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NOTE

Diastaphenazine, a new dimeric phenazine from an endophytic *Streptomyces diastaticus* subsp. ardesiacus

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Phenazines are a large group of natural and synthesized nitrogen-containing heterocycles, including more than 100 different compounds of natural origin.[1] The group of phenazine compounds exhibit a broad range of biological activities, such as antibacterial, antimalarial, antitumor and antiparasitic activities.[2] Natural phenazines are mainly isolated from different microorganisms, including *Pseudomonas*, *Streptomyces* and miscellaneous genera.[2] Even though large numbers of phenazines have been isolated from natural sources,[1,3–6] the dimeric phenazines were relatively rare. Only esmeraldines A–B,[7] phenazostatins A–D,[8–11] izumiphenazines A–B[12] and phenazinolines A–E[13] were found till now and all of them were isolated from streptomycete. In the course of screening for new bioactive compounds from endophytic actinomycete sources, a new cytotoxic dimeric phenazine, diastaphenazine (**1**), along with a known compound, izumiphenazine C (**2**) (Figure 1) was isolated from the fermentation broth of *Streptomyces diastaticus* subsp. ardesiacus. In this paper, we report the fermentation, isolation, structural elucidation, and cytotoxic and antibacterial activities of **1**.

The producing organism was isolated from surface-sterilized tissue of *Artemisia annua* collected from Xishuangbanna, Yunnan province, China. The strain was identified as *Streptomyces diastaticus* subsp. ardesiacus according to the phylogenetic analysis. The genomic DNA of the strain was extracted and used as a template for PCR-mediated amplification of 16S ribosomal DNA. The amplicons were used for sequencing and the resulting 16S ribosomal DNA sequence was compared with those of the type strains of validly published species in the genus *Streptomyces*. Phylogenetic analysis showed that the strain shared a higher 16S ribosomal RNA gene sequence similarity with the closely related strain *S. diastaticus* subsp. ardesiacus NRRL B-1773^T (accession number DQ026631). Meanwhile, the strain was examined for a number of key phenotypic properties known to be of value in streptomycete systematics, and the presence of L, L-diaminopimelic acid in the peptidoglycan together with its colonial characteristics supported its assignment to the genus *Streptomyces*.

A slant culture of the strain was inoculated into 500 ml Erlenmeyer flasks containing 100 ml of seed medium composed of yeast extract 0.4%, glucose 0.4%, malt extract 0.5%, multiple vitamins solution 0.35 ml l^{-1}, pH 7.2 with no adjustment and cultured for 2 days at 28 °C on a rotary shaker at 220 r.p.m. This seed culture was used to inoculate the fermentation medium with 10% volume. The fermentation was carried out in 1000 ml Erlenmeyer flasks containing 300 ml of fermentation medium containing starch 2.4%, beef extract 0.3%, glucose 0.1%, yeast extract 0.5%, peptone 0.3%, $CaCO_3$ 0.4% at pH 7.0 with no adjustment and cultured for 7 days at 28 °C on a rotary shaker at 220 r.p.m. for upscale fermentation.

The completed fermentation broth (70 l) was separated into filtrate and mycelium by centrifugation. The culture filtrate was extracted with ethyl acetate and the mycelium was extracted with acetone. After concentrating the ethyl acetate and acetone-soluble portions under reduced pressure, the two portions were combined and dried to yield 54 g extract. The dried extract was then separated by silica gel column chromatography ($CHCl_3$-MeOH, gradient 40:1–1:1 (v/v)) into seven fractions. Fraction 4 was subjected to gel chromatography on Sephadex LH-20 (Amersham Pharmacia Biotech, Uppsala, Sweden) (MeOH) to produce four fractions. Fraction 4.2 was then purified by silica gel column chromatography ($CHCl_3$-MeOH 30:1 (v/v)) and yielded 10 mg of compound **2**. Fraction 5 was applied to gel chromatography on Sephadex LH-20 (MeOH) to produce three subfractions. Fraction 5.1 was further separated by silica gel column chromatography ($CHCl_3$-MeOH 30:1 (v/v)) to yield 60 mg of a semipure material. Additional purification by gel chromatography on Sephadex LH-20 (MeOH) delivered 15 mg of compound **1**.

