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NOTE

Prajinamide, a new modified peptide from a soil-derived *Streptomyces*

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Actinomycetes are well recognized as the richest source of bioactive compounds, including clinically important antibiotics, antitumor agents and cell function modulators, and hence of high pharmacological and commercial interest.[1] Amongst this group, members of the genus *Streptomyces* are the most prolific producers of secondary metabolites, accounting for up to 80% of the bioactive small molecules discovered from actinomycetes.[2] Meanwhile, it is quite notable that further discovery of unknown metabolites from *Streptomyces* is predicted by the genome analysis: the number of metabolites actually isolated is far more below the number of secondary metabolite biosynthetic gene clusters identified in the whole genomes of *S. avermitilis* and *S. coelicolor*.[3,4] As a part of our chemical investigation on microbial secondary metabolites, we reported plant-growth promoting spiroacetals of polyketide origin,[5] a linear polyketide with a δ-lactone terminus with cytotoxic activity,[6] a polycyclic tetronate with antiinvasive activity[7] and a biosynthetically unprecedented heterocyclic polyketide with antibacterial and antiinvasive activities[8] from *Streptomyces*. During the course of our continuing effort to discover structurally unique secondary metabolites from these organisms, a new modified peptide was isolated from the culture broth of a soil-derived actinomycete strain *Streptomyces* sp. SPMA113 collected in Thailand. The strain was cultured in A-11M liquid medium, and the whole culture broth was extracted with 1-butanol. The HPLC/UV analysis of the extract using our in-house metabolite database indicated the presence of an unknown compound showing a UV absorption maximum at 260 nm, along with geldanamycins[9] and elaiophylins.[10] Guided by HPLC/UV, several steps of chromatographic purification resulted in the isolation of a new compound, prajinamide (**1**, Figure 1).

Compound **1** was obtained as a pale yellow oil that analyzed for a molecular formula of $C_{16}H_{25}N_3O_3$ (6 degrees of unsaturation) by interpretation of HR–ESI–TOF–MS (observed $[M+Na]^+$ at *m/z* 330.1788, calculated $[M+Na]^+$ 330.1788). This molecular formula was corroborated by ^1H and ^{13}C NMR spectral data (Table 1). Analysis of the combined 1D and 2D NMR data established that **1** possessed three carbonyl, four olefinic methine, two sp^3 methine, five sp^3 methylene and two methyl carbons, in addition to three exchangeable protons. The IR absorptions at 1647, 1599 and 1538 cm^{-1} indicated the presence of amide functionalities, which was supported by the resonances of carbonyl carbons at δ 165.6, 169.9 and 170.1 observed in the ^{13}C NMR spectrum. As three carbonyls and two double bonds accounted for five of six double-bond equivalents, **1** must be a monocyclic compound.

Further analysis of ^1H-^1H COSY and HMBC spectra provided three substructures (Figure 2). The first was an ornithine lactam that was established by the sequential COSY correlations from an NH proton at δ 7.61 (2-NH) to another NH proton at δ 8.07 (5-NH) through a methine proton (H-2) and three methylene protons (H-3, H-4 and H-5) and HMBC correlations from H-2, H-3, H-5 and 2-NH to a carbonyl carbon C-1 (δ 169.9). The second substructure, a β-alanine, was assigned on the basis of COSY correlations between an NH proton at δ 7.98 (8-NH) and H-8 and between H-8 and H-7 and HMBC correlations from H-7 and H-8 to C-6 (δ 170.1). Two and three-bond C–H correlations from 2-NH and H-2 to C-6 allowed the β-alanine residue being connected to the ornithine lactam through an amide linkage. COSY correlations between four olefinic protons H-10, H-11, H-12 and H-13 provided a conjugated diene, which was then extended to include a carbonyl carbon C-9 (δ 165.6) at C-10 on the basis of HMBC correlations from H-10 and H-11 to C-9. A vinyl methine H-13 showed a COSY correlation to H-14, which showed in turn correlations to two equivalent methyl protons H-15 and H-16, thereby establishing an isopropyl terminus attached to the diene fragment. The geometries of C-10–C-11 and C-12–C-13 double bonds were assigned as *Z* and *E*, respectively, on the basis of the vicinal coupling constants ($J_{H10,H11}$=11.3 Hz, $J_{H12,H13}$=15.5 Hz). The third substructure was thus

