Brief Report

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A new phenoxazine derivative isolated from marine sediment actinomycetes, *Nocardiopsis* sp. 236

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ABSTRACT: During screening of marine actinomycetes for anti-mycobacterial activity, a new phenoxazine derivative (1) was isolated, along with 6-phenazinediol (2), 6-methoxy-1-phenazinol (3), nocardamin (4), and 3-pyridinecarboxylic acid (5), from a culture of *Nocardiopsis* sp. 236 collected from the west Pacific. The chemical structure of 1 was established on the basis of 1D-, 2D-NMR, and HR-Q-TOF MS data. All compounds were evaluated for their anti-mycobacterial activity in vitro, and only compounds 2 and 3 exhibited weak activity.

Keywords: *Nocardiopsis* sp., phenazine, nocardamin, pyridine carboxylic acid

1. Introduction

Marine microorganisms are widely recognized as a rich source of novel natural products (*1*) and numerous novel compounds from marine actinomycetes have been discovered (*2-4*). During screening of antibacterial compounds for activity against *Mycobacterium smegmatis*, strain 236 was identified because of its remarkable activity. Further screening resulted in the isolation of five compounds. This paper describes the isolation, characterization, and antibacterial activity of compounds **1-5** (Figure 1).

2. Materials and Methods

2.1. *General experimental procedures*

Mass spectra were measured using a Bruker BioTOF-Q spectrometer; NMR spectra were measured on Bruker DRX-600 NMR spectrometers with tetramethylsilane (TMS) as an internal standard. Reversed-phase (RP) C18 silica gel for column chromatography (CC) was obtained from Merck and Sephadex LH-20 was obtained from Amersham Biosciences. Silica gel (200-300 mesh) for CC and silica gel GF_{254} for TLC were purchased from Qingdao Marine Chemical Ltd., Qingdao, Shandong, China.

2.2. *Microorganism specimens*

Strain 236 was isolated from sediment collected in the West Pacific Ocean. The strain was identified as *Nocardiopsis* sp. according to its 16s rDNA sequence (accession No. JQ355005) and BLAST search of the NCBI database (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*).

2.3. *Culture and isolation*

The strain was seeded on SYP medium (starch 10%, yeast extract 4%, peptone 2% and agar 2% agar plates, pH 7.2) in an inclined test tube and cultured for 7 d at 28°C to yield seed cultures. Fermentation (10 l) was done on SYP medium for 14 d at 28°C.

The culture was diced and extracted three times with an equal volume of EtOAc/MeOH/AcOH 80:15:5 (*v/v/v*) at room temperature. The organic solutions were collected by filtration and removed under a vacuum at 40°C to obtain the crude extract. This was partitioned between MeOH and petroleum ether (1:1) three times. The MeOH solution was concentrated under a vacuum at 40°C to obtain MeOH extract (5.0 g).

The MeOH extract was subjected to MPLC (140 g RP-18 silica gel; H_2O, MeOH/H_2O 30%, 50%, 70%, and 100% resp., 2 l each) to yield 8 fractions: Fr.A - H. Fr.A (872.8 mg) obtained from 30% MeOH was subjected to Sephadex LH-20 (120 g; MeOH) to obtain Fr.A1 (109.8 mg) and Fr.A2 (134.6 mg). Fr.A1 was purified by CC (SiO_2; chloroform /MeOH 100:1) to yield **3** (7.0 mg). Fr.A2 was further purified by MPLC (40 g RP-18 silica gel; 25% MeOH with 0.5% formic acid) to yield **5** (5 mg). Fr.C (125.2 mg) obtained from 50% MeOH was subjected to CC (30 g RP-18 silica gel; H_2O, MeOH/H_2O 45%, 50%) to obtain Fr.C1 (25.4 mg). Fr.C1 was further subjected to Sephadex

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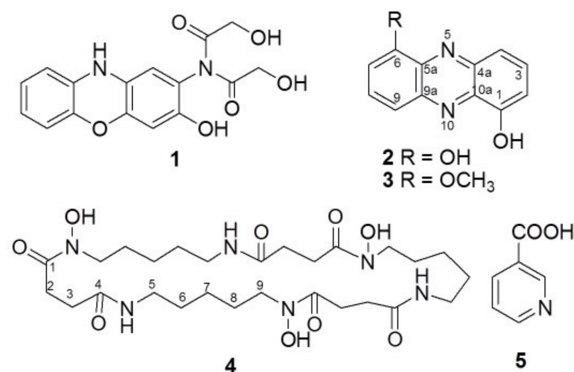

Figure 1. The chemical structures of compounds 1 - 5.

