ORIGINAL ARTICLE

New phenazine analogues from *Streptomyces* sp. IFM 11694 with TRAIL resistance-overcoming activities

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Two new phenazine derivatives, aotaphenazine (1) and 5,10-dihydrophencomycin (2), were isolated from the ethyl acetate extract of *Streptomyces* sp. IFM 11694. In addition, the known 1-phenazinecarboxylic acid (3), phencomycin (4) and 1,6-phenazinedicarboxylic acid (5) were identified. The structures of the isolated compounds (1 – 5) were characterized by spectroscopic methods including NMR and mass spectrometry data. Compound 1 showed the ability to overcome tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistance at concentration of 12.5 μM. Aotaphenazine (1) enhanced the levels of apoptosis inducing proteins DR4, DR5, p53 and also decreased the levels of cell survival protein Bcl-2 in TRAIL-resistant human gastric adenocarcinoma (AGS) cells in a dose-dependent manner.

INTRODUCTION

Actinomycetes are one of the most efficient groups of secondary metabolite producers and are a focal point in the search for novel anticancer agents.[1] Therefore, they are widely recognized as a promising resource that can potentially supply new drug discovery lead or seed compounds. As part of our ongoing effort to isolate and identify novel bioactive secondary metabolites from different actinomycetes, our laboratory reported the isolation of chromomycins A2 and A3,[2] naphthopyridazone alkaloid,[3] azaquinone-phenylhydrazone,[4] pyranonaphthoquinones and different phenazine derivatives[5–8] from *Streptomyces* sps. These compounds showed the ability to overcome TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) resistance in human gastric adenocarcinoma cells.

In our continuing search for bioactive compounds from actinomycetes, we investigated the crude extract of the *Streptomyces* sp. IFM 11694 because it exhibited relatively non-polar blue band on TLC. This band gave a dark brown color after spraying with the Dragendorff's reagent. We herein report fermentation, isolation, structure elucidation and biological activities of two new phenazines designated as aotaphenazine (1) and 5,10-dihydrophencomycin (2).

RESULTS AND DISCUSSION

Fermentation of *Streptomyces* sp. IFM 11694 on Waksman medium[9] at 28 °C for 7 days followed by extraction, evaporation and working up of the crude extract led to the isolation of two new compounds (1 and 2; Figure 1) along with the known 1-phenazinecarboxylic acid (3),[10] phencomycin (4)[11] and 1,6-phenazinedicarboxylic acid (5).[5]

Compound 1 was obtained as a blue solid with long-wavelength UV absorptions maxima at 546, 347, 287 and 231 nm. The IR spectrum of 1 suggested absorption bands of NH (3216 cm^{-1}) and carbonyl groups

(1722 cm^{-1}). The molecular formula was established as $C_{21}H_{12}N_2O_3$ by the (+)-HRESI-MS *m/z* 363.0767 [M+Na]$^+$ (calcd 363.0746, Δ+2.1 mmu). The ^{1}H NMR of 1 (Table 1, Supplementary Figures S1 and S2) and ^{1}H-^{1}H-COSY allowed the construction of three spin systems (Figure 2): H-5–H-6, [(δ$_H$ 7.37 (d, 9.1) and 7.27 (d, 9.1)]; H-8–H-9–H-10–H-11 [δ$_H$ 7.97 (d, 8.0), 7.58 (t, 8.0), 7.71 (t, 8.0) and 7.56 (d, 8.0)]; and phenyl protons H-14/H-18 [δ$_H$ 7.36 (d, 7.9)], H-15/H-17 [δ$_H$ 7.44 (t, 7.9)] and H-16 [δ$_H$ 7.38 (t, 7.9)]. In addition, the ^{1}H NMR spectrum showed one broad singlet assigned as NH group [δ$_H$ 13.90 (brs)]. The ^{13}C NMR spectrum of 1 (Supplementary Figure S3), aided by HMQC, revealed 21 signals, which were assigned to 2 carbonyl groups [δ$_C$ 165.3 and 161.5], 11 sp^2 methine carbons [δ$_C$ 133.2, 133.0, 131.7, 130.9 (2C), 130.1, 128.6 (2C), 128.4, 127.6 and 117.4] and 8 sp^2 quaternary carbons [δ$_C$ 148.6, 142.2, 138.6, 138.2, 132.8, 128.1, 115.4 and 90.1]. The HMBC correlations of H-11 to C-9 and C-7a; H-10 to C-11a and C-8; H-9 to C-11 and C-7a; and H-8 to C-11a and C-10 confirmed the structure of benzene ring in 1. The HMBC correlations from H-6 to C-12a and from H-5 to C-12b and C-6a determined the positions of C-12a, C-12b and C-6a relative to the aromatic protons H-6 and H-5. The presence of carboxylic anhydride moiety in 1 was confirmed through constant time inverse-detection gradient accordion rescaled (CIGAR)-HMBC experiment.[12]

