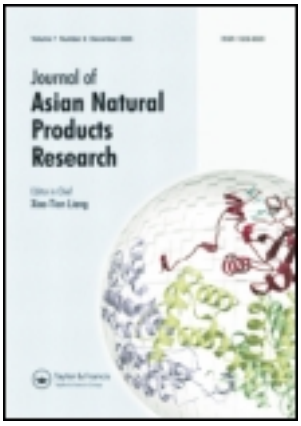

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A bisamide and four diketopiperazines from a marine-derived *Streptomyces* sp.

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A new bisamide N_1-acetyl-N_7-phenylacetyl cadaverine (**1**) and a series of diketopiperazines including a new diketopiperazine cyclo(2-hydroxy-Pro-*R*-Leu) (**2**), together with a new natural product cyclo(4-hydroxy-*S*-Pro-*S*-Trp) (**3**) and two known leucine-based diketopiperazines cyclo(4-hydroxy-*R*-Pro-*S*-Leu) (**4**) and cyclo (*S*-Pro-*R*-Leu) (**5**), were isolated from ethyl acetate extract of a fermentation broth of a marine-derived *Streptomyces* sp. Their structures were elucidated by the interpretation of spectroscopic analysis. The antitumor activities of compounds **1**–**3** against HL-60 cell lines were tested by MTT assay.

Keywords: marine-derived *Streptomyces* sp.; bisamide; diketopiperazine; antitumor activity

1. Introduction

Marine organisms are an important and productive source of new natural products. Growing attention has been paid to antibiotic and cytotoxic natural products of marine origin during the last few decades [1]. In order to isolate some bioactive compounds from marine organisms, we investigated the marine-derived *Streptomyces* sp. strain WuXin isolated from a marine sediment sample collected at the intertidal zone of Bohai Bay of China in August 2008. In this paper, we report the isolation and structural elucidation of two new compounds and the known compounds from the ethyl acetate (EtOAc) extract of the fermentation broth of the strain as well as antitumor activities of compounds **1**–**3** (Figure 1).

2. Results and discussion

Compound **1** was obtained as a colorless oil (MeOH). The molecular formula was determined to be $C_{15}H_{22}O_2N_2$ by HR-ESI-MS at *m/z* 263.1764 $[M + H]^+$. The IR absorptions were observed at 3444, 3320, 1697, and 1638 cm^{-1}, which indicated the presence of two —NH groups and two carbonyl groups. The ^{13}C NMR and DEPT spectra indicated the presence of 15 carbons as 1 methyl, 6 methylenes, 5 methines, and 3 quaternary carbons. The ^1H NMR spectrum showed aromatic proton signals at δ 7.49 (2H, m, H-4″, 8″), 7.31 (2H, m, H-5″, 7″), and 7.21 (1H, m, H-6″). The ^{13}C NMR spectrum showed six aromatic carbon signals at δ 137.3, 129.7 × 2, 128.9 × 2, and 127.0. Hence, compound **1** was considered to contain a monosubstituted

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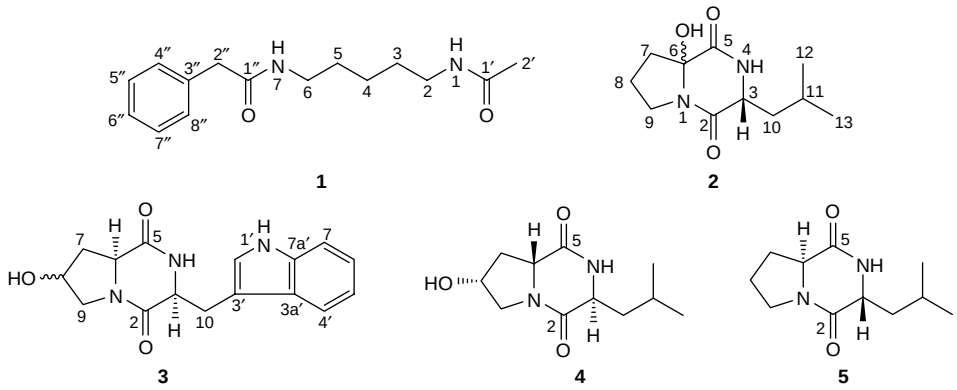

Figure 1. The structures of compounds **1**–**5**.

