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BRIEF COMMUNICATION

New phenoxazinone-related alkaloids from strain *Streptomyces* sp. KIB-H1318

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Abstract

Chemical investigation of a strain *Streptomyces* sp. KIB-H1318 isolated from soil sample led to the discovery of three new phenoxazinone-related alkaloids **1**–**3**, as well as two known analogs exfoliazone (**4**) and viridobrunnine A (**5**). Their structures were determined on the basis of extensive spectroscopic analysis. The antimicrobial activity and cytotoxicity of the isolates were assayed. Exfoliazone and viridobrunnine A exhibited minor antibacterial activity against *Escherichia coli* ATCC 8099, *Bacillus subtilis* ATCC 6633, and *Staphylococcus aureus* ATCC 6538. Compound **2** exhibited low cytotoxicity against two human cancer cell lines HeLa and SW480 with the IC_{50} values of 36.8 and 37.8 μM, respectively.

Among all known natural products producers, microorganisms represent a prolific source of structurally novel and biologically active metabolites which not only promise significant advances in chemistry and biochemistry, but also continue to serve an important role in the discovery and development of new therapeutic agents. Therefore, there is a general recognition that microbial natural product research should be expanded significantly [1]. With the aim of discovering new biologically active agents from soil and endophytic actinomycetes [2–4], we isolated a *Streptomyces* strain KIB-H1318 from soil sample collected in Yuxi, Yunnan province, China, in 2015. In the pre-fermentation work, we observed that extracts of the strain had distinct UV absorption compared with other strains, based on the physicochemical properties of compounds using HPLC-UV/vis diode array detection screening. Full characterization of metabolites from KIB-H1318 was enabled by large scale cultivation of this strain. Subsequent chemical investigations of the ethyl acetate extract of fermentation broth and mycelia of strain KIB-H1318 led to the identification of three new phenoxazinone-related alkaloids **1**–**3** and two known analogs exfoliazone (**4**) and viridobrunnine A (**5**) (Fig. 1). In this report, we describe the isolation, structural elucidation, antimicrobial activity, and cytotoxicity of compounds **1**–**5**.

Compound **1** has a molecular formula of $C_{20}H_{15}N_3O_5$ as determined by HRESIMS (*m/z* 376.0935 $[M\text{-}H]^-$, calcd 376.0939), requiring 15 degrees of unsaturation. Its IR spectrum showed absorption bands of benzene skeleton (1630, 1587, and 1528 cm^{-1}), and the broad IR absorption at 3440 cm^{-1} indicated the presence of N–H bond and hydroxyl. The ^1H NMR spectrum (Table 1, Table S3) and COSY correlations exhibited the resonances for eight aromatic protons, of which two singlets at δ_H 6.54 (1 H, s, H-1) and 6.46 (1 H, s, H-4), two ABX spin systems at δ_H 7.62 (1 H, s, H-9), 7.43 (1 H, overlapped, H-7), 7.49 (1 H, overlapped, H-6), and 8.20 (1 H, s, H-2′), 6.91 (1 H, d, $J = 8.4$ Hz, H-5′), 6.95 (1 H, d, $J = 8.4$ Hz, H-6′) were observed. These findings in association with ^{13}C NMR data (Table 1, Table S3) identified 18 aromatic resonances consistent with the molecule containing three aromatic rings δ_C 97.4 (C-1), 143.6 (C-2), 179.9 (C-3), 103.5 (C-4), 149.3 (C-4a), 148.2 (C-10a), 140.9 (C-5a), 115.7 (C-6), 127.6 (C-7), 140.1 (C-8), 125.5 (C-9), 133.6 (C-9a), 129.6 (C-1′), 115.7 (C-2′), 126.5 (C-3′), 144.5 (C-4′), 115.3 (C-5′), 119.6 (C-6′). In the COSY spectrum of compound **1**, correlations between the protons δ_H 5.38 (br s, 1 H) and H-11 were observed