The long-range HMBC correlations from H-5 (δ$_H$ 7.37) and H18/H14 (δ$_H$ 7.36) to C-3 (δ$_C$ 165.3) were observed (Supplementary Figures S10 and S11). In addition, cross-peaks from H-6 (δ$_H$ 7.27) to C-1 (δ$_C$ 161.5) through ^5J coupling were detected. These correlations fix the positions of C-1 and C-3 as shown in Figure 2. The HMBC couplings of H18/H14 and H-5 to C-4 revealed the connectivity of the phenyl ring to C-4. By considering the presence

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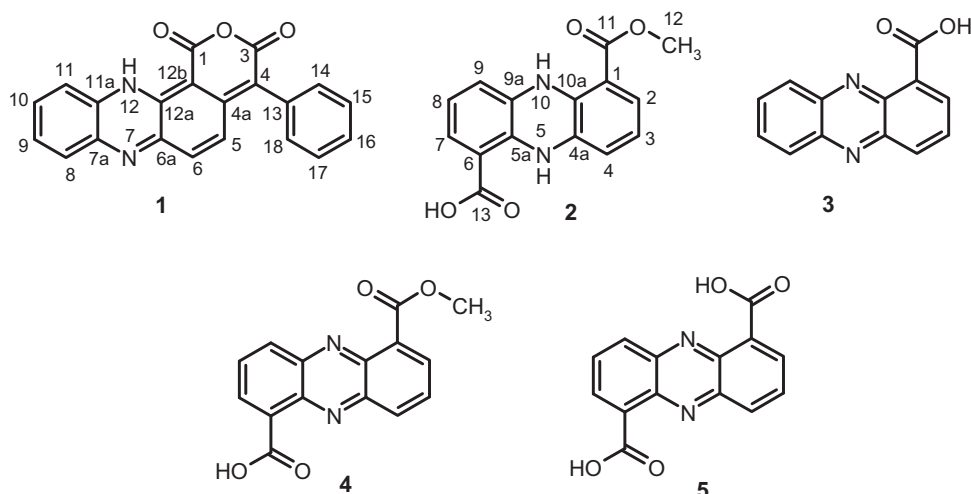MS Abdelfattah *et al*

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Figure 1 Structures of isolated compounds **1**–**5**.

Table 1 ^{13}C (150MHz) and ^{1}H (600 MHz) NMR spectroscopic data for compounds 1 and 2

|	**1**[a]		**2**[b]	
Position	δ_C (p.p.m.)	δ_H, mult. (J in Hz)	δ_C (p.p.m.)	δ_H, mult. (J in Hz)
1	161.5		109.3	
2			122.1	6.88, d (8.0)
3	165.3		119.6	6.30, t (8.0)
4	115.4		115.7	6.36, d (8.0)
4a	142.2		137.3	
5	133.2	7.37, d (9.1)		8.58, s
5a			132.1	
6	131.7	7.27, d (9.1)	108.3	
6a	148.6			
7			123.0	6.90, d (8.0)
7a	138.6			
8	130.1	7.97, d (8.0)	119.4	6.29, t (8.0)
9	127.6	7.58, t (8.0)	115.8	6.41, d (8.0)
9a			137.4	
10	133.0	7.71, t (8.0)		8.81, s
10a			132.5	
11	117.4	7.56, d (8.0)	167.3	
11a	128.1			
12			51.8	3.07, s
12a	138.2			
12b	90.1			
13	132.8		169.1	12.90, brs
14	130.9	7.36, d (7.9)		
15	128.6	7.44, t (7.9)		
16	128.4	7.38, t (7.9)		
17	128.6	7.44, t (7.9)		
18	130.9	7.36, d (7.9)		
12-NH		13.90, brs		

