New streptophenazines from marine *Streptomyces* sp. 182SMLY

Ying Liang\(^a\), Lu Chen\(^a\), Xuewei Ye\(^a\), Komal Anjum\(^a\), Xiao-Yuan Lian\(^b\) and Zhizhen Zhang\(^a\)

\(^a\)Ocean College, Zhoushan Campus, Zhejiang University, Zhoushan, China; \(^b\)College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, China

**1. Introduction**

Phenazine natural products consist of two benzene rings linked through two nitrogen atoms and are primarily isolated from *Pseudomonas* and *Streptomyces* strains from soil or marine habitats. Phenazines from *Pseudomonas* are mostly simple hydroxyl- and carboxyl-substituted structures, while phenazines isolated from *Streptomyces* usually have more complex structures with one or more C-substituents on the phenazine backbone. Some of phenazines had antibiotic, antitumor, antimalaria, and antiparasitic activities (Laursen & Nielsen 2004).

Streptophenazines are 1,6-disubstituted phenazines with a long alkyl chain at C-6 position. This type of phenazines (streptophenazines A-H) was first isolated from the culture of sponge-derived *Streptomyces* sp. strain HB202 (Mitova et al. 2008). However, the stereochemistry of the side chain of these streptophenazines was not determined therein. The
total synthesis and the absolute configuration of streptophenazines A, B, E, and G were investigated, resulting in the structural revisions of streptophenazines A, B, E, and G to (-)-streptophenazines A, B, E, and G (Yang et al. 2011; Yang et al. 2012). In the structures of (-)-streptophenazines A, B, E, and G, the alkyl chain at C-6 had a hydroxyl at C-1′ and a carbomethoxy at C-2′, instead of the hydroxyl at C-2′ and the carbomethoxyl at C-1′ in the structures of streptophenazines A, B, E, and G (Figure S1). Recently, more new streptophenazine derivatives such as streptophenazines I-L were further obtained from *Streptomyces* strain HB202 (Kunz et al. 2014) and *Streptomyces* sp. BCC21835 (Bunbamrung et al. 2014).

Actinomycetes from the genus *Streptomyces* are still the important resources of novel bioactive natural products (Yang et al. 2014; Zhang et al. 2014; Chen et al. 2015; Yang et al. 2015; Hu et al. 2016; Luo et al. 2016; Ou et al. 2016). Marine actinomycete *Streptomyces* sp. 182SMLY isolated from a sediment sample was recently reported to produce two new anthraquinones of N-acetyl-N-demethylmayamycin and streptoanthraquinone A with potent activity against the proliferation of different glioma cells (Liang et al. 2016). Further chemical investigation on the culture of this marine strain 182SMLY resulted in the isolation of six phenazines including three new streptophenazine analogues. In this study, we report the isolation and structural elucidation of these phenazines and their activities in inhibiting the proliferation of glioma cells and the growth of methicillin-resistant *Staphylococcus aureus* ATCC 43300 and *Escherichia coli* ATCC 25922.

2. Results and discussion

The isolated marine actinomycete 182SMLY was scaled up in the Gause’s liquid medium to 60 L. The culture broth was extracted by EtOAc and the EtOAc extract was fractionated by ODS column chromatography, following by HPLC purification, to yield compounds 1–6 (Figure 1).

Compounds 1–6 had characteristic UV absorption of phenazines at around 250 and 365 nm. Compound 2 was proved to be 1-carbomethoxyphenazine, a simple phenazine (Roemer, 1982; Roemer 1983), while compounds 4 and 6 were 1,6-disubstituted phenazines

*Figure 1. Structures of compounds 1–6.*
with a long alkyl chain at C-6 and were identified as (-)-streptophenazine A (4) and (-)-streptophenazine B (6) based on their NMR spectra, HRESIMS data, negative optical rotation values, and negative Cotton effects at around 251 nm (Mitova et al. 2008; Yang et al. 2011; Yang et al. 2012; Kunz et al. 2014).

