Brief Report

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Oxoprothracarcin, a novel pyrrolo[1,4]benzodiazepine antibiotic from marine *Streptomyces* sp. M10946

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ABSTRACT: A novel pyrrolo[1,4]benzodiazepine antibiotic, designated oxoprothracarcin (3), was isolated from the marine strain *Streptomyces* sp. M10946 along with three known secondary metabolites, cyclo(D)-Pro-(D)-Val (1), cyclo(D)-Pro-(D)-Leu (2), and limazepine A (4). The chemical structures of these substances were elucidated by spectroscopic analyses, including 1D- and 2D-NMR and ESI-MS. Antitumor and antibacterial assays indicated that compounds 1-4 weakly inhibit the growth of MDA-MB-231 and A549 cells. The isolation of compound 3 with a high yield (36 mg/10 L) indicated that this marine *S.* sp. M10946 may provide new lead compounds for structural modification and drug screening.

Keywords: Marine Streptomyces, cyclo(D)-Pro-(D)-Val, cyclo(D)-Pro-(D)-Leu, oxoprothracarcin, limazepine A

1. Introduction

Traditionally, secondary metabolites produced by microorganisms, and especially terrestrial actinomycetes, are remarkable sources of lead compounds for drug discovery (*1*). However, the rate at which new metabolites from terrestrial actinomycetes are discovered has decreased, and the rate of re-isolation of known compounds has increased due to the replication of isolating microbial strains (*2*). Compared to terrestrial actinomycetes, marine actinomycetes are relatively unexploited sources (*3*). The fact that marine *Streptomyces* in particular are rich in novel bioactive metabolites is just becoming apparent (*4-6*). Moreover, genetic analysis has indicated that some marine-derived *Streptomyces* form an independent clade (*7*). The present study obtained four metabolites, i.e. cyclo(D)-Pro-(D)-Val (**1**) (*8*), cyclo(D)-Pro-(D)-Leu (**2**) (*8*), oxoprothracarcin (**3**) (*9-11*), and limazepine A (**4**) (*12*) (Figure 1), from the metabolites of marine *Streptomyces* sp. M10946. Reported here are the isolation, structural determination, and antitumor and antibacterial activities of compounds **1-4**.

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2. Materials and Methods

2.1. *General*

NMR spectra were recorded on a Bruker Advance 600 spectrometer (Bruker, Fällanden, Switzerland) at 600/150 MHz. Mass spectra were obtained on ABI-4000 mass spectrometers (AB SCIEX, Framingham, MA, USA). An Agilent 1260 HPLC system (Agilent, Santa Clara, CA, USA) with a C-18 column (9.4 × 250 mm, 5 μm) was also used. Column chromatography included a RP-18 (Merck, Darmstadt, Germany) column and a Sephadex LH-20 column (GE healthcare, Uppsala, Sweden). TLC analyses were performed with precoated silica gel GF254 plates (0.20-0.25 mm, Qingdao Marine Chemical Factory, Qingdao, China). All general chemical reagents were purchased from Sinopharm Chemical Reagent Company (Beijing, China).

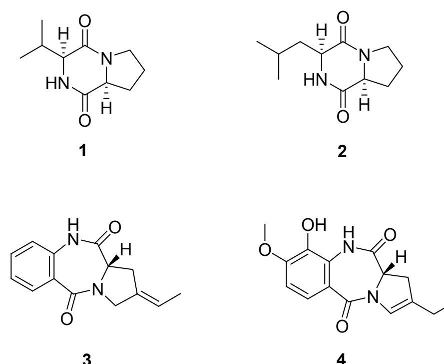

Figure 1. Chemical structures of compounds 1–4.

2.2. *Microorganism sample*

The strain *Streptomyces* sp. M10946 was isolated from mangrove sediment collected from Hut Bay and grown on medium containing trehalose as the sole carbon source [trehalose 10g, $(NH_4)_2SO_4$ 2.64 g, KH_2PO_4 2.38 g, K_2HPO_4 5.65 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, $CuSO_4 \cdot 5H_2O$ 0.0064 g, $FeSO_4 \cdot 7H_2O$ 0.0011 g, $MnCl_2 \cdot 4H_2O$ 0.0079 g, $ZnSO_4 \cdot 7H_2O$ 0.0015 g, distilled water 1 L, pH 7.2-7.4] at 28ºC. This strain was identified as *Streptomyces* sp. by partial 16S rRNA gene sequencing analysis.