LH-20 (60 g; MeOH) and CC (0.5 g silica gel, chloroform /MeOH 10:1) to yield **1** (1.8 mg). Fr.D (290.8 mg) obtained from 50% MeOH was subjected to Sephadex LH-20 (120 g; MeOH) to obtain Fr.D1-D4. Fr.D1 (49.2 mg) was further purified by CC (0.5 g silica gel, petroleum ether / acetone 30:1) to yield **2** (2.3 mg). Fr.E (400.8 mg) obtained from 50% MeOH was subjected to Sephadex LH-20 (120g; MeOH) to obtain Fr.E1 (15 mg). Fr.E1 was subjected to CC (0.5 g silica gel, petroleum ether/chloroform 30:1) to obtain **4** (3.6 mg).

2.4. *Antibacterial activity*

An antibacterial activity test was performed with *Mycobacterium smegmatis*. The culture was maintained on LB medium (yeast extract 1%, tryptone 0.5%, NaCl 1%, pH 7.2). The antibacterial activity of **1-5** was tested against *Mycobacterium smegmatis* by the disk diffusion assay on agar plates with rifampicin as a positive control.

3. Results and Discussion

3.1. *Elucidation of structures*

Compound **1** was obtained as a yellow amorphous powder and had a positive color reaction with modified Dragendorff's reagent on TLC. The molecular formula of **1** was determined to be $C_{16}H_{14}N_2O_6$ on the basis of HR-Q-TOF MS and NMR data.

The ^{13}C-NMR (DEPT) spectrum of **1** (Table 1) had 16 signals, corresponding to two O-bearing CH_2, six CH (two of them O-bearing), and eight quaternary C-atoms (including two carbonyl groups at δ 172.9 and 173.3). Six olefinic proton signals in the ^{1}H-NMR spectrum (Table 1), including two doublet-doublet and two triplet-doublet signals from δ 7.15 - 7.57 and two singlet protons at δ 6.70 and 8.29, were noted, indicating the presence of a di- and a tetra- substituted benzene ring in the structure. The phenoxazine skeleton of **1** was identified by comparison of the NMR data to those of **3b** and **3d** according to Maskey *et al*. (*5*).

In HMBC, two oxymethylene groups (δ_H 4.17, δ_C 63.0; δ_H 4.43, δ_C 61.9) had long-range coupling to the carbonyls at δ 172.9 and 173.3, respectively. In order to determine the binding of the hydroxyacetyl groups, **1** was allowed to react with acetic anhydride in pyridine at room temperature. After this reaction, the main product was obtained as a colorless powder and

Table 1. ^{1}H- and ^{13}C-NMR data for compounds 1 and 1a. Recorded at 600/150MHz (δ in ppm, J in Hz)

Position	**1** (in CD_3OD)			**1a** (in $CDCl_3$)	
	^{1}H	^{13}C	HMBC	^{1}H	^{13}C
1	8.29 (s)	117.4d	C2, C3, C4a, C10a	7.56 (s)	118.3d
2	/	122.8s			126.7s
3	/	147.9s			145.8s
4	6.70 (s)	104.3d	C2, C3, C4a, C10a	7.13 (s)	112.9d
4a	/	120.6s			125.2s
5a	/	152.4s			151.8s
6	7.15 (dd, 7.8, 1.2)	117.8d	C5a, C8, C9a	7.21 (d, 7.8)	125.0d
7	7.25 (td, 7.8, 1.2)	128.4d	C5a, C9	7.33 (t, 7.8)	128.8d
8	7.18 (td, 7.8, 1.2)	124.6d	C6, C9a	7.22 (t, 7.8)	124.1d
9	7.57 (dd, 7.8, 1.2)	126.3d	C7, C5a	7.36 (d, 7.8)	126.8d
9a	/	130.0s			127.3s
10a	/	149.1s			150.8s
1'		172.9s			171.0s
2'	4.17 (s, 2H)	63.0t	C1’	5.20 (AB d, 17.1) 5.02 (AB d, 17.1)	65.5t
1"		173.3s			173.3s
2"	4.43 (s, 2H)	61.9t	C1”	5.10 (AB d, 15.1) 4.83 (AB d, 15.1)	61.7t
-OAC				2.34 (s, 3H),	30.2 q,
-OAC				2.27 (s, 3H),	26.2 q,
-NAC				2.18 (s, 3H),	21.1 q,
-NAC				2.16 (s, 3H)	21.0 q,
					170.9 s
					170.1 s
					168.9 s
					166.4 s

Table 2. ^{1}H- and ^{13}C-NMR data for compounds 2 and 3. Recorded at 600/150MHz (δ in ppm, J in Hz)