Compound 1 had a [M + H]+ ion at m/z 439.2228 and a [M + Na]+ ion at m/z 461.2049, corresponding to a molecular formula of C_{25}H_{30}N_{2}O_{5}. In the HSQC spectrum, six aromatic protons at δ_H 8.28 (dd, J = 8.7, 1.3 Hz, H-2), 7.88 (m, H-3), 8.39 (dd, J = 8.7, 1.3 Hz, H-4), 7.78 (d, J = 6.9 Hz, H-7), 7.84 (m, H-8), and 8.27 (dd, J = 6.9, 1.3 Hz, H-9) were correlated with six carbons at δ_C 130.3 (C-2), 129.6 (C-3), 133.6 (C-4), 129.7 (C-7), 130.8 (C-8), and 132.5 (C-9). These data, in combination with six aromatic quaternary carbons (δ_C 129.8, 131.6, 139.2, 141.1, 141.5, and 144.3) observed in the 13C NMR spectrum, indicated that compound 1 was a 1,6-disubstituted phenazine (Mitova et al. 2008; Yang et al. 2011). The NMR spectra of 1 also displayed signals for two carbomethoxys. The carbomethoxy group at C-1 resonated at δ_C 167.2, 53.0 and δ_H 4.13, while the signals at δ_C 176.0, 51.9 and δ_H 3.68 were contributed to the carbomethoxy at C-2', based on the chemical shifts for these two carbomethoxys in the known streptophenazines (Mitova et al. 2008; Yang et al. 2011; Yang et al. 2012). The 13C NMR spectrum of 1 showed 25 carbon signals, from which 12 were attributed to the phenazine backbone, four to the two carbomethoxys and the remaining nine to the alkyl chain. The structure of this alkyl chain was constructed based on the HSQC and 1H–1H COSY correlations. The absolute configurations of C-1' and C-2' of 1 could be assigned based on the 3J_{1'-2'} coupling constant, the optical rotation value, and the CD data (Yang et al. 2011; Yang et al. 2012; Kunz et al. 2014). A large 3J_{1'-2'} coupling constant of 8.1 Hz, a negative optical rotation value, and a negative Cotton effect at 251 nm indicated that compound 1 had 1'S,2'R configurations (Yang et al. 2011; Yang et al. 2012; Kunz et al. 2014). From the above evidence, the structure of compound 1 was identified as (-)-streptophenazine M, a new member of streptophenazine family.

Compound 3 displayed [M + H]+ ion at m/z 425.2072 and [M + Na]+ ion at m/z 447.1894, 14 mass units lower than that of 1. Detailed 1H NMR spectroscopic comparison of compounds 1 and 3 concluded that the major difference of the 1H NMR signals for these two phenazines was the absence of a signal at δ_H 3.68 (3H, s) for the methoxyl C-10'. This difference, in combination with the HRESIMS data of 3, suggested that compound 3 is an analogue of 1 but had no methoxyl at C-10'. Just like (-)-streptophenazines A (4), B (6), and M (1), the absolute configuration of 3 were also assigned as 1'S, 2'R based on its large 3J_{1'-2'} coupling constant (8.0 Hz), negative optical rotation value, and negative Cotton effect at 251 nm. Therefore, the structure of 3 was elucidated as (-)-streptophenazine N, a new analogue of streptophenazines.

Compounds 5 had the same molecular formula C_{24}H_{28}N_{2}O_{5} as that of (-)-streptophenazine N (3) based on their HRESIMS data, but different retention times from HPLC analysis. Detailed 1H NMR spectroscopic analysis indicated that the structures of phenazines 5 and 3 were different in the terminal substitute of the long alkyl chain. A triplet signal at δ_H 0.84 (3H, t, J = 6.9 Hz) observed in the 1H NMR spectrum of 5 suggested that the long alkyl chain of 5 had a methyl as the terminal group, instead of an isopropyl in 3. The 1'S,2'R absolute configurations of 5 were also deduced from its large 3J_{1'-2'} coupling constant (8.1 Hz), negative optical rotation value, and negative Cotton effect at 251 nm. The structure of 5 was thus identified as (-)-streptophenazine O, a new member of streptophenazine family.
The isolated phenazines 1–6 were tested for their activity in inhibiting the proliferation of four glioma cell lines of C6, U87-MG, SHG-44, and U251 by Sulforhodamine B (SRB) assay (Liang et al. 2016). Doxorubicin (DOX, one of the chemotherapeutic drugs) (Tacara et al. 2013) was used as a positive control. Unfortunately, none of these phenazines showed activity against the tested glioma cells.