2.3. *Tumor cell lines*

The MDA-MB-231 human breast cancer cell line and A549 human lung cancer cell line were purchased from the American Type Culture Collection (ATCC). All cells were maintained in DMEM with 10% fetal bovine serum (FBS, Gibco) in a humidified CO_2 incubator in 5% CO_2 at 37°C.

2.4. *Fermentation and isolation*

Fermentation took place for 14 d on YMG (10 L) agar plates at 28°C. The culture was diced and extracted with AcOEt/MeOH/AcOH (80:15:5). The organic solution was collected through filtration and the remaining agar residue was extracted exhaustively with the same solvent until the filtrate was colorless. Upon evaporation, the combined filtrate yielded a crude extract. The crude extract was partitioned between water and EtOAc (1:1, v/v) until the EtOAc layer was colorless. The EtOAc extract was partitioned between MeOH and petroleum ether. The MeOH layer was concentrated in a vacuum to yield a brown syrupy extract (1.4 g). The extract was subjected to MPLC (30 g RP-18 silica gel; 30%, 50%, 70%, and 100% MeOH, 1 L for each gradient) to yield 4 fractions, Fr. a–d.

Fraction a (192 mg) was separated by column chromatography (CC) over Sephadex LH-20 (in MeOH) to yield one subfraction (Fr.a.1). Fr.a.1 (33 mg) was subjected to HPLC (C-18 column, 9.4 × 250 mm, 5 μm; 30% MeOH) to yield 1 (3 mg) and 2 (9 mg). Fraction b (240 mg) was subjected to CC over Sephadex LH-20 (in MeOH) to yield two subfractions (Fr.b.1 and Fr.b.2). Fr.b.1 (53mg) was purified by Sephadex LH-20 (in MeOH) to yield 3 (36 mg). Fr.b.2 (28 mg) was purified by recrystallization (MeOH) to yield 4 (20 mg).

2.5. *Biological study*

The cytotoxicity of compounds **1**-**4** was assessed using a 3-(4,5-dimethylthiazol-2-yl) –2,5-diphenyltetrazolium bromide (MTT) cell survival assay (*13*), Briefly, 3000 - 5000 MDA-MB-231 and A549 cells were seeded in 96-well plates overnight and treated three times with increasing concentrations of compounds. After the cells were treated for 72 h, a 10-μL of aliquot of MTT solution (5 mg/mL) was added and cells were incubated for 4 h at 37°C. Two hundred μL of DMSO was then added to dissolve formazan crystals. The color density was measured with a microplate reader (M-3350, Bio-Rad) at 570 nm. Growth inhibition rates were calculated as follows: $(A570_{\text{control cells}} - A570_{\text{treated cells}})/A570_{\text{control cells}} \times 100\%$.

The antibacterial activity of compounds **1** - **4** was tested against *Bacillus subtilis* (CMCC (B) 63501), *Bacillus pumilus* (CMCC (B) 63202), and *Penicillium avellaneum* (UC 4376) using the filter paper method. Growth inhibition was calculated as the radius of the inhibition zone.

3. Results and Discussion

3.1 *Elucidation of the structures of compounds*

ESI-MS revealed the molecular weight of compound **1** to be 196 Da. The ^{13}C-NMR spectrum of **1** (Table 1) displayed 10 signals. The ^{1}H-NMR spectrum of **1** (Table 1) displayed 15 signals. ^{1}H-NMR signals at δH 4.19 (t, 7.3 Hz, 1H) and 4.04 (s, 1H) and ^{13}C-NMR signals at δC 172.6 and 167.6 revealed the presence of two acylamino groups, indicating that **1** is a cyclic dipeptide. ^{1}H-NMR signals at δH 2.47-2.52 (m, 1H), 1.09 (d, J = 7.3, 3H), and 0.93 (d, J = 6.9, 3H) and ^{13}C-NMR signals at δC 29.5, 18.8, and 16.7 indicated the presence of an isopropyl group. The HMBC correlation from CH(10) to C(1), along with the ^{1}H-^{1}H COSY correlation between CH(9)↔CH_2(10)↔CH_3(11), indicated the presence of fragment **1A** (Figure 2), which was a valine residue. ^{1}H-NMR signals at δH 3.43-3.58 (m, 2H), 1.95-1.96 (m, 2H), 2.01-2.06 (m, 1H), and 2.31-2.34 (m, 1H) and ^{13}C-NMR signals at δ 46.2, 23.3,

Table 1. ^{1}H-NMR and ^{13}C-NMR spectroscopic data for compounds 1 and 2 (MeOD).

