

NOTE

Rakicidin D, an inhibitor of tumor cell invasion from marine-derived *Streptomyces* sp.

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Tumor metastasis is the leading cause of death in cancer patients. It is the process by which a tumor cell leaves the primary tumor, disseminates to a distant site through the circulatory system and establishes a secondary tumor.¹ During the metastatic cascade, tumor cells must pass through the extracellular matrix barriers to accomplish the metastasis. Although the genetic basis of tumorigenesis can vary greatly, the steps required for metastasis are similar for all tumor cells. Therefore, interruption of metastasis is a promising approach to the treatment of cancers of various genetic origins. In our continuing search for anti-invasive compounds from microbial secondary metabolites,^{2–5} rakicidin D (**1**) was isolated from the culture broth of an actinomycete strain of the genus *Streptomyces*. Rakicidins are the 15-membered depsipeptides consisting of three amino acids and a 3-hydroxyfatty acid (Figure 1). To date, three congeners, rakicidins A (**2**) and B (**3**) from *Micromonospora* and rakicidin C (**4**) from *Streptomyces*, have been reported.^{6,7} Rakicidins contain a rare unusual amino acid, 4-amino-2,4-pentadienoate, which has been found only in the secondary metabolites from actinomycetes. Except for rakicidins, only two classes of cyclic peptides, BE43547⁸ and vinylamycin,⁹ are reported to date to contain this unusual amino acid. Herein, we describe the isolation, structure elucidation and biological properties of rakicidin D (**1**).

The producing strain *Streptomyces* sp. MWW064 was isolated from a marine sediment sample collected in Samut Sakhon province, Thailand. The strain was cultured in our standard medium for actinomycetes and the whole culture broth was extracted with 1-butanol. The extract showed inhibitory activity toward tumor cell invasion into Matrigel, the reconstituted extracellular matrix proteins.¹⁰ Bioassay-guided fractionation of the extract led to the isolation of a new compound, rakicidin D (**1**).

Compound **1** was obtained as a colorless amorphous powder. The high-resolution ESITOFMS indicated a molecular formula of

C₂₄H₃₈N₄O₇, which was consistent with the ¹H and ¹³C NMR data. The IR spectrum of **1** indicated the presence of OH or NH (3356 cm⁻¹) and carbonyl (1688 and 1648 cm⁻¹) functionalities. The UV spectrum and the ¹H and ¹³C NMR spectra of **1** showed high similarity to those for rakicidins A and B.⁷ The ¹³C NMR and HMQC analysis confirmed the presence of 24 carbons attributable to six deshielded signals including carbonyl carbons, two sp² methines, one sp² methylene, five sp³ methines, six sp³ methylenes, four methyl carbons and five exchangeable protons.

2-Amino-2,4-pentadienoate moiety was elucidated by a COSY correlation for H-9/H-10 and a series of HMBC correlations from H-12 to C-10 and C-11, from H-10 to C-8, C-11 and C-12, and from H-9 to C-8 and C-10 (Figure 2). An NOE between H-10 and an exomethylene proton at δ_H 5.41 indicated that the latter proton and C-10 were located on the same side of the C-11/C-12 double bond. *E* configuration for the double bond between C-9 and C-10 was confirmed by a large coupling constant for H-9 and H-10 (³J_{HH}=15.0 Hz). HMBC correlations from H-7 to C-6 and C-8, and from H-6 to C-5 and C-8 established the connectivity of *N*-methylglycine and the pentadienoate. COSY correlations for 2-NH/H-2/H-3 and HMBC correlations from H-2 to C-1, H-3 to C-4 and 4-NH₂ to C-3 confirmed the presence of a β-hydroxyasparagine moiety. Connection of this amino acid to the glycine moiety was established by an HMBC correlation from 2-NH to C-5. Three additional fragments, H-23/H-14/H-15, H-24/H-16 and H-21/H-22, were recognized from the COSY spectrum. HMBC correlations from H-23 to C-13, C-14 and C-15, and from H-24 to C-15, C-16 and C-17, established the 2,4-dimethyl-3-hydroxyalkanoate substructure. Connectivity of this fragment to the peptide unit was elucidated by HMBC correlations from 11-NH to C-13 and from H-15 to C-1. Finally, the fragment C-22/C-21 containing the triplet methyl group, the methylene fragment C-20 that had an HMBC correlation from H-22 and

