, 2H), 3.60 (s, 3H), 3.51 (s, 3H), 3.39 (s, 3H), 2.86 (m, 2H), 2.72 (m, 2H), 2.37 (m, 2H), 2.06–2.01 (m, 3H), 1.55 (m, 1H), 1.24 (d, 1H, J=13.0 Hz), 1.22 (d, 3H, J=7.0 Hz), 1.19 (m, 1H), 0.95 (d, 3H, J=6.8 Hz), 0.86 (d, 3H, J=6.8 Hz); ESI-MS: m/z 1095 [M+H]$^+$, ESI-MS m/z 1117 [M+Na]$^+$.

3.7. Mono-MTPA esters of actinoramide A

Actinoramide A (**1**, 3.0 mg) was dissolved in freshly distilled dry pyridine (1 mL) with several dry crystals of dimethylaminopyridine. The mixture was stirred for 15 min at room temperature. Treatment with *R*(-)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) at room temperature yielded the *S*-MTPA ester after 11 h. The reaction was quenched with 1 mL of methanol and extracted with EtOAc. After removal of solvent under vacuum, the residue was purified by reversed-phase HPLC (Phenomenex Luna C18 (2), 5 μm, 100 Å, 250×100 mm, 2.0 mL/min, UV=254 nm) using an isocratic solvent system with 60% aqueous CH$_3$CN. The *S*-MTPA esters were obtained at 42 and 48 min, respectively. *R*-MTPA esters were prepared with *S*-MTPA-Cl in the same manner. The $\Delta\delta_{S-R}$ values for *S*- and *R*-MTPA esters of actinoramide A were recorded in ppm in methanol-d_4.

3.7.1. Ahmppa-S-MTPA ester of actinoramide A (5a). ^{1}H NMR (600 MHz, methanol-d_4): δ 7.51 (d, 1H, J=8.0 Hz), 7.46 (d, 2H, J=8.0 Hz), 7.38–7.36 (m, 2H), 7.17 (dd, 2H, J=8.0, 8.0 Hz), 7.13 (d, 2H, J=8.0 Hz), 7.05 (dd, 1H, J=8.0, 8.0 Hz), 5.45 (dd, 1H, J=8.0, 4.0 Hz), 5.24 (d, 1H, J=8.0 Hz), 3.83 (m, 1H), 3.79 (m, 1H), 3.51 (dd, 1H, J=8.0, 4.0 Hz), 3.47 (dd, 1H, J=10.0, 4.0 Hz), 3.37 (s, 3H), 3.36 (m, 1H), 3.29 (s, 3H), 3.28 (m, 1H), 3.19 (m, 1H), 2.90 (m, 1H), 2.71–2.69 (m, 2H), 2.55 (m, 1H), 2.47 (m, 1H), 2.25 (m, 1H), 2.06 (d, 1H, J=14.0 Hz), 1.91 (t, 1H, J=14.0 Hz), 1.63 (m, 1H), 1.46–1.41 (m, 2H), 1.18–1.16 (m, 2H), 0.96 (d, 3H, J=8.0 Hz), 0.90 (d, 3H, J=6.8 Hz), 0.84 (d, 3H, J=6.8 Hz); ESI-MS: m/z 878 [M+H]$^+$, ESI-MS m/z 900 [M+Na]$^+$.

3.7.2. Ahmppa-R-MTPA ester of actinoramide A (5b). ^{1}H NMR (600 MHz, methanol-d_4): δ 7.51 (d, 1H, J=8.0 Hz), 7.47 (d, 2H, J=8.0 Hz), 7.38 (m, 1H), 7.17 (dd, 2H, J=8.0, 8.0 Hz), 7.11 (d, 2H, J=8.0 Hz), 7.05 (dd, 1H, J=8.0, 8.0 Hz), 7.05 (d, 1H, J=8.0), 5.42 (dd, 1H, J=8.0, 8.0 Hz), 5.35 (d, 1H, J=8.0 Hz), 4.55 (dd, 1H, J=8.0, 8.0 Hz), 4.49 (m, 1H), 3.76 (m, 1H), 3.46 (m, 3H), 3.44 (s, 3H), 3.31 (m, 1H), 3.30 (s, 3H), 2.76 (dd, 2H, J=14.0, 2.0 Hz), 2.75 (dd, 1H, J=8.0, 8.0 Hz), 2.58 (m, 2H), 2.48 (d, 1H, J=8.0 Hz), 2.23 (m, 1H), 1.96 (d, 1H, J=14.0 Hz), 1.90 (t, 1H, J=14.0 Hz), 1.59 (m, 1H), 1.39 (m, 1H), 1.13 (m,

2H), 1.10 (d, 3H, J=8.0 Hz), 0.85 (d, 3H, J=6.8 Hz), 0.80 (d, 3H, J=6.8 Hz); ESI-MS: m/z 878 [M+H]$^+$, ESI-MS m/z 900 [M+Na]$^+$.