Compound **1** was obtained as a yellow amorphous powder. $[\alpha]_D^{20}$ −1248 (c 0.11, dimethyl sulfoxide). UV (MeOH) λ_{max} (log ε) 371 (3.69), 250 (4.26) nm. IR (KBr) ν_{max} 3427, 1708, 1627, 1579 cm^{-1}. ESI-MS *m/z* 437 $[M+H]^+$, 459 $[M+Na]^+$, 895 $[2M+Na]^+$, 435 $[M-H]^-$, 471 $[M+Cl]^-$, 893 $[2M+Na-2H]^-$. High resolution

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electron impact-MS *m/z* 436.1168 (calcd for $C_{25}H_{16}N_4O_4$, 436.1172). For ^1H and ^{13}C NMR data see Table 1.

The molecular formula of **1** was established as $C_{25}H_{16}N_4O_4$ by high resolution electron impact-MS. The IR spectrum of **1** indicated the presence of a hydroxyl group (3427 cm^{-1}), a carboxyl group (1708 cm^{-1}) and aromatic rings (1627, 1579 cm^{-1}). The ^1H NMR spectrum of **1** (Table 1) showed eight aromatic proton signals at $\delta = 8.43$ (1H, d, $J = 8.0$ Hz), 8.40 (1H, d, $J = 8.0$ Hz), 8.11 (1H, d, $J = 8.8$ Hz), 8.00 (1H, t, $J = 8.0$ Hz), 7.93 (1H, d, $J = 8.8$ Hz), 7.46 (1H, t, $J = 8.0$ Hz), 7.25 (1H, d, $J = 8.0$ Hz), 7.05 (1H, d, $J = 8.0$ Hz) as well as three methine signals at $\delta = 5.06$ (1H, t, $J = 4.5$ Hz), 4.58 (1H, d, $J = 4.5$ Hz) and 4.36 (1H, brt). In addition, a methylene AB signals appeared at $\delta = 3.68$ (1H, dd, $J = 17.6$, 4.9 Hz) and 3.05 (1H, d, $J = 17.6$ Hz). The ^{13}C NMR data suggested twenty-five carbons altogether, and the DEPT experiment showed eight aromatic methines ($\delta = 133.2$, 133.0, 130.7, 129.8, 128.8, 128.6, 117.7 and 111.7), three methines ($\delta = 75.0$, 54.9 and 40.2) and one methylene ($\delta = 31.9$), respectively. Furthermore, thirteen quaternary carbons were presented, including one carboxyl carbon ($\delta = 166.7$) and twelve aromatic quaternary carbons. ^1H and ^{13}C NMR data suggested that **1** possesses a dimeric phenazine skeleton like those related compounds, phenazinolins A–E.[13] 2D NMR (HMBC, HSQC, COSY and ROESY) correlations suggested two phenazine structural units A and B (Figure 2). The linkage between fragments A and B were determined by HMBC experiments. HMBC correlations were observed between H-8 ($\delta = 7.93$) and C-9′ ($\delta = 54.9$); H-9′ ($\delta = 4.58$) and C-6 ($\delta = 140.4$), C-7 ($\delta = 147.0$), C-5′a ($\delta = 153.9$), C-9′a ($\delta = 152.0$); H-7′ ($\delta = 4.36$) and C-6 ($\delta = 140.4$), C-7 ($\delta = 147.0$), C-5′a ($\delta = 153.9$) showed C-7 linking with C-9′ ($\delta = 54.9$), and C-6 ($\delta = 140.4$) bonding to C-7′ ($\delta = 40.2$) (Figure 2). The planar structure of **1** was also confirmed by ROESY spectrum. NOE correlations between H-4′ ($\delta = 7.25$) and H-6′ ($\delta = 3.05$ and 3.68) confirmed the hydroxyl group was located at C-1′. NOE correlations among H-8′ ($\delta = 5.06$) / H-6′β ($\delta = 3.05$) determine the relative configurations of C-8′. NOE correlations observed between H-8 ($\delta = 7.93$)/H-9′ ($\delta = 4.58$), H-4 ($\delta = 8.43$)/ H-6′β ($\delta = 3.05$) and H-7′ ($\delta = 4.36$) supported the connectivities between units A and B. Therefore, the structure of **1** was determined (Figure 1) and was named as diastaphenazine considering its microbiological origin *S. diastaticus* subsp. ardesiacus.

