CYTOTOXIC METABOLITES OF *Streptimonospora salina*

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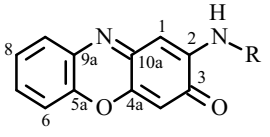
Streptimonospora salina *gen. nov., sp. nov. was found to produce three phenoxazinone antibiotics, 2-amino-3*H*-phenoxazin-3-one (**1**), 2-methylamino-3*H*-phenoxazin-3-one (**2**), 2-acetylamino-3*H*-phenoxazin-3-one (**3**), and one phenazine antibiotic, phenazine-1-carboxylic acid (**4**). The chemical structures of the compounds were determined using 1D and 2D NMR spectrometry and electrospray mass spectrometry (ESMS). Compounds **1–4** exhibited modest cytotoxicity against a human renal carcinoma cell line ACHN with IC_{50} values of 35.4, 12.4, 65.4, and 82.9 μM, respectively. Compound **2** was discovered for the first time from a biological origin.*

Key words: phenoxazinoes, *Streptimonospora salina* gen. nov., sp. nov., cytotoxic.

A number of phenoxazinones have been isolated from various microorganisms, such as *Lepiota americana* [1], *Chaetosphaeria* sp. [2], *Pseudomonas putida* [3], *Streptomyces thioluteus* [4], *Streptomyces exfoliatus* [5], *Streptomyces griseus* [6], *Actinomadura* sp. [7], and *Halomonas* sp. [8]. Many of them have been described to exhibit antitumor activity against various human cancer cell lines [6–10]. In the course of our search for anticancer agent, we have isolated three phenoxazinone antibiotics **1–3** and one phenazine antibiotic **4** from the culture of *Streptimonospora salina* gen. nov., sp. nov. (YIM 90002^T), which was isolated from soil samples collected from Aibi Lake, Jinghe County, Xinjiang Province, China (89°10′32″ N, 42°32′10″ E) [11]. Herein we described the fermentation, isolation, structure determination, and anticancer activity of **1**–**4**.



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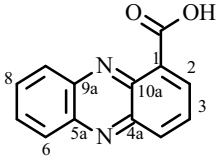

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1: R = H; **2:** R = CH_3; **3:** R = $COCH_3$

The total methanol extract of the culture filtrate was separated over silica gel using $CHCl_3$–CH_3OH systems of increasing polarity. Tests for anticancer activity showed that fractions eluted from silica gel by $CHCl_3$–CH_3OH (95:5) (fraction 1) and (85:15) (fraction 2) contained active compounds. Gel-filtration chromatography of fraction 1 over Sephadex LH-20 ($CHCl_3$–CH_3OH, 50:50) produced three chromatographically pure compounds **1**, **2**, and **3**.

The molecular weight of **1** is 212 Da according to quasimolecular ions produced by electrospray mass spectrometry (ESMS) performed in two regimes: $[M+H]^+$ 213 and $[M-H]^-$ 211. The ^1H and ^{13}C NMR spectra and the HSQC spectrum (in DMSO-d_6, δ, ppm) of **1** showed six Ar protons (δ_H 6.36 → δ_C 98.4, H-1; δ_H 6.37 → δ_C 103.4, H-4; δ_H 7.49 → δ_C 115.9, H-6; δ_H 7.46 → δ_C 128.8, H-7; δ_H 7.39 → δ_C 125.2, H-8; δ_H 7.71 → δ_C 127.9, H-9). These data for **1** show that they are identical to those for 2-amino-3*H*-phenoxazin-3-one, which was isolated previously from *Streptomyces thioluteus* [4].

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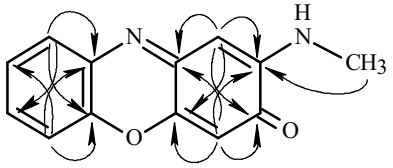

Fig. 1. Key HMBC correlations of **2**.

The molecular weight of **2** is 226 Da according to quasimolecular ions produced by ESMS performed in two regimes: $[M+H]^+$ 227 and $[M-H]^-$ 225. The ^1H and ^{13}C NMR spectra and the HSQC spectrum (in DMSO-d_6, δ, ppm) of **2** showed one methyl (δ_H 2.85 → δ_C 29.0), six methine protons (δ_H 6.09 → δ_C 95.1, H-1; δ_H 6.37 → δ_C 103.4, H-4; δ_H 7.50 → δ_C 115.9, H-6; δ_H 7.46 → δ_C 128.7, H-7; δ_H 7.40 → δ_C 125.3, H-8; δ_H 7.70 → δ_C 127.8, H-9), and one secondary amino group (δ_H 7.19, -NH). These data and HMBC spectrum of **2** (Fig. 1) indicate that the compound is 2-methylamino-3*H*-phenoxazin-3-one (**2**), which was chemically synthesized previously by condensation of 2-bromo-7-methoxytropone and *o*-aminophenol [12]. Compound **2** is discovered for the first time from a biological origin.

The molecular weight of **3** is 254 Da according to quasimolecular ions produced by ESMS performed in two regimes: $[M+Na]^+$ 277 and $[M-H]^-$ = 253. The ^1H and ^{13}C NMR spectra and the HSQC spectrum (in DMSO-d_6, δ, ppm) of **3** showed one methyl (δ_H 2.29 → δ_C 24.9), six methine protons (δ_H 8.44 → δ_C 113.9, H-1; δ_H 6.46 → δ_C 104.1, H-4; δ_H 7.42 → δ_C 116.1, H-6; δ_H 7.56 → δ_C 131.8, H-7; δ_H 7.44 → δ_C 125.7, H-8; δ_H 7.89 → δ_C 130.1, H-9), and one acetyl amino group (δ_H 8.60, ¯NH). The data for **3** show that they are identical to those for 2-acetylamino-3*H*-phenoxazin-3-one, which was isolated previously from *Streptomyces exfoliatus* [5].