benzene moiety. Moreover, the ^{1}H NMR spectrum showed a methylene signal at δ 3.71 (2H, s, H-2″), and the ^{13}C NMR spectrum showed the corresponding carbons of the methylene and a carbonyl at δ 43.8 and 171.0, respectively. On the basis of the above evidence, compound **1** was considered to contain a phenylacetyl group (**1a**) as shown in Figure 2. The ^{1}H NMR spectrum showed a methyl signal at δ 2.59 (3H, s, H-2′), and the ^{13}C NMR spectrum showed the presence of the corresponding carbons of the methyl and a carbonyl at δ 28.7 and 177.8, respectively. Hence, compound **1** was considered to contain an acetyl group (**1b**). In the HMBC experiment of **1** (Figure 2), the correlations of H-2 (δ 3.44) with C-3 (δ 27.2) and C-4 (δ 24.5); H-3 (δ 1.47) with C-2 (δ 38.6), C-4 (δ 24.5), and C-5 (δ 29.6); H-4 (δ 1.22) with C-2 (δ 38.6), C-3 (δ 27.2), C-5 (δ 29.6), and C-6 (δ 39.6); H-5 (δ 1.47) with C-3 (δ 27.2), C-4 (δ 24.5), and C-6 (δ 39.6); H-6 (δ 3.31) with C-4 (δ 24.5) and C-5 (δ 29.6) revealed the presence of a cadaverine moiety (**1c**). The conjunction of moiety **1c** with moiety **1a** and **1b** was deduced by the HMBC correlations of H-2 (δ 3.44) with C-1′ (δ 177.8), H-6 (δ 3.31) with C-1″ (δ 171.0), and H-2′ (δ 2.59) with C-2 (δ 38.6). On the basis of the above evidence, compound **1** was characterized as N_1-acetyl-N_7-phenylacetyl cadaverine.

Compound **2** was obtained as a colorless oil with $[\alpha]_D^{20}$ +40 (c = 0.05, MeOH). The molecular formula was determined to be $C_{11}H_{18}O_3N_2$ by HR-ESI-MS at *m/z*

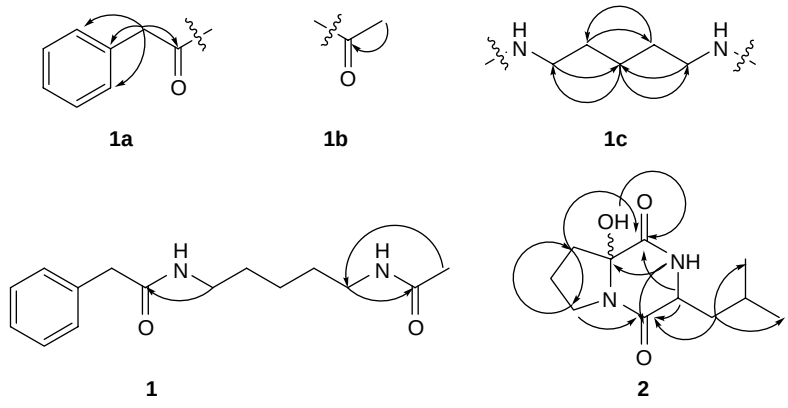

Figure 2. The key HMBC correlations of **1** and **2** and the moieties of **1**.

249.1210 $[M + Na]^+$. The IR absorptions were observed at 3448, 1657, and 1626 cm^{-1}, which indicated the presence of a hydroxyl group and two carbonyl groups. The ^{13}C NMR spectrum displayed the characteristic structure of a diketopiperazine ring system, which included two amide carbonyl signals at δ 167.7 and 167.8 and a methine signal at δ 55.4. The ^1H NMR spectrum displayed the characteristic structure of a leucine residue, which included two methyl signals at δ 0.87 (3H, d, $J = 6.3$ Hz, H-12) and 0.90 (3H, d, $J = 6.3$ Hz, H-13); a methylene signal at δ 1.77 (1H, m, H-10_a) and 1.55 (1H, m, H-10_b); and two methine signals at δ 3.66 (1H, m, H-3) and 1.77 (1H, m, H-11). And it also displayed the characteristic structure of a proline residue, which included three methylene signals at δ 3.42 (2H, m, H-9), 2.01 (1H, m, H-8_a), 1.77 (1H, m, H-8_b), and 2.01 (2H, m, H-7). The HMBC correlations (Figure 2) of hydroxyl proton with C-5 (δ 167.8) and the chemical shift value of C-6 (δ 86.2) suggested the presence of a hydroxyl attached to C-6. The assignment of all protonated carbon was established by HSQC experiment. In the HMBC experiment, the correlations of H-9 (δ 3.42) with C-2 (δ 167.7) and H-3 (δ 3.66) with C-5 (δ 167.8) established a leucine–proline diketopiperazine molecular structure. The stereochemistry of compound **2** at C-3 was determined as *R*-configuration by its downfield shift of C-10 (δ 44.6) [2]. However, the stereochemistry at C-6 was not determined by Marfey's method [3] because the hydroxylproline residue decomposed under acidic conditions. On the basis of the above evidence, compound **2** was determined as cyclo(2-hydroxy-Pro-*R*-Leu).