	Compound **1**		Compound **2**	
	Proton	Carbon	Proton	Carbon
	Pro		Pro	
3	3.43-3.58 (m, 2H)	46.2t	3.50-3.52 (m, 2H)	46.4t
4	1.95-1.96 (m, 2H)	23.3t	1.93-1.96 (m) 1.99-2.06 (m)	23.6t
5	2.01-2.06 (m) 2.31-2.34 (m)	29.9t	1.99-2.06 (m) 2.28-2.33 (m)	29.1t
6	4.04 (m)	60.0d	4.27 (t, J = 7.5)	60.3d
7		172.6s		172.8s
8	Val		Leu	
9	4.21 (t, J = 7.3)	61.5d	4.13-4.16 (m)	54.6d
10	2.47-2.52 (m)	29.5d	1.87-1.92 (m, 2H)	39.4t
11	1.09 (d, J = 7.3, 3H)	18.8q	1.60-1.64 (m)	25.8d
12	0.93 (d, J = 6.9, 3H)	16.7q	0.96 (d, J = 4.3, 3H)	23.3q
13			0.97 (d, J = 4.2, 3H)	22.2q
1		167.6s		168.9s

δ in ppm. J in Hz.

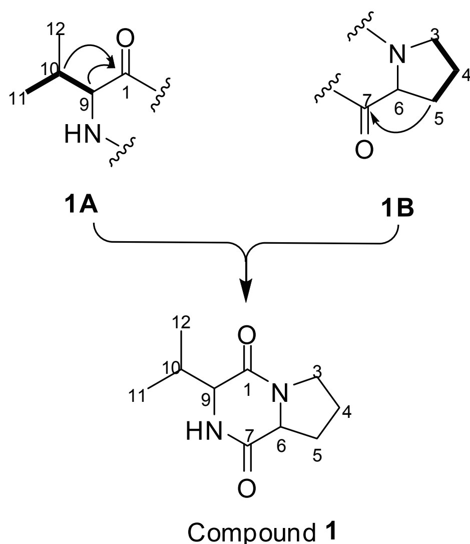

Figure 2. Selected HMBC (H→C) and ^{1}H-^{1}H COSY (—) correlations, and the structures of fragments 1A and 1B of compound 1.

and 29.9 indicated the presence of three CH_2 groups. Furthermore, the HMBC spectrum showed that $CH_2(5)$ was correlated with C(7). In combination with the ^{1}H-^{1}H COSY correlation between $CH_2(3)$↔CH(4)↔CH(5), these findings indicated the presence of fragment **1B** (Figure 2), which was a proline residue (Figure 2). The spectral data for **1** were consistent with those reported in the literature (*8*). Thus, compound 1 was determined to be cyclo(D)-Pro-(D)-Val.

ESI-MS revealed the molecular weight of compound **2** to be 210 Da. The chemical structure of **2** was determined by comparing NMR data for **2** with those for **1**. Both compounds had similar spectroscopic data (Table 1), except for C(11), C(12), and C(13). ^{1}H-NMR signals at δH 1.87-1.92 (m, 2H), 1.60-1.64 (m, 1H), 0.96 (d, J = 4.3, 3H), and 0.97 (d, J = 4.2, 3H) and ^{13}C-NMR signals at δC 39.4, 25.8, 23.3, and 22.2 indicated the presence of an isobutyl group. The HMBC spectrum showed that CH(9) was correlated with C(1). Along with the ^{1}H-^{1}H COSY correlation between CH(9)↔$CH_2(10)$↔CH(11)↔$CH_3(12)$, these findings indicated that **2** had a leucine residue. The spectral data of **2** were consistent with those reported in the literature (*8*). Therefore, compound **2** was determined to be cyclo(D)-Pro-(D)-Leu.

