Organic LETTERS

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Juanlimycins A and B, Ansamycin Macrodilactams from *Streptomyces* sp.

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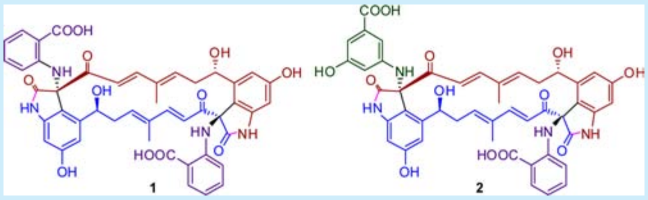
Supporting Information



ABSTRACT: Ansamycins are a family of macrolactams characterized by an aromatic chromophore with an aliphatic chain (*ansa* chain) connected back to a nonadjacent position through an amide bond. This family has shown a high degree of druggability exemplified by rifamycins, maytansinoids, and geldanamycins. In this study, the isolation of two novel ansamycin macrodilactams with unprecedented features, juanlimycins A (**1**) and B (**2**), from *Streptomyces* sp. LC6 were reported. The structures of **1** and **2** were assigned on the basis of analysis of NMR spectroscopic data and X-ray single crystal diffraction.

Ansamycins are a family of macrolactam antibiotics with remarkable potential of druggability as exemplified by the antituberculosis rifamycins,[1] antitumor agents maytansinoids[2] and geldanamycins.[3] By analogy to many macrolactones, ansamycins are synthesized by type I polyketide synthase (PKS). The striking differences are that the biosynthesis of ansamycins is started by loading 3-amino-5-hydroxybenzoic acid (AHBA) to the chain-initiation module consisting of an acyl carrier protein (ACP) and an adenyltransferase, and it is terminated by releasing polyketide chains via intramolecular amidation.[4] The last step of the AHBA biosynthetic pathway is carried out by AHBA synthase, which is conserved in various ansamycin pathways.[5,6] We have approached novel ansamycins through PCR screening AHBA synthase genes of plant-associated and marine-derived actinomycetes,[7] which was followed by characterizing the biosynthesis of hygrocins,[8,9] divergolides[10] and cuevaene A.[11] Moreover, among these AHBA synthase gene-positive strains, *Streptomyces* sp. LC6 showed the potential of producing pentaketide-type ansamycins on the basis of bioinformatic analysis of the AHBA synthase gene cluster.[7] Pentaketide-type ansamycins belong to a small group of the ansamycin family. Before this study, only three types including nine members were reported, i.e., Q-1047-A, Q-1047-B, Q-1047R-A and Q-1047R-B,[12] ansaetherone[13] and tetrapetalones A–D.[14,15] Herein, the isolation and structure elucidation of two novel macrodilactams of pentaketide-type ansamycins, namely, juanlimycins A and B, from *Streptomyces* sp. LC6 were reported (Figure 1), which were determined by analysis of the high-resolution ESIMS, 1D and 2D NMR spectroscopic data and X-ray single crystal diffraction study.

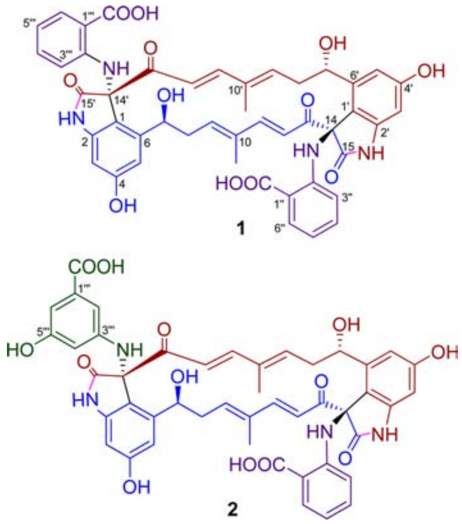


Figure 1. Structures of juanlimycins A (**1**) and B (**2**).

The strain *Streptomyces* sp. LC6, isolated from the leaves of *Kandelia candel* (Linn.) Druce collected from Longhai, Zhangzhou, Fujian Province, China, was cultured for 7 d on soybean powder medium (soybean powder 20 g, mannitol 20 g, agar 15 g, pH 7.2) at 28 °C. The fermentated agar cakes were diced and extracted three times overnight with EtOAc–