Compound **3** was obtained as colorless needles with $[\alpha]_D^{20}$ −139 ($c = 0.10$, MeOH). The NMR spectrum of **3** also showed typical signals for a diketopiperazine ring system. Comparison of the reference data assigned to cyclo(Pro-Trp) [4] suggested that they were very similar, except that one of the H-8 protons was replaced by the hydroxyl signal at δ 5.09. So, compound **3** was elucidated as cyclo(4-hydroxy-*S*-Pro-*S*-Trp). To date, this compound was only known as an intermediate in the synthesis of diazabicyclo alkane derivatives [5]. Thus, the discovery of compound **3** as a natural product is new.

Two known compounds cyclo(4-hydroxy-*R*-Pro-*S*-Leu) with $[\alpha]_D^{20}$ +32 ($c = 0.40$, MeOH) (**4**) and cyclo(*S*-Pro-*R*-Leu) with $[\alpha]_D^{20}$ −86 ($c = 0.10$, MeOH) (**5**) were identified by comparison of their NMR spectral data and optical rotation values with those reported in the literature [6,7].

The antitumor activities of compounds **1**–**3** were studied using HL-60 cell lines. As a result, all these tested compounds exhibited moderate cytotoxic activities ($IC_{50} = 58.43$, 98.49, 64.34 μM, respectively) *in vitro*.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a PerkinElmer 241 polarimeter. IR spectra were measured on a Bruker IFS-55 infrared spectrophotometer. The NMR spectral data were recorded on Bruker AV-600 (600 MHz for ^1H and 150 MHz for ^{13}C) with TMS as an internal standard. The HR-ESI-MS data were obtained on the Micross Mass Autospec-UltimaE TOF mass spectrophotometer. Chromatography was carried out on silica gel (200–300 mesh, Qingdao Haiyang Chemical Factory, Qingdao, China), Sephadex LH-20 (Pharmacia, Uppsala, Sweden), and reversed-phase HPLC (Shimadzu LC-8A vp, Kyoto, Japan).

3.2 Strain isolation and cultivation

Actinomycete strain, WuXin, was isolated from a marine sediment sample collected at the intertidal zone of Bohai Bay of China in August 2008 and showed a 99% similarity of its 16S rDNA gene sequence to

Streptomyces sp. MS-2 [8]. However, they had some differences in physiological and biochemical properties. It was suggested that this strain may be a novel species of genus *Streptomyces* and proposed *Streptomyces* sp. WuXin (GenBank accession number: JF779684) for the moment. The strain was identified by teacher Xin Wu and has been deposited in the School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University.

The strain was propagated on Gause′s synthetic agar solid medium at 28°C. Two weeks later, plugs of agar supporting mycelia growth were cut and transferred aseptically to a 500 ml flask containing 150 ml liquid medium (glucose 1.5%, peptone 1%, soy flour 1.4%, NaCl 3%, $CaCO_3$ 0.1%, pH 7). After incubation at 28°C at 180 rpm for 3 days, a 7.5 ml culture liquid was transferred as a seed into each of 500 ml flask containing 150 ml of the same liquid medium. The flasks were subsequently incubated at the same conditions for 8 days before extraction.

3.3 Extraction and isolation

The fermentation broth of the strain WuXin (about 80 l) was concentrated and extracted with EtOAc. The EtOAc crude extract (35.9 g) was subjected to silica gel column, eluted with $CHCl_3$–CH_3OH (100:0 → 0:100), yielding seven fractions. Fraction 4 (4.8 g) and fraction 5 (4.5 g) were purified by silica gel column eluted with petroleum–acetone (100:0 → 0:100) and Sephadex LH-20 column chromatography eluted with CH_3OH–$CHCl_3$ 1:1. Then, subfractions were subjected to preparative HPLC (flow rate 1 ml/min, wavelength 210 nm) by eluting with CH_3OH–H_2O to yield compounds **1** (2.8 mg), **2** (2.9 mg), **3** (2.3 mg), **4** (3.4 mg), and **5** (14.4 mg), respectively.

3.3.1 Compound *1*

Colorless oil (MeOH); IR (KBr) ν_{max} (cm^{-1}): 3444, 3320, 1697, 1638; ^1H and ^{13}C NMR spectral data, see Table 1; HR-ESI-MS *m/z*: 263.1764 $[M + H]^+$ (calcd for $C_{15}H_{23}O_2N_2$, 263.1760).