[a]Data were measured in CDCl$_3$.
[b]Data were measured in DMSO-d_6.

of phenazine moiety and the NOE correlation between the NH group [δ_H 13.90 (brs)] and the aromatic proton H-11 [δ_H 7.56 (d, 8.0)] (Supplementary Figure S12), structure of compound **1** was assigned as
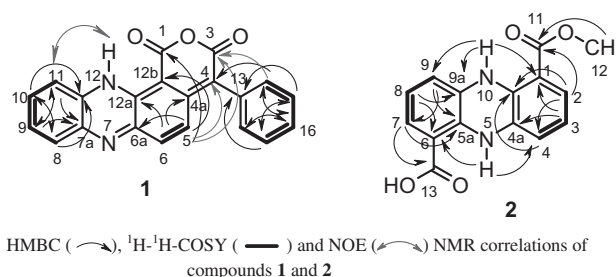
Figure 2 HMBC (⌒→), ^{1}H-^{1}H-COSY (—) and NOE (↙→) NMR correlations of compounds **1** and **2**. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

shown in Figure 2. Finally, the structure was supported by comparison of the spectral data with Dermacozine F.[13] According to the best of our knowledge; aotaphenazine (**1**) is one of the rare natural blue phenazine pigments. Pyocyanin (5-*N*-methylphenazine-1-one) was the first blue phenazine derivative described by Fordos.[14]

Compound **2** was obtained as a yellow solid. It gave a yellow fluorescent spot under UV light at 366 nm. The molecular weight of **2** was determined as 284 Dalton based on the (+)-ESI-MS. The molecular formula was estimated as C$_{15}$H$_{13}$N$_2$O$_4$ by HRESIMS *m/z* 285.0921 [M+H]$^+$ (calcd 285.0875, Δ+4.6 m.m.u.). The UV spectrum recorded in MeOH (maxima at 447, 366, 244 and 215 nm) suggested a phenazine nucleus.[15] The IR absorption data showed the presence of hydroxy (3736 cm^{-1}), NH (3326 cm^{-1}) and carbonyl groups (1736 cm^{-1}). The ^{1}H NMR data of **2** (Table 1, Supplementary Figures S12 and S13) recorded in DMSO-d_6 indicated one broad peak for hydroxyl group [δ_H 12.90 (brs)], two singlets assigned as two NH groups [δ_H 8.81(s) and 8.58 (s)], six aromatic protons [between δ_H 6.29 and 6.90] and one singlet attributable to –OCH$_3$ group [δ_H 3.07 (s)]. From the ^{1}H-^{1}H COSY, the aromatic protons of **2** constituted two ABC systems (Figure 2). The ^{13}C NMR spectrum of **2** (Supplementary Figures S14) showed two carbonyl groups (δ_C 169.1 and 167.3), six sp^2 methine carbons (δ_C 123.0 122.1, 119.6, 119.4, 115.8 and 115.7), six quaternary sp^2 carbons (δ_C 137.4, 137.3, 132.5, 132.1, 109.3 and 108.3) and one methoxy group (δ_C 51.8). The substitution pattern of the individual benzene ring of **2** is well-established by HMBC correlations

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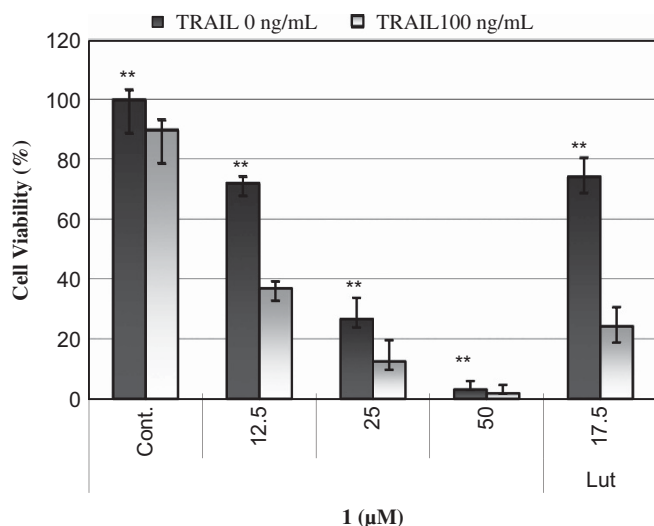
Figure 3 Effects of the isolated compound **1** on the cell viability of AGS cells in the presence and absence of TRAIL. The s.e. bar represents the means ($n=3\pm$s.d.). The significance of differences was determined with Student's *t*-test (**$P<0.01$ vs control for luciferase activity). A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Figure 4 Western blot analysis of DR4, DR5, p53 and Bcl-2 protein levels in AGS cells after 24-h treatment with **1**. The AGS cells were seeded in culture plate for 24 h and then treated with the indicated concentration of **1** for 24 h and then analyzed by western blotting. The β-actin was used as an internal control.