Received: April 13, 2014
Published: May 5, 2014

MeOH–AcOH (80:15:5, v/v/v) at room temperature. After the removal of solvents under a vacuum, the crude extract was partitioned between H_2O and EtOAc. EtOAc-soluble partition was dissolved in 70 mL of 95% methanol and extracted five times with an equal volume of petroleum ether to afford the defatted MeOH extract (4.2 g). The MeOH extract was fractionated by column chromatography over silica gel (200–300 mesh) eluted with gradient CH_2Cl_2–MeOH mixtures (200:1 to 0:100, v/v) to give fractions 1–6. Fraction 6 (3.3 g) was purified by column chromatography over Sephadex LH-20 eluted with MeOH to give six subfractions (Fr. A–F). In accordance with the TLC results, Fr. D (1.0 g) was subjected to medium pressure liquid chromatography (MPLC) over RP18 silica gel (30 g) eluted with gradient aqueous methanol (30, 40, 50, and 100% MeOH, 600 mL each) to produce Fr. D1–D6. Fr. D2 (107 mg) and Fr. D3 (74 mg) were purified by semipreparative HPLC (ZORBAX Eclipse XDB-C_{18}, 9.4 × 250 mm, 4 mL/min, UV 274 nm) eluted with 35% methanol and 29% acetonitrile in 0.05% formic acid to afford **1** (7 mg) and **2** (10 mg) (Figure 1), respectively.

Compound **1** was obtained as a pale-yellow crystal (from aqueous methanol), decomposed under 146 °C with $[\alpha]_D^{25}$ −145.9 (*c* 0.22, MeOH) and UV λ_{max} 220, 255, 290, and 335 nm. Interpretation of the ^1H, ^{13}C NMR and HSQC spectroscopic data (Table 1) showed the presence of a ketone (188.8 ppm) and one acyl (177.1 ppm) carbon, four olefinic carbons, one oxymethine, one sp^3 quaternary (76.2 ppm), a methyl and a methylene carbons, and a tetrasubstituted and an *ortho*-disubstituted benzene rings. The presence of an *ortho*-disubstituted benzene ring was further confirmed by the ^1H–^1H COSY correlations of H-3″-6″, and the HMBC correlations from H-6″ (δ_H 7.93) to C-2″ (δ_C 148.6), C-4″ (δ_C 134.7), HOOC-1″ (δ_C 173.0, estimated), as well as that from H-4″ (δ_H 7.06) to C-2″ (δ_C 148.6), and H-3″ (δ_H 5.91) and H-4″ (δ_H 7.06) to C-1″ (δ_C 115.5, estimated) (Figure 2).

Table 1. ^1H and ^{13}C NMR Data for Juanlimycin A (1) Recorded in CD_3OD at 600 and 150 MHz, Respectively (δ in ppm, *J* in Hz)

position	δ_H, mult., (*J* in Hz)	δ_C, type
1/1′		114.5, C
2/2′		144.4, C
3/3′	6.51, s	99.8, CH
4/4′		161.5, C
5/5′	6.73, s	109.1, CH
6/6′		145.5, C
7/7′	5.03, d, (10.8)	66.6, CH
8α/8′α	2.43, d, (11.9)	41.0, CH_2
8β/8′β	2.90, q, (11.8)	
9/9′	5.74, dd, (10.7, 4.1)	140.3, CH
10/10′		135.2, C
11/11′	7.15, d, (15.2)	152.2, CH
12/12′	5.66, d, (15.2)	116.9, CH
13/13′		188.8, C
14/14′		76.2, C
15/15′		177.1, C
1″/1‴		115.5[a], C
2″/2‴		148.6, C
3″/3‴	5.91, d, (8.4)	114.4, CH
4″/4‴	7.06, t, (7.7)	134.7, CH
5″/5‴	6.57, t, (7.4)	117.1, CH
6″/6‴	7.93, d, (7.6)	133.4, CH
Me-10/Me-10′	1.18, s	11.6, CH_3
COOH-1″/-1‴		173.0[a], C

[a] Estimated from the corresponding HMBC correlations.

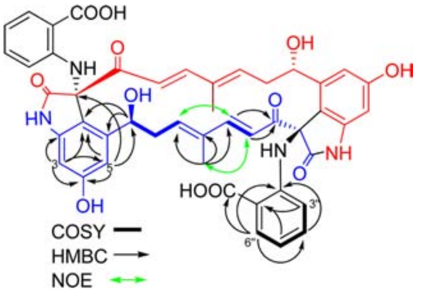


Figure 2. Selected ^1H–^1H COSY, HMBC, and NOE correlations for **1**.

The presence of a tetrasubstituted benzene ring was further confirmed the HMBC correlations of the two aromatic protons at δ_H 6.51 and 6.73 to the carbons at δ_C 114.5 (C-1), 144.4 (C-2), 161.5 (C-4) and 109.1 (C-5), and the carbons at δ_C 114.5 (C-1), 99.8 (C-3), 161.5 (C-4) and 66.6 (C-7), respectively, indicating the *meta*-positions of these two protons and the connection between C-6 and C-7 (Figure 2 and Table S1). The eight-carbon fragment from C-7 to C-13 was established on the basis of ^1H–^1H COSY correlations between H-7 and H-8, H-8 and H-9, and between H-10 and H-11, along with the HMBC correlations from the protons of Me-10 to C-9, C-10 and C-11, and that from H-11 and H-12 to C-13 (Figure 2 and Table S1). It is noteworthy that the four-bond HMBC correlations from H-3 and H-5 to the carbon at δ_C 7.62 (C-14/C-14′) were observed, respectively, indicating that C-14/C-14′ was connected to C-1/C-1′. Additionally, the assignments of chemical shifts δ_C 144.4 for C-2 and δ_C 161.5 for C-4 suggested that C-2 and C-4 was substituted by an amino and an oxy group, respectively. Hence, a structure of pentaketide-type ansamycin could be suggested though no HMBC correlations were observed for the acyl carbon at δ_C 177.1.