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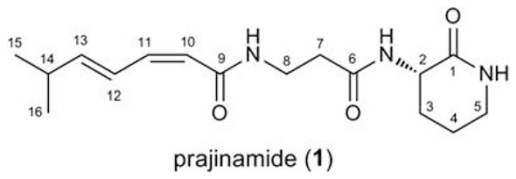

Figure 1 Structure of prajinamide (**1**).

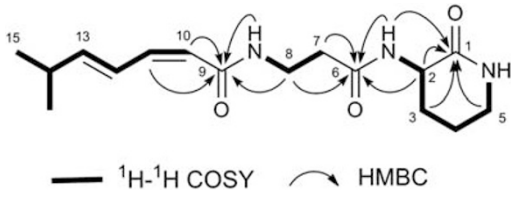

Figure 2 ^{1}H-^{1}H COSY and key HMBC correlations for **1**.

Table 1 ^{1}H and ^{13}C NMR data for prajinamide (1) in DMSO-d_6

Position	δ_C[a]	δ_H *mult (J in Hz)*[b]	*HMBC*[b,c]
1	169.9, qC		
2	48.8, CH	4.14, ddd (10.7, 8.3, 6.0)	1, 6
3	27.6, CH_2	1.58, dddd (12.5, 11.0, 10.7, 4.0)	1, 2, 5
		1.92, m	1, 2, 5
4	21.0, CH_2	1.71, m	
		1.75, m	2, 5
5	40.9, CH_2	3.11, m	1
6	170.1, qC		
7	35.3, CH_2	2.25, ddd (17.0, 6.8, 6.8)	6, 8
		2.27, ddd (17.0, 6.8, 6.8)	6, 8
8	35.1, CH_2	3.27, dddd (19.3, 6.8, 6.8, 6.3)	6, 7, 9
		3.31, dddd (19.3, 6.8, 6.8, 6.3)	6, 7, 9
9	165.6, qC		
10	119.7, CH	5.59, d (11.3)	9, 12
11	140.1, CH	6.32, dd (11.3, 11.0)	9, 13
12	124.3, CH	7.45, dd (15.5, 11.0)	11, 14
13	148.5, CH	5.91, dd (15.5, 6.7)	11, 14, 15, 16
14	30.7, CH	2.37, dqq (6.7, 6.3, 6.3)	12, 13, 15, 16
15	21.8, CH_3	0.98, d (6.3)	13, 14, 16
16	21.8, CH_3	0.98, d (6.3)	13, 14, 15
2-NH		8.07, d (8.3)	1, 2, 6
5-NH		7.61, s	
8-NH		7.98, t (6.3)	7, 8, 9

[a]Recorded at 100 MHz.
[b]Recorded at 500 MHz.
[c]HMBC correlations are from proton(s) stated to the indicated carbon.

established as 6-methyl-(2*Z*, 4*E*)-hepta-2,4-dienoate. This unit was connected to the β-alanine residue via an amide bond on the basis of HMBC correlations from 8-NH and H-8 to C-9, providing the planar structure of **1** as depicted in Figure 2. The absolute configuration of the ornithine lactam residue in **1** was determined to have the L configuration by Marfey's analysis.[11] The acid hydrolysate of **1** was derivatized with L-FDLA (1-fluoro-2,4-dinitrophenyl-5-L-leucinamide), and the HPLC retention time was compared with D- and L-ornithine standards that were similarly derivatized with L-FDLA. The derivatized D- and L-ornithine standards eluted at 9.8 and 13.4 min, respectively, while the L-FDLA derivative of the acid hydrolysate of **1** eluted at 13.5 min.