(H-2/C-11, C-10a, C-4; H-3/C-4a, C-1; H-4/C-10a, C-2; H-7/C-13, C-9, C-5a; H-8/C-9a, C-6; H-9/ C-7, C-5a and H$_3$-12/C-11), and due to the molecular formula both parts should be connected by NH bridges forming dihydrophenazine system. The presence of 1,6-disubstituted ring system in **2** was established by the HMBC couplings of the NH-10 (δ_H 8.81) and NH-5 (δ_H 8.58) to the C-9, C-1, and C-6 and C-4, respectively. The structure of **2** was also confirmed by comparing the spectral data with the structurally related 5,10-dihydrophencomycin methyl ester.[16] From literature, compounds containing dihydrophenazine moiety are not common in nature. A few examples have been isolated from bacteria such as 5,10-dihydro-4,9-dihydroxy- phencomycin methyl ester[17] and endophenazine D.[18]

Biological activity

We evaluated the bioactivity of aotaphenazine (**1**) for their activity in overcoming TRAIL resistance in AGS cells. This cell line has been widely used as a model system for evaluating cancer cell apoptosis and is reported to be refractory to apoptosis induction by TRAIL.[19] To assess the effects of compounds **1** and **2** on cell viability, in the presence and absence of TRAIL, AGS cells were treated with the indicated agents and subjected to fluorometric microculture cytotoxicity assay method (FMCA). Luteolin was used as a positive control at 17.5 μM.[20] The assay results (Figure 3) showed that compound **1** at 12.5 μM exhibited 35.1% decreases in cell viability in the presence of TRAIL (100 ng ml^{-1}) compared with in the absence of TRAIL. Compound **2**, however, did not show TRAIL-resistance overcoming activity. These results suggest that aotaphenazine (**1**) had a synergistic effect in combination with TRAIL against AGS cells. To assess the molecular mechanism underlying the synergistic induction of apoptosis by the combined treatment of compound **1** and TRAIL in AGS cells, we found that western blotting analysis after 24 h treatment of AGS cells with **1** induced a dose-dependent increase of DR4 and DR5 protein levels (Figure 4). The p53 protein also plays a critical role in response to various cellular stresses by modulating
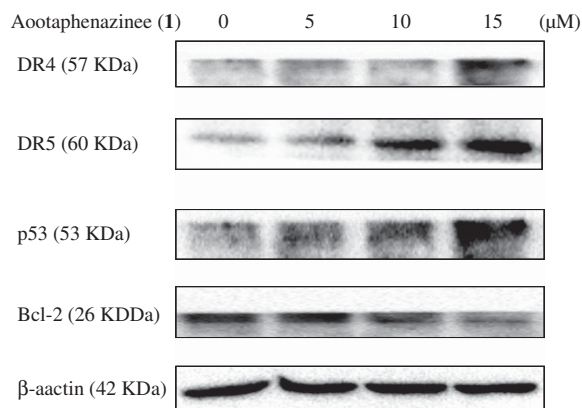
transformation, cell growth, DNA synthesis and repair, differentiation and apoptosis.[21] It acts as a transcriptional factor of the proteins involved in the TRAIL signal.[22] Accordingly, the protein level of p53 was investigated by western blot analysis in the present study. As shown in Figure 4, the treatment of AGS cells with **1** for 24 h increased p53 protein levels in a dose-dependent manner. In addition, **1** significantly triggered the downregulation of cell survival protein Bcl-2 by increasing the concentration of **1**.