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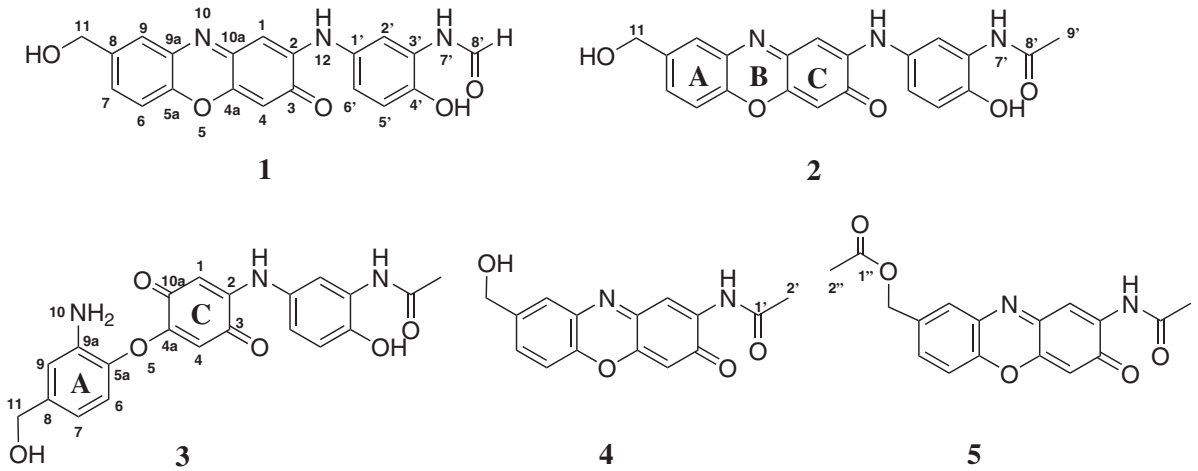

Fig. 1 The structures of compounds **1**–**5**

(Table S3). Additional NMR data were attributed to a formamido (δ_C 160.2, C-8′, δ_H 8.31, 1 H, s, H-8′) and a hydroxymethyl (δ_C 62.1, C-11, δ_H 4.56, 2 H, s, H-11; δ_H 5.38, 1 H, br s, 11-OH). The HMBC correlations from H-1 to C-3, C-4a, C-2, C-10a, from H-4 to C-2, C-10a, C-3, C-4a, from H-6 to C-8, C-9a, C-5a, from H-9 to C-7, C-5a, C-11, from hydroxylmethyl protons H-11 to C-7, C-9, and from NH-12 to C-1, C-3 led to the assignment of a phenoxazinone nucleus (Fig. 2), which is structurally related to exfoliazone except for a different substituent group at NH-12 [5]. The HMBC correlations from NH-12 to C-2′, C-6′, C-1′ and from H-8′ to C-3′ indicated the presence of a 3′-formamido-4′-hydroxyphenyl group. The remaining ^{1}H NMR resonance at 9.73 (br s) was ascribed to 4′-OH. From the foregoing evidence, compound **1** was established as shown in Fig. 1.

The molecular formula of compound **2** was established as $C_{21}H_{17}N_3O_5$ on basis of HRESIMS (*m/z* 390.1104 $[M\text{-}H]^-$, calcd. 390.1095) data, which pointed to 15 double-bond equivalents. The UV spectrum of **2** (208, 247, 284, 442, 516 nm) was similar to those of **1** (206, 247, 283, 439, 514 nm), suggesting that **2** is similar to **1**. Comparing NMR spectra of **2** with **1**, the significant difference was the replacement of formamido C-8′ (δ_C 160.2, δ_H 8.31, s) at 7′-NH in compound **1** by those of acetamido in **2** (δ_C 169.1, C-8′; δ_C 23.8, C-9′, δ_H 2.12, 3 H, s, H-9′). This was further confirmed by the HMBC correlation from H-9′ to C-8′. A 7′-NH proton at 9.40 (s) can be confirmed by ROESY correlations from H-7′ to H-9′ and from H-7′ to H-2′ (Table S4). Thus, the structure of **2** was identified as shown in Fig. 1.

Compound **3** was determined to have a molecular formula of $C_{21}H_{19}N_3O_6$ by HRESIMS (*m/z* 408.1202 $[M\text{-}H]^-$, calcd. 408.1201), requiring 14 degrees of unsaturation. Its IR spectrum showed absorption bands of benzene skeleton (1643, 1575, and 1502 cm^{-1}), and the broad IR absorption at 3425 cm^{-1} indicated the presence of N–H bond and hydroxyl. Similar to compounds **1** and **2**, the ^{1}H and COSY NMR spectra of compound **3** exhibited resonances for eight aromatic protons, of which two singlets at δ_H 5.66 (1 H, s, H-1) and 5.76 (1 H, s, H-4), two ABX spin systems at δ_H 7.26 (1 H, s, H-9), 6.99 (1 H, d, $J = 8.0$ Hz, H-7), 6.89 (1 H, d, $J = 8.0$ Hz, H-6) and 7.84 (1 H, d, $J = 1.8$ Hz, H-2′), 6.89 (1 H, d, $J = 8.0$ Hz, H-5′), 6.93 (1 H, dd, $J = 8.5$, 1.8 Hz, H-6′) were observed. The ^{13}C NMR spectrum showed two resonances 178.9 and 179.5, indicating the presence of *p*-quinone group. It is likely that N–C double bond is cleaved. Through the HRESIMS of **3**, which was 18 mass units (H_2O) larger than **2**, one double-bond equivalent **<2**, suggesting that **3** is a hydrolyzed product of **2**. In the HMBC spectrum of **3**, correlations from H-1 to C-3, C-4a, C-2, from H-4 to C-10a, C-2, C-4a, from H-9 to C-5a, C-7, C-11, C-8, from H-6 to C-8, C-9a, C-5a, C-4a (weak), and from NH-12 to C-1, C-3, C-2′, C-6′ proved the deduction of cleavage C–N bond at B ring of **2**. In the COSY spectrum of compound **3**, correlations between the protons δ_H 5.16 (br s, 1 H, 11-OH) and H-11 were observed. The ^{1}H NMR resonance at 9.36 (s) was ascribed to 7′-NH proton based on δ_H 9.36 interacted with C-2′, C-3′, C-4′, C-8′ in HMBC spectrum (Table S5). Compared with **2**, C-4a is shifted upfield from 149.4 to 146.0, C-5a is shifted downfield from 140.9 to 148.9, C-9a is shifted upfield from 133.6 to 124.9, and C-10a is shifted downfield from 148.2 to 178.9 (Table 1). The spectroscopic feature mentioned above determined the structure of compound **3** (Fig. 1).