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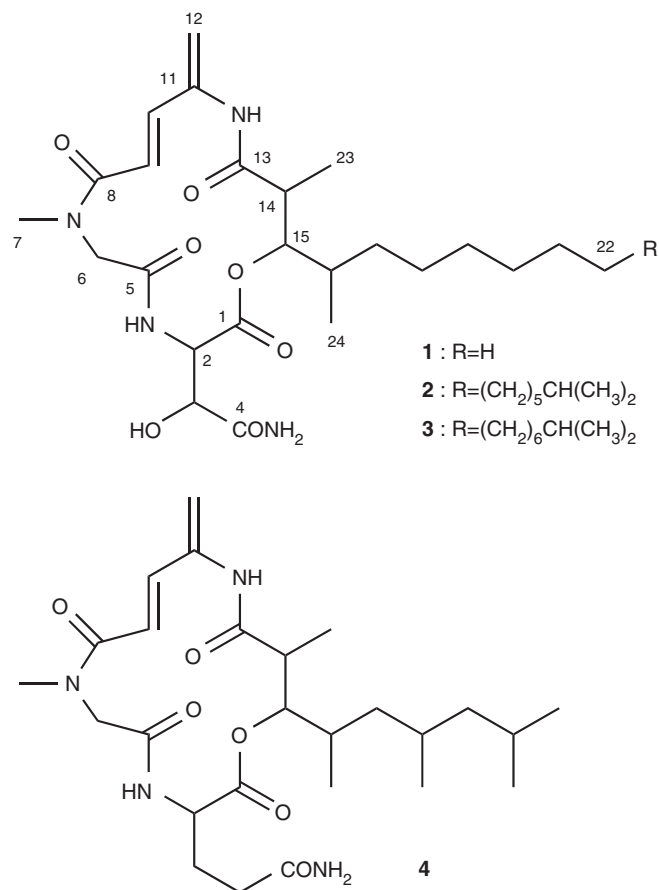


Figure 1 Structure of rakicidins D (1), A (2), B (3) and C (4).

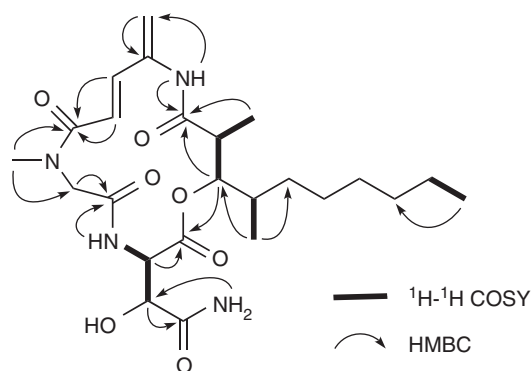


Figure 2 ¹H-¹H COSY and key HMBC correlations for **1**.

the remaining two CH₂ fragments (C-18 and C-19) were assigned to the terminal linear moiety of the alkanolate to complete the planar structure of **1**. Compound **1** has the same amino-acid components as rakicidins A and B but a shorter side chain with no terminal methyl branching in the fatty acid moiety.

Rakicidin D (**1**) inhibited the invasion of murine carcinoma colon 26-L5 cells with an IC₅₀ of 3.3 μg ml⁻¹ (=6.7 μM), whereas it showed no growth inhibition toward the same cell line at 10 μg ml⁻¹.¹⁰ Rakicidins A (**2**) and B (**3**), which were separately obtained from *Micromonospora* sp. TP-A0860. Rakicidin B was described to have an

isopropyl terminus in the fatty acid moiety but also described to have 3-hydroxy-2,4,16-trimethyloctadecanoic acid (*anteiso*-terminus), while the NMR data for rakicidin B were not provided in the literature. We thus reisolated rakicidin B and unambiguously confirmed that it had an *iso*-terminus on the basis of NMR analysis (see Supplementary Information). Compounds **2** and **3** showed much higher cytotoxicity toward the same cell line (IC₅₀=0.44 μg ml⁻¹ and 0.17 μg ml⁻¹), giving a suggestion that the alkyl side chain of the fatty acid moiety has a key role in cytotoxic effect. Anti-invasive effects of **2** and **3** were not observed at noncytotoxic concentrations.

EXPERIMENTAL PROCEDURE

General experimental procedure

Optical rotations were measured using a JASCO DIP-3000 polarimeter (JASCO, Tokyo, Japan). UV spectrum was recorded on a Hitachi U-3210 spectrophotometer (Hitachi, Ibaraki, Japan). IR spectrum was measured on a PerkinElmer Spectrum 100 (PerkinElmer, Waltham, MA, USA). NMR spectra were obtained on a Bruker AVANCE 400 or a Bruker AVANCE 500 spectrometer (Bruker, Bremen, Germany) in dimethyl sulfoxide-*d*₆ using the signals of the residual solvent protons (δ_H 2.49) and carbons (δ_C 39.8) as internal standard. HRESITOFMS were recorded on a Bruker microTOF focus.

Microorganism

Strain MWW064 was isolated from a sediment sample collected in Samut Sakhon province, Thailand. The strain was identified as a member of the genus *Streptomyces* on the basis of 99.0% 16S rRNA gene sequence (1431 nucleotides; DDBJ, DNA Data Bank of Japan, accession number GU295447) identity with *Streptomyces argenteolus* subsp. *toyonakensis* NBRC13847 (accession number AB184528.1).