Overall, our search for new bioactive natural products from actinomycetes yielded the terrestrial *Streptomyces* sp. IFM 11694, which produced two new phenazine derivatives named aotaphenazine (**1**) and 5,10-dihydrophencomycin (**2**). Aotaphenazine (**1**) showed TRAIL-resistance overcoming activity in AGS cells at concentration of 12.5 μM. To understand the molecular mechanism underlying apoptosis produced by combined treatment of TRAIL and compound **1** in AGS cells, we investigated changes in the levels of the death receptor pathway-related proteins. Western blotting analysis showed that **1** upregulate DR4 and DR5 protein levels, but downregulate the levels of cell survival protein Bcl-2 in a dose-dependent manner.

METHODS

General experimental procedures

The NMR spectra were recorded on a ECZ600 spectrometer (JEOL, Tokyo, Japan) with deuterated solvents, the chemical shifts of which were used as an internal standard. Low resolution ESI-MS were obtained on a LCMS-2020 spectrometer (Shimadzu, Kyoto, Japan). HR-ESI-MS were recorded on AccuTOF-T100LP mass spectrometer (JEOL). IR spectra were measured on attenuated total reflection on a FT-IR 230 spectrophotometer (JASCO, Tokyo, Japan). UV spectra were measured on a Shimadzu UV mini-1240 spectrometer (Shimadzu). Preparative reversed-phase HPLC was performed using COSMO-SIL Cholester Packed Column (Nacalai Tesque, Tokyo, Japan).

Identification of the microbial strain

The *Streptomyces* sp. IFM 11694 was isolated from a soil sample collected from Namegata city, Ibaraki prefecture, Japan. The identification was carried out by Professor Tohru Gonoi at the Medical Mycology Research Center, Chiba University, where a voucher specimen is deposited with code IFM 11964. Identification of the strain was carried out by sequence analysis of 16S ribosomal RNA gene using the DDBJ-BLAST search. The strain was found to have 98.7% similarity with the 16S ribosomal RNA of *Streptomyces hyderabadensis*.

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Fermentation and isolation of compounds

The isolated strain was cultivated on Waksman agar plates at 28 °C for 3 days. To prepare the seed culture, chunks of well grown agar plate were used to inoculate 5×500 cm^3 Sakaguchi flasks each containing 100 ml of Waksman liquid medium consisting of glucose (2.0 g per 100 ml), meat extract (0.5 g per 100 ml), peptone (0.5 g per 100 ml), dried yeast (0.3 g per 100 ml), NaCl (0.5 g per 100 ml) and CaCO$_3$ (0.3 g per 100 ml). The cultures were grown at 28 °C for 3 days with reciprocal shaking at 200 r.p.m.. An aliquot of seed culture (20 ml) was subsequently used to inoculate 16×3 l flasks each containing 750 ml of liquid Waksman medium. Fermentation was continued at 28 °C with shaking (200 r.p.m.) for 7 days. The culture broth (12 l) was centrifuged at 6000 r.p.m. for 20 min then extracted four times with ethyl acetate. The mycelial cake was extracted three times with acetone. After removal of acetone, the aqueous solution was extracted three times with ethyl acetate. The extracts from water phase and mycelial cake were combined together to yield 5.32 g of solid crude extract.

The crude extract was fractionated using silica gel flash column chromatography through a stepwise gradient solvent system of increasing polarity starting from 100% CHCl$_3$ to 100% MeOH to obtain six fractions. Fraction **II** (980 mg) was fractionated using silica gel column chromatography (φ 15×600 mm) eluted with CHCl$_3$: MeOH = 98:2 to give three sub-fractions named **IIa** (250 mg), **IIb** (485 mg) and **IIc** (245 mg). Purification of sub-fractions **IIb** by preparative thin layer chromatography (5 plates, 20×20 cm, CHCl$_3$/1% MeOH) followed by preparative HPLC (Develosil ODS HG-5, 10×250 mm; Nomura Chemical, Seto, Japan) afforded compound **1** (6.4 mg, $R_t = 28.2$ min) as a blue solid. The mobile phase was a gradient of CH$_3$CN/H$_2$O at flow rate of 5.0 ml min^{-1}. Sub-fraction **IIc** was purified two times by Sephadex LH-20 (φ 15×600 mm, CHCl$_3$: MeOH = 3:2) followed by preparative HPLC, using CH$_3$CN/H$_2$O as mobile phase, to yield compound **2** (10.2 mg, $R_t = 16.1$ min) as a yellow solid. Fraction **III** (480 mg) was applied two times to Sephadex LH-20 (φ 15×600 mm) eluted with CHCl$_3$/MeOH (3:2) to give compound **3** (95.2 mg) as a yellow–green solid. Fraction **IV** (220 mg) was chromatographed on silica gel column chromatography, eluted with CHCl$_3$/MeOH (85:15), followed by preparative thin layer chromatography (4 plates, 20×20 cm, CHCl$_3$/15% MeOH) to afford **4** (124.9 mg) as a yellow–green solid. Compound **5** (65.8 mg) was precipitated from fractions **V** and **VI** as a green solid.