3.7.3. Hle-S-MTPA ester of actinoramide A (6a). ^{1}H NMR (600 MHz, methanol-d_4): δ 7.34 (d, 2H, J=8.0 Hz), 7.31–7.28 (m, 3H), 7.15 (d, 2H, J=8.0 Hz), 7.12 (d, 1H, J=8.0 Hz), 7.10 (d, 1H, J=8.0 Hz), 7.03 (dd, 1H, J=8.0, 8.0 Hz), 5.41 (dd, 1H, J=12.0, 2.0 Hz), 4.54 (dd, 1H, J=11.4, 10.4 Hz), 4.49 (m, 1H), 4.19 (m, 1H), 3.79 (s, 3H), 3.77 (m, 2H), 3.48 (d, 1H, J=10 Hz), 3.45 (d, 1H, J=12 Hz), 3.41 (m, 2H), 3.40 (s, 3H), 3.17 (m, 1H), 2.84 (t, 1H, J=14.0 Hz), 2.77 (dd, 1H, J=14.0, 2.0 Hz), 2.44 (t, 1H, J=14.0 Hz), 2.27 (m, 2H), 2.18 (m, 1H), 1.96 (m, 1H), 1.91 (m, 2H), 1.77 (d, 1H, J=14.0 Hz), 1.00 (d, 3H, J=8.0 Hz), 0.93 (d, 3H, J=6.8 Hz), 0.89 (d, 3H, J=6.8 Hz), 0.88 (m, 1H); ESI-MS m/z 878 [M+H]$^+$, ESI-MS m/z 900 [M+Na]$^+$.

3.7.4. Hle-R-MTPA ester of actinoramide A (6b). ^{1}H NMR (600 MHz, methanol-d_4): δ 7.46 (d, 1H, J=8.0 Hz), 7.33 (d, 2H, J=8.0 Hz), 7.24 (d, 2H, J=8.0 Hz), 7.11 (d, 2H, J=8.0 Hz), 7.05 (dd, 2H, J=8.0, 8.0 Hz), 6.97 (dd, 1H, J=8.0, 8.0 Hz), 5.63 (d, 1H, J=8.0 Hz), 4.97 (dd, 1H, J=8.0, 2.0 Hz), 4.50 (dd, 1H, J=14.0, 8.0 Hz), 4.16 (m, 1H), 3.77 (m, 1H), 3.74 (s, 3H), 3.44 (d, 1H, J=2.0 Hz), 3.42 (d, 1H, J=2.0 Hz), 3.40 (dd, 1H, J=8.0, 2.0 Hz), 3.33 (m, 1H), 3.29 (s, 3H), 3.10 (m, 1H), 2.79 (m, 1H), 2.73 (dd, 1H, J=14.0, 2.0 Hz), 2.51 (d, 1H, J=14.0 Hz), 2.26 (t, 1H, J=14.0 Hz), 2.20 (m, 1H), 2.15 (m, 1H), 1.96 (m, 1H), 1.87 (m, 2H), 1.37 (m, 1H), 1.21 (d, 1H, J=14.0 Hz), 1.01 (d, 3H, J=6.9 Hz), 0.94 (d, 1H, J=14.0 Hz), 0.76 (d, 3H, J=6.8 Hz), 0.68 (d, 3H, J=6.8 Hz); ESI-MS: m/z 878 [M+H]$^+$, m/z 900 [M+Na]$^+$.

3.8. Analytical-scale advanced Marfey's reaction

Actinoramide A (**1**, 0.2 mg) was hydrolyzed in 0.4 mL of 6 N HCl at 110 °C for 1 h; the HCl was removed under vacuum; and the dry material was resuspended in 0.4 mL of water and dried three times to remove residual acid. The hydrolyzate was divided into two portions and dissolved in 1 N NaHCO$_3$ (100 μL). To the first portion was added 50 μL of 1% L-FDLA (1-fluoro-2,4-dinitrophenyl-5-L-leucinamide) in acetone; to the second portion was added 25 μl of 1% D- and L-FDLA mixtures in acetone. The reaction mixtures were incubated at 60 °C for 30 min, and quenched with 100 μL of 2 N HCl. A small portion (100 μL) of CH$_3$CN was added to the solution to dissolve the reaction mixture. The resulting products were analyzed by reversed-phase LC–MS with a gradient solvent system (95% aqueous CH$_3$CN to 50% aqueous CH$_3$CN (0.1% TFA) over 50 min; 50% aqueous CH$_3$CN to 100% aqueous CH$_3$CN (0.1% TFA) over 20 min; 100% CH$_3$CN (0.1% TFA) for 15 min).