Fermentation

Strain MWW064 cultured on a slant agar medium was inoculated into 500-ml K-1 flasks 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%, triptone (Difco Laboratories, Sparks, MD, USA) 0.5%, K₂HPO₄ 0.1%, MgSO₄·7H₂O 0.05% and CaCO₃ 0.3% (pH 7.0). The flasks were cultivated 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-3M production medium consisting of glucose 0.5%, glycerol 2%, soluble starch 2%, Pharmamedia (Traders Protein, Memphis, TN, USA) 1.5%, yeast extract 0.3% and Diaion HP-20 (Mitsubishi Chemical, Kanagawa, Japan) 1%. The pH of the medium was adjusted to 7.0 before sterilization. The inoculated flasks were cultured 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 was added to each flask, and they were allowed to shake for 1 additional hour. The mixture was centrifuged at 6000 r.p.m. for 10 min and the organic layer was separated from the aqueous layer containing the mycelium. Evaporation of the solvent gave 27.9 g of crude extract from 3 liter of culture. The crude extract (27.9 g) was subjected to silica gel column chromatography with a step gradient of CHCl₃-MeOH (1:0, 20:1, 10:1, 4:1, 2:1, 1:1 and 0:1, v/v). Fractions six and seven were concentrated to provide 0.79 g of dark viscous oil, which was further purified by reversed phase octadecyl silane column chromatography with a gradient of MeCN-0.1% HCOOH (2:8, 3:7, 4:6, 5:5, 6:4, 7:3 and 8:2, v/v). Fraction 4 (50:50) was evaporated and the remaining aqueous solution was lyophilized to afford **1** (66 mg) as a colorless amorphous solid. Production of metabolites analogous to rakicidins was not detected in the culture extract.

Rakicidin D (**1**). Colorless amorphous solid; [α]_D²³ -140 (c 0.20, dimethyl sulfoxide); UV (30% MeCN) λ_{max} (log ε) 207 (4.57), 261 (4.18); IR (neat) ν_{max} 3371, 2930, 1688, 1648 cm⁻¹; ¹H and ¹³C NMR data, see Table 1 (¹H and ¹³C NMR spectra are shown in Supplementary Figures S1 and S2 of Supplementary Information); HRESITOFMS [M-H]⁻ 493.2665 (calcd for C₂₄H₃₇N₄O₇, 493.2668).

Table 1 ^1H and ^{13}C NMR data for rakicidin D (1) in $\text{DMSO-}d_6$

Position	δ_C^a	δ_H mult (J in Hz) ^b	HMBC ^{b,c}
1	169.2, qC		
2	54.9, CH	4.88, dd (10.0, 1.8)	1, 3
3	72.5, CH	4.18, br.s	2, 4
4	172.7, qC		
5	167.6, qC		
6	52.5, CH ₂	4.46, d (18.2) 3.68, d (18.2)	5, 7 5, 7, 8
7	36.5, CH ₃	2.94, s	6, 8
8	166.0, qC		
9	118.8, CH	6.16, d (15.0)	8, 10
10	137.9, CH	6.86, d (15.0)	8, 11, 12
11	138.4, qC		
12	117.1, CH ₂	5.43, s 5.32, s	11 10, 11
13	172.5, qC		
14	41.7, CH	2.89, dq (9.9, 7.0)	13, 15, 23
15	78.1, CH	5.11, d (9.9)	1, 13, 14, 24
16	33.9, CH	1.69, m	24
17	32.8, CH ₂	1.35, m 1.13, m	
18	27.0 ^d , CH ₂	1.32, m 1.22, m	
19	28.9 ^d , CH ₂	1.22, m	
20	31.3, CH ₂	1.22, m	
21	22.1, CH ₂	1.26, m	
22	14.0, CH ₃	0.85, t (6.6)	20, 21
23	15.4, CH ₃	1.04, d (7.0)	13, 14, 15
24	13.3, CH ₃	0.93, d (6.8)	15, 16, 17
2-NH		8.09, d (10.0)	5
4-NH ₂		7.31, s 7.29, s	3 3
11-NH		8.90, s	12, 13

^aRecorded at 100 MHz.^bRecorded at 500 MHz.^cHMBC correlations are from proton(s) stated to the indicated carbon.^dInterchangeable.

Isolation of rakicidins A and B. Rakicidins A (2) and B (3) were obtained from the culture extract of *Micromonospora* sp. TP-A0860.¹¹

Rakicidin A (2). Colorless amorphous solid; $[\alpha]_D^{25}$ -126 (c 0.10, dimethyl sulfoxide); ^1H and ^{13}C NMR data were identical with those reported in the literature;⁶ HRESITOFMS $[\text{M-H}]^-$ 605.3927 (calcd for $\text{C}_{32}\text{H}_{53}\text{N}_4\text{O}_7$, 605.3920).

Rakicidin B (3). Colorless amorphous solid; $[\alpha]_D^{25}$ -90 (c 0.10, dimethyl sulfoxide); ^1H NMR and ^{13}C NMR data, see Supplementary Table S1 in Supplementary Information (^1H and ^{13}C NMR spectra are shown in Supplementary Figures S3 and S4 of Supplementary Information; described earlier). HRESITOFMS $[\text{M-H}]^-$ 619.4072 (calcd for $\text{C}_{33}\text{H}_{55}\text{N}_4\text{O}_7$, 619.4076).

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)