However, the molecular formula of **1** was determined to be $C_{46}H_{40}N_4O_{12}$ on the basis of analysis of the high-resolution ESIMS *m/z* 841.2682 $[M + H]^+$ and 863.2491 $[M + Na]^+$ (calcd for $C_{46}H_{41}N_4O_{12}$, 841.2715; $C_{46}H_{40}N_4O_{12}Na$, 863.2535). Moreover, the double bonds C-9/C-10 and C-11/C-12 were assigned to be *E*-configuration by the NOE correlations between H-9 and H-11, and Me-10 and H-12, which was consistent with the large coupling constant (*J* = 15.2 Hz) between H-11 and H-12. Both the high-resolution ESIMS and NOE data, along with the NMR assignments suggested that **1** must be a C2-symmetric dimer. Finally, the structure of **1** with relative configuration was fully determined by X-ray diffraction analysis of a single crystal obtained from aqueous methanol (Figure 3 and Supporting Information).

Compound **2** was obtained as a pale yellow amorphous powder with $[\alpha]_D^{25}$ −162.0 (*c* 0.10, MeOH) and UV λ_{max} 220, 255, 290, and 335 nm. The ^{13}C NMR spectrum of **2** is similar to that of **1**, but 13 resonance signals showed in pairs. Interpretation of the ^1H, ^{13}C NMR, HSQC and HMBC spectroscopic data (Table S2) revealed the presence of two *ansa* chains, an *ortho*-disubstituted and two tetrasubstituted benzene rings, the same as **1**, but additional *meta*-trisubstituted benzene ring, suggesting that **2** was an asymmetric dimer with

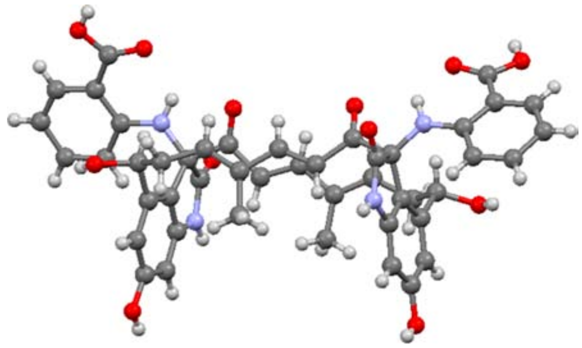


Figure 3. Single-crystal X-ray structure for juanlimycin A (**1**).

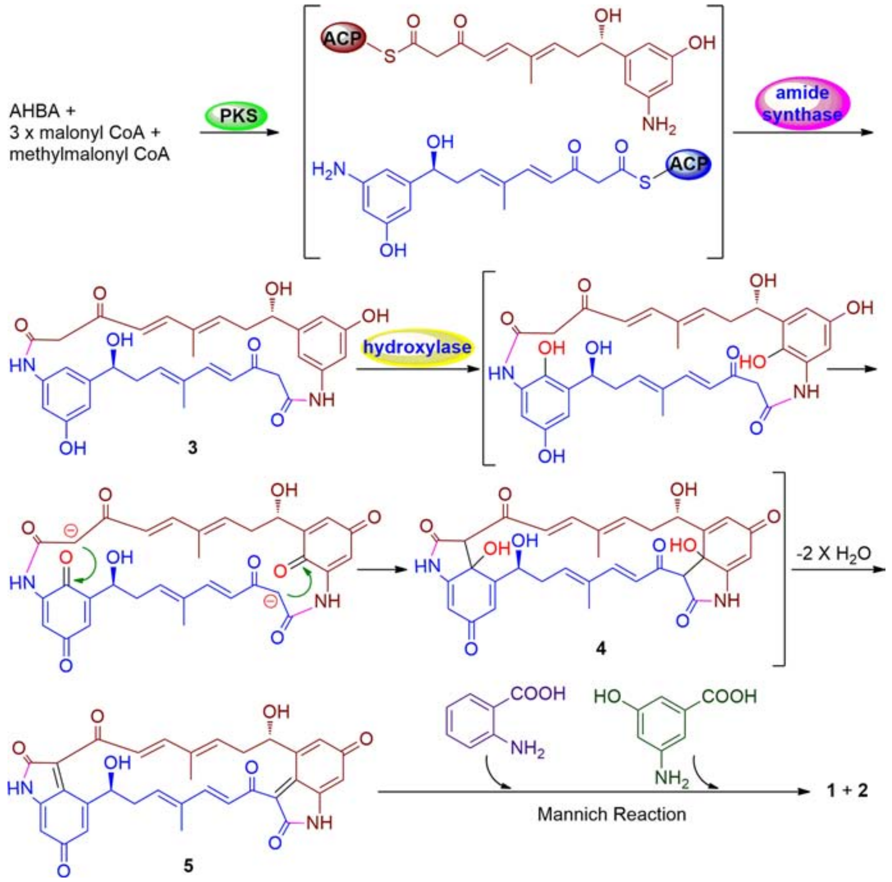
different substitutions at C-14 and C-14′. Interestingly, the *meta*-trisubstituted benzene ring turned out to be an AHBA residue on the basis of NMR assignment and HR-ESIMS data (m/z 857.2636 $[M + H]^+$ and 879.2445 $[M + Na]^+$, calcd for $C_{46}H_{41}N_4O_{13}$, 857.2665; $C_{46}H_{40}N_4O_{13}Na$, 879.2484). Accordingly, the anthranilate and AHBA residues were assumed to link with C-14 and C-14′ via amino group, respectively.