3.3.2 Compound *2*

Colorless oil, $[\alpha]_D^{20}$ +40 ($c = 0.05$, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3448, 1657, 1626; ^1H and ^{13}C NMR spectral data, see Table 2; HR-ESI-MS *m/z*: 249.1210 $[M + Na]^+$ (calcd for $C_{11}H_{18}O_3N_2Na$, 249.1210).

Table 1. ^1H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of **1** (in C_5D_5N).

Position	δ_H (*J*, Hz)	δ_C	HMBC (H → C)
1	8.45 (1H, br s)[a]	–	
2	3.44 (2H, m)	38.6	C-3, C-4, C-1′
3	1.47 (2H, m)[a]	27.2	C-2, C-4, C-5
4	1.22 (2H, m)	24.5	C-2, C-3, C-5, C-6
5	1.47 (2H, m)[a]	29.6	C-3, C-4, C-6
6	3.31 (2H, m)	39.6	C-4, C-5, C-1″
7	8.45 (1H, br s)[a]	–	
1′	–	177.8	
2′	2.59 (3H, s)	28.7	C-2
1″	–	171.0	
2″	3.71 (2H, s)	43.8	C-1″, C-4″, C-8″
3″	–	137.3	
4″/8″	7.49 (2H, m)	129.7	C-2″, C-6″, C-8″/4″
5″/7″	7.31 (2H, m)	128.9	C-3″, C-7″/5″
6″	7.21 (1H, m)	127.0	C-4″, C-8″

[a] Note: Overlapping signals.

Table 2. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of **2** (in DMSO-d_6).

Position	δ_H (J, Hz)	δ_C	HMBC (H → C)
2	–	167.7	
3	3.66 (1H, m)	55.4	C-2, C-10
4	8.33 (1H, d, $J = 4.2$)	–	C-2, C-3, C-6
5	–	167.8	
6	–	86.2	
7	2.01 (2H, m)[a]	36.5	C-5, C-8, C-9
8	1.77 (1H, m)[a]	19.2	
	2.01 (1H, m)[a]		
9	3.42 (2H, m)	44.9	C-2, C-7, C-8
10	1.55 (1H, m)	44.6	C-2, C-3, C-12, C-13
	1.77 (1H, m)[a]		
11	1.77 (1H, m)[a]	23.9	
12	0.87 (3H, d, $J = 6.3$)	21.6	C-10, C-11, C-13
13	0.90 (3H, d, $J = 6.3$)	23.1	C-10, C-11, C-12
6-OH	6.56 (1H, br s)	–	

[a] Note: Overlapping signals.

3.3.3 *Compound* ***3***

Colorless needles, $[\alpha]_D^{20}$ −139 ($c = 0.10$, MeOH); ^{1}H NMR (DMSO-d_6) δ: 1.63 (1H, m, H-7$_b$), 1.95 (1H, m, H-7$_a$), 3.09 (1H, m, H-9$_b$), 3.04 (1H, m, H-10$_b$), 3.20 (1H, m, H-9$_a$), 3.27 (1H, m, H-10$_a$), 4.07 (1H, m, H-6), 4.20 (1H, m, H-8), 4.30 (1H, m, H-3), 5.09 (1H, br s, OH-8), 6.97 (1H, m, H-6′), 7.05 (1H, d, $J = 1.2$ Hz, H-2′), 7.18 (1H, m, H-5′), 7.27 (1H, d, $J = 8.1$ Hz, H-4′), 7.56 (1H, d, $J = 7.2$ Hz, H-7′), 7.73 (1H, br s, NH-4), 10.87 (1H, br s, NH-1′). ^{13}C NMR (DMSO-d_6) δ: 26.4 (C-10), 53.7 (C-9), 36.2 (C-7), 55.3 (C-3), 56.4 (C-6), 67.6 (C-8), 108.6 (C-3′), 111.2 (C-4′), 118.7 (C-6′), 119.3 (C-7′), 121.0 (C-5′), 124.5 (C-2′), 127.9 (C-3a′), 135.9 (C-7a′), 165.8 (C-5), 169.4 (C-2).

3.4 *Cytotoxic activity assay*

HL-60 cell lines obtained from the American Type Culture Collection (Rockville, MD, USA) were used to evaluate the cytotoxic activities of compounds **1**–**3** *in vitro* by means of the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay. 5-FU (5-fluorouracil) and 0.1% DMSO were used as a positive control and a negative control, respectively.

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