The known compound izumiphenazine C (**2**) was identified by comparison of the MS, ^1H and ^{13}C NMR data with the reported spectroscopic data.[12]

To evaluate the cytotoxic activity of **1**, we measured its cytotoxic effects on five human tumor cell lines including human large-cell lung carcinoma cell line (H460), human hepatocellular liver carcinoma cell line (HepG2), human colon carcinoma cell line (HCT116), human cervix carcinoma cell line (HeLa) and human gastric carcinoma cell line (BGC-823) with an MTT assay procedure.[14] Compound **1** showed weak cytotoxicity against five human tumor cell lines BGC-823, HeLa, HCT116, HepG2 and H460 with IC_{50} values of 14.9, 28.8, 65.2, 82.5, and >100 μM, respectively. Adriamycin as a positive control showed IC_{50} values 1.48, 0.97, 1.38, 0.50 and 0.98 μM against BGC-823, HeLa, HCT116, HepG2 and H460 cell lines, respectively.

The antimicrobial effects of **1** were also assayed against *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), and

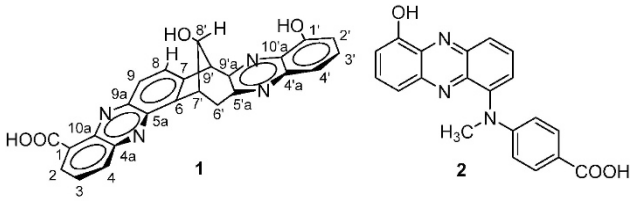

Figure 1 Structures of compounds **1** and **2**.

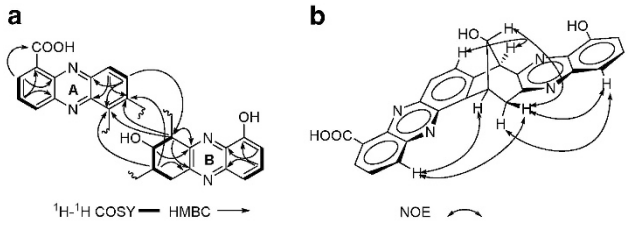

Figure 2 (**a**) COSY and HMBC correlations of units A and B of compound **1**. (**b**) Key NOE correlations of **1**.

Table 1 ^1H (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6) data of compound 1

No.	δc	δ_H	*No.*	δc	δ_H
1	129.8	—	**1′**	153.3	—
2	133.2	8.40 (1H, d, J=8.0 Hz)	**2′**	111.7	7.05 (1H, d, J=8.0 Hz)
3	130.7	8.00 (1H, t, J=8.0 Hz)	**3′**	129.8	7.46 (1H, t, J=8.0 Hz)
4	133.0	8.43 (1H, d, J=8.0 Hz)	**4′**	117.7	7.25 (1H, d, J=8.0 Hz)
4a	142.4	—	**4a′**	142.3	—
5a	139.6	—	**5a′**	153.9	—
6	140.4	—	**6′**	31.9	α 3.68 (1H, dd, J=17.6, 4.9 Hz)
					β 3.05 (1H, d, J=17.6 Hz)
7	147.7	—	**7′**	40.2	4.36 (1H, brt)
8	128.6	7.93 (1H, d, J=8.8 Hz)	**8′**	75.0	5.06 (1H, t, J=4.5 Hz)
9	128.8	8.11 (1H, d, J=8.8 Hz)	**9′**	54.9	4.58 (1H, d, J=4.5 Hz)
9a	140.7	—	**9a′**	152.0	—
10a	139.3	—	**10a′**	131.5	—
1-COOH	166.7	—	**1′-OH**		10.33 (1H, brs)
			8′-OH		6.26 (1H, brs)

Abbreviation: DMSO, dimethyl sulfoxide.

Candida albicans (ATCC 10231) using the micro broth dilution method.[15] **1** showed antibacterial activity against *S. aureus* with a MIC value of 64 μg ml^{-1} and was inactive against *E. coli* and *C. albicans* at 128 μg ml^{-1}. Ciprofloxacin and amphothericin B were used as positive controls with MIC values 0.5, 0.12 and 0.5 μg ml^{-1} against *S. aureus*, *E. coli* and *C. albicans*, respectively.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)