Compounds **1** and **2** were tested for the antimicrobial activities against *Staphylococcus aureus* ATCC 25923, *Mycobacterium smegmatis* mc^2 155 and *Candida albicans* 5314. Both **1** and **2** showed no evident activities on these strains. In *Salmonella enterica* serovar Typhimurium UK-1χ8956 (ΔP rpoS183::TT araC PBAD rpoS) assay, **1** showed moderate inhibition on the secretion of SPI-1 (Salmonella Pathogenicity Island-1) effectors, SipA/B/C/D, compared to the vehicle control DMSO and the positive control cytosporone B (Figure S1). Using SRB assay, **1** and **2** showed no obvious cytotoxicities against the proliferations of HepG2, HL-7702, HeLa, SW480 and HL7702 cell lines (Supporting Information).

The biosynthesis of **1** and **2** was proposed to be assembled by type I PKS using 3-amino-5-hydroxybenzoic acid (AHBA) as the starter unit, three malonyl-CoAs and one methylmalonyl-CoAs as the extender units. The putative amide synthase probably catalyzes the intermolecular amidation of two polyketide intermediates to form the C2-symmetric macrodilactam (**3**) (Scheme 1). The intermediate **3** may be subjected to oxidation by a 3-(3-hydroxyphenyl)propionate hydroxylase similar to that in the biosynthesis of many other ansamycins, e.g., rifamycins,[16] geldanamycins,[17] hygrocins,[18] divergolides,[19] ansalactam A,[20] rubradirin,[21] and naphthomycins.[22] The formation of the γ-lactam rings in **4** may proceed through aldol condensation as that in divergolide D[19] and hygrocins,[9,23,24] which underwent dehydration to afford **5**. With availability of the rare amino acids anthranilate and AHBA, the Mannich reaction could transform **5** into **1** and **2**, respectively (Scheme 1).

In most instances, the type I polyketide chain extension was terminated by a thioesterase, which releases the polyketide chain from the ACP domain of PKS and allows cyclization.[25] For ansamycins, the enzyme homologous to *N*-acyltransferase, namely, amide synthase, releases the nascent polyketide chain from ACP through intramolecular amidation.[16,18−20,26,27] Juanlimycins A (**1**) and B (**2**) are the first macrodilactams among the reported ansamycins. Indeed, macrodilactones are

Scheme 1. Proposed Biosynthetic Pathway and Post-PKS Modifications of 1 and 2



not rare and were suggested to be produced by intermolecular esterizations likely carried out with thioesterases.[28,29] However, the mechanism of intermolecular macrocyclization remains elusive.[28] Therefore, it is of great interest to investigate the biosynthetic mechanism of **1** and **2**, particularly, the mechanism of intermolecular amidation. Having verified this amide synthase, its unique function would be a useful tool to make macrodilactams from many other ansamycins, which shall increase the structure diversity of ansamycins and accordingly the bioactivity diversity.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H, ^{13}C NMR, HSQC, HMBC, and HRESIMS spectra of **1** and **2**, and X-ray crystallographic data of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was supported in part by the National Natural Science Foundation of China (81373304), 973 Programs (2010CB833802, 2012CB721005), the Fundamental Research Funds of Shandong University (2014JC027), and Program for Changjiang Scholars and Innovative Research Team in University (IRT13028).

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■ NOTE ADDED AFTER ASAP PUBLICATION

Scheme 1 contained errors in the version published ASAP May 5, 2014; the correct version reposted May 7, 2014.