Phenoxazinones are olefinically rearranged derivatives of phenoxazine which contain a tricyclic core heterocyclized by nitrogen and oxygen atoms [6, 7]. Previous studies showed that phenoxazine-based analogs possess a wide range of pharmaceutical properties, such as cytotoxic [8],

Table 1 ^{1}H and ^{13}C NMR data of compounds **1–3** (DMSO-d_6, δ in ppm)

No.	1 (DMSO-d_6)		2 (DMSO-d_6)		3 (DMSO-d_6)	
	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	97.4, CH	6.54, s	97.5, CH	6.57, s	94.1, CH	5.66, s
2	143.6, C		143.4, C		148.3, C	
3	179.9, C		179.9, C		179.5, C	
4	103.5, CH	6.46, s	103.5, CH	6.46, s	95.6, CH	5.76, s
4a	149.3, C		149.4, C		146.0, C	
5a	140.9, C		140.9, C		148.9, C	
6	115.7, CH	7.49, overlapped	115.7, CH	7.49, d (8.4)	115.8, CH	6.89, d (8.0)
7	127.6, CH	7.43, overlapped	127.7, CH	7.43, d (8.4)	124.7, CH	6.99, d (8.0)
8	140.1, C		140.1, C		133.7, C	
9	125.5, CH	7.62, s	125.5, CH	7.62, s	120.7, CH	7.26, s
9a	133.6, C		133.6, C		124.9, C	
10-NH_2						8.81, s
10a	148.2, C		148.2, C		178.9, C	
11	62.1, CH_2	4.56, s	62.2, CH_2	4.57, s	62.6, CH_2	4.41, s
11-OH		5.38, br s		5.39, br s		5.16, br s
12-NH		8.67, s		8.67, s		9.29, s
1′	129.6, C		129.9, C		128.5, C	
2′	115.7, CH	8.20, s	116.8, CH	7.90, d (2.3)	117.6, CH	7.84, d (1.8)
3′	126.5, C		126.9, C		126.8, C	
4′	144.5, C		145.2, C		145.9, C	
4′-OH		9.73, br s				10.16, br s
5′	115.3, CH	6.91, d (8.4)	116.0, CH	6.90, d (8.5)	115.5, CH	6.89, d (8.0)
6′	119.6, CH	6.95, d (8.4)	119.8, CH	6.97, dd (8.5, 2.3)	120.5, CH	6.93, dd (8.0, 1.8)
7′-NH				9.40, s		9.36, s
8′	160.2, CH	8.31, s	169.1, C		169.1, C	
9′			23.8, CH_3	2.12, s	23.8, CH_3	2.11, s

antimicrobial [9], antiviral [10], and antiinflamnatory [11] activities. Thus, antimicrobial activity and cytotoxicity were investigated for each compound. First, the antibacterial and antifungal activity of compounds **1–5** were evaluated in a primary screen by the disc diffusion assay against four bacteria, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8099, and *Xanthomonas oryzae* pv. *oryzae* RS105, and eight different pathogenic fungi (Supplementary materials). The tested compounds (30 μg/disc) were significantly less potent than the positive control, kanamycin (10 μg/disc) and nystatin (10 μg/disc). Compound **4** showed minor antibacterial activity against *B. subtilis* and *S. aureus* with inhibition zone 8 mm and 12 mm, respectively. Compound **5** showed minor antibacterial activity against *E.coli*, *B. subtilis*, and *S. aureus* with inhibition zone 7 mm, 8 mm, and 10 mm, respectively (Supplementary Table S1). No antimicrobial activity was observed for compounds **1–3**. None of tested compounds displayed any antifungal activity. The cytotoxicity of compounds **1–5** was tested against cervical cancer HeLa, hepatocellular carcinoma SMMC-7721, lung cancer A-549, breast adenocarcinoma MCF-7 and colon carcinoma SW480 using the MTS method (DDP and Taxol were used as a positive control)[12]. Compound **2** showed minor inhibitory activity against two human cancer cell lines HeLa and SW480 with the IC_{50} values of 36.8 and 37.8 μM, respectively (Supplementary Table S2), while other compounds showed no significant cytotoxic activity.