Phenazines 1–6 were also assayed for their activity against the growth of methicillin-resistant S. aureus and E. coli using micro broth dilution method (Ye et al. 2015). Norfloxacin was used as a positive control. The result indicated that only (-)-Streptophenazine B (6) exhibited activity against the growth of methicillin-resistant S. aureus with MIC 4.2 μg/mL. The positive control norfloxacin inhibited the growth of both S. aureus and E. coli with MIC values of 20.0 and 10.0 μg/mL, respectively.

3. Experimental

3.1. General experimental procedures

IR spectra were recorded on an AVATAR 370 FT-IR spectrometer (Thermo Nicolet). Optical rotations were measured on a JASCO DIP-370 digital polarimeter. CD spectra were recorded on a JASCO J 715 spectropolarimeter. NMR spectra were acquired on a Bruker 500 spectrometer and chemical shifts were expressed in δ (ppm). HRESIMS data were acquired on an Agilent 6230 TOF LC/MS spectrometer. Octadecyl-functionalized silica gel (ODS, Cosmosil 75C18 Prep, Nacalai Tesque Inc., Kyoto, Japan) was used for column chromatography. HPLC purification was performed on an Agilent 1260 HPLC system with DAD detector. Human glioma U251, U87-MG, SHG-44 cells and rat glioma C6 cells were obtained from the Cell Bank of the Chinese Academy of Sciences. The methicillin-resistant S. aureus ATCC 43300 and E. coli ATCC 25922 were gifts from Professor Zhongjun Ma and Dr Pinmei Wang. Nutrient Broth (NB), Mueller Hinton Broth (MHB), and Gause’s-agar were purchased from Hangzhou Microbial Reagent Co. Ltd. (Hangzhou, China), Thermo Fisher Scientific Inc (USA), and Guangdong Huankai Microbial Science and Technology Co. Ltd. (Guangzhou, China), respectively. Doxorubicin (DOX, ≥ 98.0%) was obtained from Sigma-Aldrich and norfloxacin (98%) from Saen Chemical Technology Co. Ltd. (Shanghai, China).

3.2. Experimental materials

A total of 60.0 L culture broth of strain Streptomyces sp. 182SMLY was prepared according to the previously reported method (Liang et al. 2016).

3.3. Isolation of compounds 1–6

The 60.0 L culture broth was extracted with EtOAc three times and the EtOAc extract was fractionated by column chromatography of ODS successively eluting with 70, 85, and 100% MeOH to yield three fractions (Fr. 1–3). Fr. 2 was further separated by HPLC using an Agilent column (Zorbax SB-C18, 250 × 9.4 mm, 5 μm) with isocratic mobile phase of MeOH and H2O (80:20) at a flow rate of 1.0 mL/min and UV detection wavelength of 256 nm to afford 2 (2.8 mg, \( t_R \) 9.32 min), 3 (0.6 mg, \( t_R \) 12.17 min), 4 (1.8 mg, \( t_R \) 21.72 min), 5 (0.5 mg, \( t_R \) 23.87 min),
and 6 (5.0 mg, $t_R$ 25.36 min). Similarly, Fr. 3 was separated by HPLC using an isocratic mobile phase of MeOH and H$_2$O (85:15) to give 1 (4.8 mg, $t_R$ 28.65 min).