Cell culture and FMCA

The AGS cells were derived from the Institute of Development, Aging and Cancer, Tohoku University, Japan.[23] AGS cells were also seeded in a 96-well culture plate (6×10^3 cells per well) in 200 μl of RPMI medium containing 10% fetal bovine serum. Cells were incubated at 37 °C in a 5% CO$_2$ incubator for 24 h. Test samples with or without TRAIL (Wako Pure Chemical Industries, Osaka, Japan, 100 ng ml^{-1}) at different doses were added to each well. After 24 h incubation, cells were washed with phosphate-buffered saline (PBS), and 200 μl of PBS containing fluorescein diacetate (10 μg ml^{-1}; Wako) was added to each well. The plates were incubated at 37 °C for 1 h and fluorescence was detected after 1 h.

Western blot analysis

The lysates of AGS cells were prepared using lysis buffer (Tris-HCl (20 mM, pH 7.4), NaCl (150 mM), Triton X-100 (0.5%), sodium deoxycholate (0.5%), EDTA (10 mM), sodium orthovanadate (1 mM) and NaF (0.1 mM)), containing protease inhibitor cocktail (1%, Nacalai Tesque). Proteins were separated on a 12.5% SDS–polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). After blocking with 5% skimmed milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h at room temperature, the membrane was incubated at room temperature with primary antibodies (anti-DR4 (1:750, Sigma-Aldrich); anti-DR5 (1:750, Sigma-Aldrich); anti-p53 (1:4000, Sigma-Aldrich, St. Louis, MO, USA); anti-Bcl-2 (1:1000, Sigma-Aldrich) and anti-β-actin (1:4000, Sigma-Aldrich) overnight at room temperature). The anti-β-actin antibody served as an internal control. After being washed with TBST, the membrane was incubated with either horseradish peroxidase conjugated anti-mouse IgG (1:4000, GE Healthcare, Little Chalfont, UK) or anti-rabbit (1:4000, Jackson

Immuno Research, West Grove, PA, USA) for 1 h at room temperature. After further washing with TBST, immunoreactive bands were detected using the ECL Advanced Western detection system (GE Healthcare) or Immobilon Western chemiluminescent HRP substrate (Merck Millipore, Billerica, MA, USA) using Molecular Imager ChemiDoc XRS+ (Bio-Rad Laboratories).

Aotaphenazine (1). Blue solid; UV (MeOH) λ_{max} (log ε) 546 (3.3), 347 (3.5), 287 and 231 (3.3) nm; IR ν_{max} (attenuated total reflection) *ca.* 3216, 2358, 1722, 1643, 1092 and 843 cm^{-1}; ^{1}H and ^{13}C NMR data in Table 1; (+)-HRESIMS *m/z* 363.0767 [M+Na]$^+$ (calcd for C$_{21}$H$_{12}$N$_2$O$_3$Na, 363.0746).

5,10-dihydrophencomycin (2). Yellow solid; UV (MeOH) λ_{max} (log ε) 447 (3.2), 366 (3.9), 244 (4.3) and 215 (4.7) nm; IR ν_{max} (attenuated total reflection) *ca.* 3736, 3326, 2923, 1736, 1365 and 1092 cm^{-1}; ^{1}H and ^{13}C NMR data in Table 1; (+)-HRESIMS *m/z* 285.0921 [M+H]$^+$ (calcd for C$_{15}$H$_{13}$N$_2$O$_4$, 285.0875).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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