The biological activity of prajinamide (**1**) is still being examined in diverse bioassays. To date, **1** was found to induce differentiation of preadipocytes into matured adipocytes. Adipocytes have ability to secrete adiponectin, a peptidic hormone that is beneficial to improve insulin-sensitivity in insulin-resistant type 2 diabetes patients.[12] Small molecules that induce adipocyte differentiation are thus expected as leads for antidiabetic agents.[13] By the treatment with 50 μM prajinamide, 60% of murine ST-13 preadipocyte cells were differentiated into matured adipocytes with accumulation of cytosolic lipid droplets. Further detailed analysis of its inducing activity as well as the induction of adiponectin secretion is under investigation. Compound **1** was inactive in a cancer cell cytotoxicity assay ($IC_{50} > 100$ μM against MCF7 human breast cancer cells) and an antimicrobial assay against *Escherichia coli*, *Micrococcus luteus* and *Candida albicans* (MIC > 50 μg ml^{-1}).

Prajimamide (**1**) is a relatively simple compound consisting of three small building blocks, but no similar metabolites are known with respect to its overall structure. Specifically, the unsaturated fatty acid unit, 6-methyl-(2*Z*, 4*E*)-hepta-2,4-dienoate, is unprecedented in natural products, whereas its 2*E* isomer has been found only in daryamide C from *Streptomyces*.[14] The discovery of **1** is the result of our comprehensive chemical screening from *Streptomyces*, providing additional evidence that this species is still a promising source of structurally novel small molecules.

EXPERIMENTAL PROCEDURE

General experimental procedures

Optical rotation was measured using a JASCO DIP-3000 polarimeter (JASCO Corporation, Tokyo, Japan). UV spectrum was recorded on a Hitachi U-3210 spectrophotometer (Hitachi, Tokyo, Japan). IR spectrum was measured on a Perkin Elmer Spectrum 100 (Perkin-Elmer, Fremont, CA, USA). NMR spectra were obtained on a Bruker AVANCE 400 or a Bruker AVANCE 500 spectrometer (Bruker, Rheinstetten, Germany) in DMSO-d_6, referenced to residual solvent signals (δ 2.49 for ^{1}H; δ 39.5 for ^{13}C). HR–ESI–TOF–MS was recorded on a Bruker microTOF focus. Silica Gel 75-C18 (Nacalai Tesque, Kyoto, Japan, 75 μM) was used for ODS column chromatography.

Microorganism

Strain SPMA113 was isolated from a soil sample collected in Prajinburi Province, Thailand. The strain was identified as a member of the genus *Streptomyces* on the basis of 98.9% similarity of 16S rRNA gene sequence (1393 nucleotides; GenBank accession number HQ340163) to the nearest type strain *Streptomyces malaysiensis* ATB-11^T (accession number AF117304).

Fermentation

Strain SPMA113 cultured on a Bn-2 slant (soluble starch 0.5%, glucose 0.5%, meat extract (Kyokuto Pharmaceutical Industrial, Tokyo, Japan) 0.1%, yeast extract (Difco Laboratories, Becton, Dickinson and Company, Sparks, MD, USA) 0.1%, NZ-case (Wako Chemicals USA, Richmond, VA, USA) 0.2%, NaCl 0.2%, $CaCO_3$ 0.1%, agar 1.5%) was inoculated into 500 ml K-1 flasks (K-Techno, Toyama, Japan) each containing 100 ml of the V-22 seed medium consisting of soluble starch 1%, glucose 0.5%, NZ-case 0.3%, yeast extract 0.2%, tryptone (Difco Laboratories) 0.5%, K_2HPO_4 0.1%, $MgSO_4{\cdot}7H_2O$ 0.05% and $CaCO_3$ 0.3% (pH 7.0). The flasks were placed on a rotary shaker (200 r.p.m.) at 30 °C for 4 days. The seed culture (3 ml) was transferred into 500 ml K-1 flasks each containing 100 ml of the A-11M production medium consisting of glucose 0.2%, soluble starch 2.5%, polypeptone N (Wako Chemicals USA) 0.5%, yeast extract 0.5%, NZ-amine (Wako Chemicals USA) 0.5%, $CaCO_3$ 0.3% and Diaion HP-20 (Mitsubishi Chemical, Tokyo, Japan) 1%. The pH of the medium was adjusted to 7.0 before sterilization. The inoculated flasks were placed on a rotary shaker (200 r.p.m.) at 30 °C for 6 days.