Position	**2** (in DMSO-d_6)		**3** (in $CDCl_3$)	
	^{1}H	^{13}C	^{1}H	^{13}C
1	/	152.2s	/	151.5s
2	7.54(dd, 5.0,7.9)	110.5d	7.11(d, 7.8)	109.3d
3	8.38(d, 6.8)	131.2d	7.76(t, 7.8)	131.4d
4	8.69 (dt, 3.7, 7.9)	123.4d	7.95(dd, 0.8,8.8)	120.6d
4a	/	149.9s	/	142.6s
5a	/	137.8s	/	135.6s
6	/	152.2s		155.2s
7	7.54(dd, 5.0, 7.9)	110.5d	7.24(dd, 0.8, 7.8)	106.8d
8	8.38(d, 6.8)	131.2d	7.78(t, 7.8)	130.5d
9	8.69 (dt, 3.7, 7.9)	123.4d	7.83(dd, 0.8, 8.7)	121.0d
9a	/	149.9s		142.0s
10a	/	137.8s		137.6s
6-OCH3	/	/	4.19(s)	56.5q
OH	9.08 (2H, br s)		8.17(1H, br s)	/

designated **1a**. The ESI-MS of **1a** indicated a quasi-molecular ion peak at *m/z* 521.6 $[M + Na]^+$, which suggested the addition of four acetyl groups in **1a**. Both of the hydroxyacetyl groups were determined to be bound to N-C(2) by comparison of the chemical shifts of C(1) and C(4), which were at δ_H 8.29, δ_C 117.4 and δ_H 6.70, δ_C 104.3 in **1** and at δ_H 7.56, δ_C 118.3 and δ_H 7.13, δ_C 112.9 in **1a**, respectively. Thus, the structure of **1** was determined to be 2-hydroxy-N-(3-hydroxy-10H-phenoxazin-2-yl)-N-(2-hydroxyacetyl)acetamide.

Compounds **2** and **3** were obtained as yellow amorphous powder. ESI-MS indicated a quasi-molecular ion peak $[M + H]^+$ at *m/z* 227.3 and 213.3, respectively. These compounds were identified as 6-methoxy-1-phenazinol (**2**) (*6, 7*) and 1,6-phenazinediol (**3**) (*7, 8*) by comparing their ^{1}H and ^{13}C-NMR data (see Table 2) with those previously reported.

Compound **4** was found to be nocardamin by comparing the ^{1}H- and ^{13}C-NMR data to those previously reported (*9, 10*). Both ^{1}H and ^{13}C NMR data (in DMSO-d_6) revealed only seven methylene protons (at δ 1.20(H-7), 1.38(H-8), 1.49(H-6), 2.28(H-2), 2.59(H-3), 3.01(H-9), and 3.47(H-5)) and nine carbons signals (at δ 172.3(C-1), 171.7(C-4), 47.1(C-5), 38.5(C-9), 30.2(C-2), 28.8(C-8), 27.7(C-3), 26.1(C-6), and 23.2(C-7)). The NMR spectra of **4** revealed an N-hydroxy-N-succinylcadaverine unit with a molecular weight corresponded to 200, while ESI-MS analysis of **4** revealed a peak at *m/z* 601.7 $[M + H]^+$. Therefore, **4** consisted of three N-hydroxy-N-succinylcadaverine units that were conjugated iteratively.

Compound **5** was found to be identical to 3-pyridinecarboxylic acid based on the NMR spectra: ^{1}H-NMR (600 MHz, methanol-d_4): δ 9.10 (s, H-2),8.40 (d, 7.8, H-4), 7.56 (dd, 4.8, 7.8, H-5), 8.71 (d, 4.8, H-6); ^{13}C NMR(150 MHz, methanol-d_4): δ 151.0 (C-2), 128.5 (C-3), 138.9(C-4), 124.9(C-5), 153.4 (C-6), 167.6(C-7).

3.2. *Biological study*

The antibacterial efficacy of **1-5** against *M. smegmatis* was studied. Compounds **2** and **3** exhibited activity against *M. smegmatis* with inhibitory zones of 1.0 and 0.8 cm, respectively, at a dose of 20 μg/disk. This result was consistent with the reported data (*6, 7*). All other tested compounds were inactive, whereas the positive control, rifampicin exhibited activity with an inhibitory zone of 2.0 cm (0.1 μg/disk).

Four type of compounds were obtained from the fermentation and culture of *Nocardiopsis* sp. 236, including phenazine, phenoxazine, nocardamin, and pyridine derivatives. As a hydroxamate siderophore, nocardamin was initially isolated as an antibacterial metabolite of a Nocardia strain (*9*). Later, its derivatives were isolated from several actinomycetes (*10, 11*) and are currently the only available therapeutic agent for chronic iron overload and acute iron intoxication. Phenazines and phenoxazines comprise a large group of nitrogen-containing heterocyclic compounds that differ in their chemical and physical properties based on the type and position of functional groups present. More than 100 different phenazine structural derivatives have been identified in nature (*12*). The diverse biological actions of these compounds include cytotoxic, antibacterial, antiparasitic, and antimalarial activities (*13*). The current study obtained 6-methoxy-1-phenazinol (**2**) and 1,6-phenazinediol (**3**) with antibacterial activity against *M. smegmatis*.

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