In summary, we have isolated and characterized three new phenoxazinone-related alkaloids **1–3**, along with two known analogs exfoliazone (**4**) and viridobrunnine A (**5**) from a soil-derived *Streptomyces* sp. KIB-H1318. Although the isolates showed weak cytotoxicity and antimicrobial activity against the selected cancer cell lines and pathogenic strains, the benzene substituents at NH-12 of **1–3** are structurally uncommon and unique in natural phenoxazinone-related alkaloids, which constitutes an important addition to the body of knowledge on phenoxazinone-derived metabolites.

Compound 1 dark brown solid; UV (MeOH) λ_{max} (log ε) 206 (4.23), 247 (4.18), 283 (3.95), 439 (3.93), 514 (3.54) nm; IR (KBr) ν_{max} 3440, 3426, 2956, 2923, 1630, 1587,

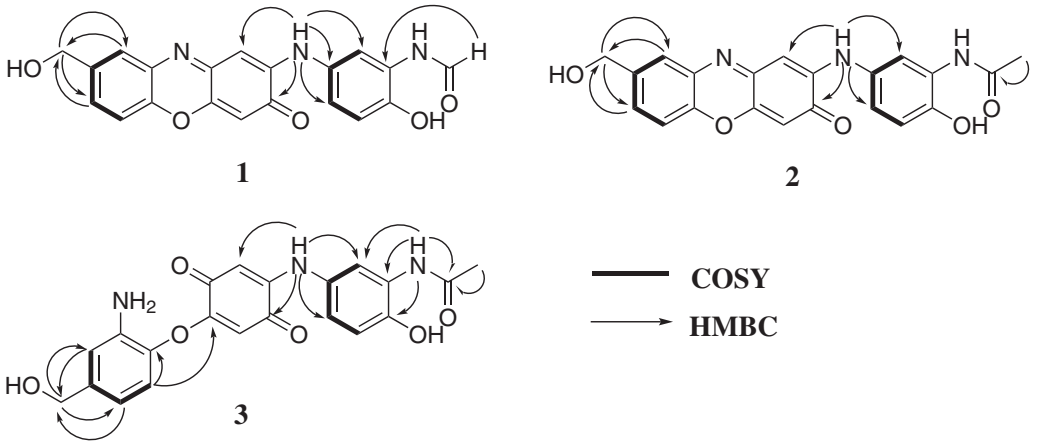

Fig. 2 Key COSY and HMBC interactions of **1**–**3**

1528, 1469, 1433, 1272, 1204, 1044, 849, 812, 594 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) and ^{13}C NMR (DMSO-d_6, 150 MHz) see Table 1. ESIMS *m/z* 376 [M-H]$^{-}$, 412 $[M+Cl]^{-}$; negative ion HRESIMS *m/z* 376.0935 (calcd for $C_{20}H_{15}N_3O_5$ $[M–H]^{-}$, 376.0939)

Compound 2 dark brown solid; UV (MeOH) λ_{max} (log ε) 208 (4.30), 247 (4.31), 284 (4.10), 442 (4.09), 516 (3.70) nm; IR (KBr) ν_{max} 3417, 3087, 2926, 2872, 1640, 1588, 1532, 1512, 1428, 1272, 1205, 1022, 848, 815, 608 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) and ^{13}C NMR (DMSO-d_6, 150 MHz) see Table 1. ESIMS *m/z* 390 $[M–H]^{-}$, 426 $[M + Cl]^{-}$; negative ion HRESIMS *m/z* 390.1104 (calcd for $C_{21}H_{17}N_3O_5$ $[M–H]^{-}$, 390.1095)

Compound 3 dark red solid; UV (MeOH) λ_{max} (log ε) 206 (4.36), 280 (3.87), 415 (3.87) nm; IR (KBr) ν_{max} 3425, 2929, 1643, 1575, 1502, 1445, 1383, 1347, 1283, 1204, 1113, 1024, 819, 594 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) and ^{13}C NMR (DMSO-d_6, 150 MHz) see Table 1. ESIMS *m/z* 408 $[M–H]^{-}$; negative ion HRESIMS *m/z* 408.1201 (calcd for $C_{21}H_{19}N_3O_6$ $[M–H]^{-}$, 408.1202)

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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