3.9. Preparatory-scale advanced Marfey's reaction and isolation of 7 and 8

Actinoramide A (**1**, 20.0 mg) was hydrolyzed in 10 mL of 6 N HCl at 110 °C for 1 h, and the HCl was removed under vacuum. The dry material was purified by reversed-phase HPLC (Phenomenex Ultracarb C30, 5 μm, 100 Å, 250×100 mm, 2.0 mL/min, detection at 254 nm) using a gradient solvent system from 5% to 40% CH$_3$CN (0.05% TFA) over 50 min. Compound **8** (5-benzyl-4-hydroxy-3-methylpyrrolidin-2-one) was obtained at 25.1 min. The residues from HPLC were dried under vacuum, dissolved in 1 N NaHCO$_3$ (2 mL), and 2 mL of 1% L-FDLA solution was added. The reaction mixture was incubated at 60 °C for 30 min and quenched by neutralization with 2 mL of 2 N HCl. The products were purified by reversed-phase HPLC (Phenomenex Ultracarb C30, 5 μm, 100 Å, 250×100 mm, 2.0 mL/min, detection at 338 nm) using a gradient solvent system from 5% to 40% CH$_3$CN (0.05% TFA) over 60 min. Auba-L-FDLA (**7**) was obtained at 43.0 min.

3.9.1. Auba-L-FDLA (7). [α]$^{25}_D$ +31 (c 0.05, methanol); ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.96 (s, 1H), 8.75 (d, NH, J=6.8 Hz), 8.51 (d, NH, J=6.8 Hz), 7.86 (s, NH), 7.40 (s, NH), 6.09 (s, NH), 5.76 (s, 1H), 5.53 (s, NH$_2$), 4.48 (m, 1H), 4.27 (m, 1H), 3.05 (m, 2H), 1.97 (m, 2H), 1.71 (m, 3H), 0.98 (d, 3H, J=6.9 Hz), 0.88 (d, 3H, J=6.9 Hz); ESI-TOF-MS: m/z 456 [M+H]$^+$; HR ESI-TOF-MS m/z 456.1832 (calcd for C$_{17}$H$_{25}$N$_7$O$_8$, 456.1837).

3.9.2. 5-Benzyl-4-hydroxy-3-methylpyrrolidin-2-one (8). [α]$^{25}_D$ −61 (c 0.1, methanol); ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.56 (s, 2−NH), 7.27 (dd, H-9 and H-9′, J=8.0, 8.0 Hz), 7.27 (d, H-8 and H-8′, J=8.0 Hz), 7.19 (d, H-10, J=8.0, 8.0 Hz), 5.39 (d, 4-OH, J=5.1 Hz), 3.84 (dd, H-4, J=11.7, 5.1 Hz), 3.65 (dd, H-5, J=14.0, 6.3 Hz), 2.95 (dd, H-6a, J=14.0, 6.3 Hz), 2.57 (dd, H-6b, J=14.0, 6.3 Hz), 2.09 (qt, H-2, J=6.9 Hz), 0.99 (d, H-3, J=6.9 Hz); ^{13}C NMR (150 MHz, DMSO-d_6) δ 176.0 (C-1), 138.7 (C-7), 129.4 (C-9 and C-9′), 128.3 (C-8 and C-8′), 125.5 (C-10), 74.8 (C-4), 57.6 (C-5), 40.6 (C-2), 35.2 (C-6), 12.9 (C-3); ESI-TOF-MS: m/z 206 [M+H]$^+$; HR ESI-TOF-MS m/z 206.1177 (calcd for C$_{12}$H$_{15}$NO$_2$, 206.1175).

Supplementary data

The ^{1}H, ^{13}C, and 2D NMR spectra of actinoramide A, and ^{1}H, and ^{13}C for actinoramides B and C, and ^{1}H NMR spectrum of bis-MTPA esters (**4a**,**b**), and mono-MTPA esters (**5a**,**b**, **6a**,**b**), and **7**, and ^{1}H, ^{13}C, and 2D NMR spectra of **8** are available. Supplementary data associated with this article can be found in online version at doi:10.1016/j.tet.2011.04.051.

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