Extraction and isolation

At the end of the fermentation period, 50 ml of 1-butanol were added to each flask, and they were allowed to shake for 1 h. The mixture was centrifuged at 5000 r.p.m. for 10 min, and the organic layer was separated from the aqueous

layer containing the mycelium. Evaporation of the solvent gave 2.9 g of extract from 2 l of culture. The crude extract (2.9 g) was subjected to silica gel column chromatography with a step gradient of $CHCl_3$/MeOH (1 : 0, 20 : 1, 10 : 1, 4 : 1, 2 : 1, 1 : 1 and 0 : 1 v/v). Fraction 5 was concentrated to provide 0.57 g of brown oil, which was further purified by reversed phase ODS column chromatography with a gradient of MeCN/0.1% HCO_2H (2 : 8, 3 : 7, 4 : 6, 5 : 5, 6 : 4, 7 : 3 and 8 : 2 v/v). Fraction 3 was evaporated and the remaining aqueous solution was extracted with EtOAc. The aqueous layer was then lyophilized to give a yellow powder (7.1 mg). Final purification was achieved by preparative C-18 HPLC using a Cosmosil 5C18-AR-II column (Nacalai Tesque, 10×250 mm) with MeCN/0.1% HCO_2H (30 : 70 for 0–5 min, 30 : 70 to 40 : 60 over 5–25 min) at 3 ml min^{-1}, followed by evaporation and lyophilization, yielding prajinamide (**1**, 4.2 mg) with a retention time of 12.2 min.

Prajinamide (1): pale yellow oil; $[\alpha]_D^{24}$ −3.0 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 260 nm (3.80); IR (ATR) ν_{max} 3267, 1647, 1599, 1538 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HR–ESI–TOF–MS $[M+Na]^+$ 330.1788 (calculated for $C_{16}H_{25}N_3O_3Na$ 330.1788).

Marfey's analysis

A portion of **1** (0.1 mg) was hydrolyzed at 110 °C with 6 M HCl (200 µl) for 6 h, and the reaction mixture was evaporated to dryness. A 0.1 M $NaHCO_3$ solution (100 µl) was added to the dried hydrolysate of **1**, as well as to standards of L- and D-ornithine (Orn). A solution of 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) in acetone (0.05 mg in 50 µl) was added to each reaction tube. Each tube was sealed and incubated at 50 °C for 30 min. To quench reactions, 2 M HCl (50 µl) was added and then diluted with MeCN/0.2% HCO_2H (100 µl, 50 : 50). The Marfey's derivatives of the hydrolysate and standards were analyzed by HPLC using a Cosmosil 5C18-AR-II column (Nacalai Tesque, 4.6×250 mm) eluted with MeCN-2% HCO_2H at a flow rate of 1.0 ml min^{-1}, monitoring at 340 nm. The gradient elution was set as follows: 0–5 min (25% MeCN), 5–45 min (25–55% MeCN). Retention times for the amino acid standards were 13.4 min for L-Orn-L-FDLA and 9.8 min for D-Orn-L-FDLA, while the L-FDLA-hydrolysate of **1** gave a peak at 13.5 min.

Biological assays

Adipocyte differentiation assay,[13] antimicrobial assay[7] and cytotoxic assay[15] were carried out according to the procedures previously described. Rosiglitazone,[16] an antidiabetic drug, was used as a positive control in the adipocyte differentiation assay. It induced differentiation in 80% of murine ST-13 preadipocyte cells at 0.02 µM.

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