Gel filtration chromatography over Sephedex LH-20 in CH_3OH of fraction 2 produced pure **4** of molecular weight 224 Da, which was established for the quasimolecular ions produced by ESMS performed in two regimes: $[M+H]^+$ 225 and $[M-H]^-$ 223. The ^1H and ^{13}C NMR spectra and the HSQC spectrum (in $CDCl_3$, δ, ppm) of **4** showed seven methine protons (δ_H 8.97 → δ_C 137.4, H-2; δ_H 8.04 → δ_C 130.2, H-3; δ_H 8.53 → δ_C 135.1, H-4; δ_H 8.33 → δ_C 130.1, H-6; δ_H 7.98 → δ_C 131.6, H-7; δ_H 8.00 → δ_C 133.1, H-8; δ_H 8.28 → δ_C 128.0, H-9). The data for **4** show that they are identical to those for phenazine-1-carboxylic acid, which was isolated previously from *Streptomyces antibioticus*, strain Tu [13].

Compounds **1–4** exhibited modest cytotoxicity against human renal carcinoma cell line ACHN with IC_{50} values of 35.4, 12.4, 65.4, and 82.9 μM, respectively.

EXPERIMENTAL

^1H and ^{13}C NMR spectra of compounds in DMSO-d_6 and $CDCl_3$ were measured on a Bruker DRX-500 spectrometer with TMS internal standard. Mass spectra were recorded on an Autospect VG-3000 spectrometer. Chromatographic monitoring was performed on Nano-Durasil-20 UV 254 plates (Macherey-Nagel & Co) using $CHCl_3$–CH_3OH (95:5 and 90:10). Compounds on chromatography plates were stained using anisaldehyde prepared as before [14]. Cytotoxic activities were studied by the published methods [15].

Cultivation of Bacteria and Isolation of 1–4. Bacteria were cultivated in 500 mL Ehrlenmeyer flasks in media containing malt extract 5 g/L, yeast extract 5 g/L, peptone 5 g/L, L-asparagine 1 g/L, glycerol 1 g/L, K_2HPO_4 1 g/L, NaCl 150 g/L, vitamin solution 7.0 mL/L, and trace element solution 1 mL/L, pH 7.8.

Fermentation was performed with continuous rocking for 7 d at 28°C. The total volume of culture medium was 20 L.

The completed fermentation broth was separated into filtrate and mycelium by centrifugation. The culture filtrate was absorbed onto the polymeric resin Amberlite XAD-16 (4 L, Rohm & Hass, France). The NaCl was washed out with water followed by 50% aq. MeOH (5 L each). Then, other absorbed organic material was eluted with 5 L 70% aq. MeOH, and finally material was eluted with 100% MeOH to yield 15 g of dried extract after removing solvent in vacuo. The extract was chromatographed over silica gel using $CHCl_3$:CH_3OH mixtures of increasing polarity. Fractions 1 and 2 were obtained. Chromatography over Sephadex LH-20 of fraction 1 using $CHCl_3$–CH_3OH (50:50) and subsequent rechromatography produced compounds **1–3**; of fraction 2 using CH_3OH, compound **4**.

Yields of compounds **1–4** per weight of total extract were 0.17, 0.12, 0.13, and 0.12%, respectively.

2-Amino-3*H*-phenoxazin-3-one (1) is a red crystal, very soluble in CH_3OH. It acquires a dark brown color on reaction with anisaldehyde. IR spectrum (KBr, ν, cm^{-1}): 3400 (NH_2), 1588 (C=O). ESMS: $[M+H]^+$ 213, $[M-H]^-$ 211. ^{13}C NMR: 180.2 (C-3), 147.3(C-4a), 141.9 (C-5a), 133.7 (C-9a), 148.2 (C-10a) ppm.

2-Methylamino-3*H*-phenoxazin-3-one (2) is a brown-red needle crystal, very soluble in CH_3OH. Reaction with anisaldehyde imparted a brown color. IR spectrum (KBr, ν, cm^{-1}): 3371 (NH), 1579 (C=O). ESMS: $[M+H]^+$ 227, $[M-H]^-$ 225. ^{13}C NMR: 179.8 (C-3), 149.4 (C-4a), 141.9 (C-5a), 133.8 (C-9a), 148.0 (C-10a) ppm.

2-Acetylamino-3*H*-phenoxazin-3-one (3) is an orange needle crystal, very soluble in CH_3OH and CH_3CH_2OH. Reaction with anisaldehyde imparted a dark brown color. IR spectrum (KBr, ν, cm^{-1}): 3343 (NH), 1676 (C=O), 1586 (C=O). ESMS: $[M+Na]^+$ 277, $[M-H]^-$ 253. ^{13}C NMR: 179.7(C-3), 149.4(C-4a), 143.3(C-5a), 134.0(C-9a), 137.1(C-10a) ppm.

Phenazine-1-carboxylic acid (4) is a yellow needle crystal, very soluble in CH_3OH and CH_3CH_2OH. Reaction with anisaldehyde imparted a dark color. IR spectrum (KBr, ν, cm^{-1}): 3430 (OH), 1740 (C=O). ESMS: $[M+H]^+$ 225, $[M-H]^-$ 223. ^{13}C NMR: 143.4 (C-4a), 144.1 (C-5a), 140.1 (C-9a), 139.9 (10a) ppm.

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