ESI-MS data revealed the molecular weight of compound **3** to be 242 Da. The ^{1}H-NMR spectrum of **3** (Table 2) displayed 14 signals. The ^{13}C-NMR spectrum of **3** (Table 2) displayed 14 signals for one methyl, two methylenes, six methines, and five quaternary carbon atoms, including two carbonyl groups (with one at δC 172.2 C(11) and the other at δC 167.7 C(5)). This suggested the presence of two acylamino groups as in compounds **1** and **2**. The ^{1}H-NMR signal at δH 5.50 (1H, m) and ^{13}C-NMR signals at δC 119.1 and 134.5 suggested a trisubstituted

Table 2. ^{1}H-NMR and ^{13}C-NMR spectroscopic data for compounds 3 and 6 (DMSO-d_6).

Position	Compound **3**		Compound **4**	
	Proton	Carbon	Proton	Carbon
1	2.59-2.63 (m) 3.26-3.28 (m)	28.2t	2.71-2.76 (m) 3.26-3.28 (m)	33.0t
2		134.5s		129.3s
3	4.03 (d, J = 15.8) 4.24 (d, J = 15.7)	52.5t	6.61 (s)	121.1d
4				
5		167.7s		161.5s
5a		127.7s		120.8s
6	7.79 (d, J = 7.8)	131.4d	7.30 (d, J = 8.7)	121.2d
7	7.16 (t, J = 8.1)	125.8d	6.97 (d, J = 8.8)	108.7d
8	7.26 (t, J = 7.6)	133.9d		150.4s
9	7.64 (d, J = 7.7)	122.5d		137.1s
9a		137.8s		125.1s
NH/OH	10.64 (s)		9.43 (s, 2H)	
11		172.2s		169.0s
11a	4.34 (dd, J = 9.3, 2.4)	58.4d	4.66 (dd, J = 10.8, 3.7)	56.6d
12	5.49-5.50 (m)	119.1d	2.16 (q, J = 7.3, 2H)	21.5t
13	1.68 (d, J = 6.7, 3H)	14.5q	1.08 (t, J = 7.4, 3H)	12.5q
MeO			3.87 (s, 3H)	56.5q

δ in ppm. J in Hz.

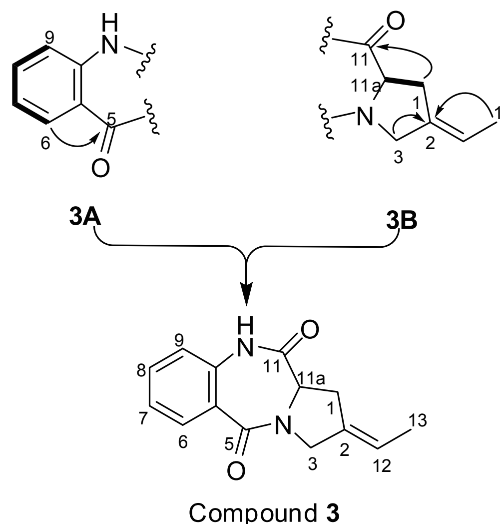

Figure 3. Selected HMBC (H→C) and 1H-1H COSY (—) correlations, and the structures of fragments 3A and 3B of compound 3.

double bond. The ^{1}H-^{1}H COSY correlation between CH(6)↔CH(7)↔CH(8)↔CH(9) indicated that **3** had a disubstituted benzene. Next, the HMBC correlation from CH(6) to C(5) indicated the presence of fragment **3A** (Figure 3). The double bond was assigned at C(2) and CH(12) in accordance with the HMBC correlation (Figure 3) of δH 1.68 $CH_3(13)$ with δC 127.5 CH(12) and δH 2.59, δH 3.28 $CH_2(1)$ and that of δH 4.03, δH 4.24 $CH_2(3)$ with δC 134.5 C(2). The HMBC correlation from CH(11a) and $CH_2(1)$ to C(5), in combination with the ^{1}H-^{1}H COSY correlation between $CH_2(1)$↔CH(11a), indicated the presence of fragment **3B** (Figure 3). Therefore, compound **3** was determined

to be (S,E)-2-ethylidene-2,3-dihydro-1H-benzo[e] pyrrolo[1,2-a][1,4]diazepine-5,11(10H,11aH)-dione, a new natural product that has been registered (CAS No.: 1052219-35-8). After consulting the names of reported analogues (*9-11*), compound **3** was designated oxoprothracarcin.

ESI-MS data revealed the molecular weight of compound **4** to be 288 Da. The ^{1}H-NMR and ^{13}C-NMR spectral data for **4** (Table 2) were similar to those for **3**. The ^{1}H-NMR spectrum of **4** (Table 2) displayed 16 signals. The ^{13}C-NMR spectrum of **4** (Table 2) displayed 15 signals for two methyls, two methylenes, four methines, and seven quaternary carbon atoms, including two carbonyl groups (with one at δC 169.0 C(11) and the other at δC 161.5 C(5)). The ^{1}H-NMR signal at δH 6.61 (1H, s) and ^{13}C-NMR signals at δC 121.1 and 129.3 suggested a trisubstituted double bond like that in compound **3**. However, the HMBC correlation from CH_2(1), CH_2(3), CH(11a), CH_2(12), and CH_3(13) to C(2) and the ^{1}H-^{1}H COSY correlation between CH_2(12)↔CH_3(13) and CH_2(1)↔CH(11a) indicated a C=C bond between C(2) and C(3). The ^{1}H-^{1}H COSY correlation between CH(6)↔CH(7), and the ^{13}C-NMR signals at δC 150.4 C(8) and 137.1 C(9) indicated the presence of a methoxyl group at C(8) and a hydroxyl group at C(9). The spectral data for compound **4** were identical to those of limazepine A reported in the literature (*8*).

3.2. *Biological study*

At a concentration of 10 μM, compounds **1** - **4** inhibited the growth of MDA-MB-231 cells at rates of 5.2%, 6.3%, 10.2%, and 3.8%, and they inhibited the growth of A549 cells at rates of 18.4%, 19.6%, 7.3%, and 0.7%. The antibacterial activity of compounds **1** - **4** was tested against *Bacillus subtilis* (CMCC (B) 63501), *Bacillus pumilus* (CMCC (B) 63202), and *Penicillium avellaneum* (UC 4376) using the filter paper method. Activity of each compound was tested twice at a concentration of 1.0 mg/mL with a loading volume of 20 μL. Results indicated that compounds **1** - **4** had no effect on the growth of the bacteria tested at 20 μg/disc.

3.3. *Conclusions and perspectives*

Genetic analysis indicated that some marine *Streptomyces* form an independent clade (*7*), so marine *Streptomyces* was surmised to potentially be rich in novel secondary metabolites. The present study succeeded in isolating only one new compound, which suggests that fermentation medium screening and/or genetic manipulation are needed to encourage the production of secondary metabolites in marine *Streptomyces*. However, compound **3** was isolated with a high yield (36 mg/10 L) and could be used as a lead compound for structural modifications.

Acknowledgement

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Appendix

Cyclo[Val-Pro] (**1**). Colorless crystal. ^{1}H- and ^{13}C-NMR spectral data: see Table 1. ESI-MS: m/z = 197.5 ([M + H]$^{+}$) and m/z = 219.5([M + Na]$^{+}$).

Cyclo[Leu-Pro] (**2**). Colorless crystal. ^{1}H- and ^{13}C-NMR spectral data: see Table 1. ESI-MS: m/z = 211.4 ([M + H]$^{+}$) and m/z = 233.4([M + Na]$^{+}$).

Oxoprothracarcin (2-ethylidene-2,3-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-5,11(10H,11aH)-dione, **3**). White crystal. ^{1}H- and ^{13}C-NMR spectral data: see Table 2. ESI-MS: m/z = 243.5 ([M + H]$^{+}$) and m/z = 265.4([M + Na]$^{+}$).

Limazepine A (**4**). White crystal. ^{1}H- and ^{13}C-NMR spectral data: see Table 2. ESI-MS: m/z = 289.4 ([M + H]$^{+}$) and m/z = 311.5 ([M + Na]$^{+}$).