(-)-Streptophenazine M (1): yellow amorphous powder; molecular formula C$_{25}$H$_{30}$N$_2$O$_5$; $t_R$ 28.65 min (85% MeOH in H$_2$O); $[\alpha]_D^{25}$ −12.4 (c 0.50, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 208 (5.91), 252 (5.47), 366 (4.79) nm; ECD (10 mg/L, MeOH) $\lambda_{max}$ ($\Delta\varepsilon$) 251 (−4.6) nm; IR (KBr) $\nu_{max}$ 3416, 2930, 2858, 1733, 1667, 1531, 1438, 1267, 1025, 953 cm$^{-1}$; $^1$H NMR data (500 MHz, in CDCl$_3$) $\delta$H 8.28 (1H, dd, $J$ = 8.7, 1.3 Hz, H-2), 7.88 (1H, m, H-3), 8.39 (1H, dd, $J$ = 8.7, 1.3 Hz, H-4), 7.78 (1H, d, $J$ = 6.9 Hz, H-7), 7.84 (m, H-8), 8.27 (1H, dd, $J$ = 6.9, 1.3 Hz, H-9), 4.13 (3H, s, H-12), 5.11 (1H, d, $J$ = 6.9 Hz, H-1'), 4.12 (3H, s, H-11'); 13C NMR data (125 MHz, in CDCl$_3$) $\delta$C 129.8 (C, C-1), 130.3 (C, C-2), 129.6 (C, C-3), 133.6 (C, C-4), 144.3 (C, C-4a), 139.2 (C, C-5), 144.3 (C, C-6), 129.7 (C, C-7), 141.5 (C, C-9), 141.5 (C, C-9a), 131.6 (C, C-10), 167.2 (C, C-11), 53.0 (CH$_3$, C-12), 75.3 (CH, C-1'), 51.9 (CH$_3$, C-9'); HRESIMS $m/z$ [M + H]$^+$ 439.2228 (Calcd for C$_{25}$H$_{31}$N$_2$O$_5$, 439.2233) and [M + Na]$^+$ 461.2049 (Calcd for C$_{25}$H$_{30}$N$_2$NaO$_5$).

Note: The data with the same labels may be interchanged.

(-)-Streptophenazine N (3): yellow amorphous powder; molecular formula C$_{24}$H$_{28}$N$_2$O$_5$; $t_R$ 12.17 min (80% MeOH in H$_2$O); $[\alpha]_D^{25}$ −3.84 (c 0.50, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 216 (5.00), 252 (4.42), 366 (3.69) nm; ECD (10 mg/L, MeOH) $\lambda_{max}$ ($\Delta\varepsilon$) 251 (−2.0) nm; IR (KBr) $\nu_{max}$ 3418, 2925, 2858, 1732, 1689, 1531, 1438, 1272, 1065, 951 cm$^{-1}$; $^1$H NMR data (500 MHz, in CDCl$_3$) $\delta$H 8.27 (1H, dd, $J$ = 8.7, 1.4 Hz, H-2), 7.88 (1H, m, H-3), 8.36 (1H, dd, $J$ = 8.7, 1.4 Hz, H-4), 7.75 (1H, dd, $J$ = 6.9, 1.4 Hz, H-7), 7.80 (1H, m, H-8), 8.25 (1H, dd, $J$ = 6.9, 1.4 Hz, H-9), 4.13 (3H, s, H-12), 5.43 (1H, d, $J$ = 8.1 Hz, H-1'), 3.31 (1H, m, H-2'), 0.84 (3H, t, $J$ = 6.9 Hz, H-9'); HRESIMS $m/z$ [M + H]$^+$ 425.2072 (Calcd for C$_{24}$H$_{29}$N$_2$O$_5$, 425.2076) and [M + Na]$^+$ 447.1890 (Calcd for C$_{24}$H$_{28}$N$_2$NaO$_5$).

Note: The data with the same labels may be interchanged.

(-)-Streptophenazine O (5): yellow amorphous powder; molecular formula C$_{24}$H$_{28}$N$_2$O$_5$; $t_R$ 23.87 min (80% MeOH in H$_2$O); $[\alpha]_D^{25}$ −19.0 (c 0.50, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 219 (4.93), 252 (4.44), 366 (3.70) nm; ECD (10 mg/L, MeOH) $\lambda_{max}$ ($\Delta\varepsilon$) 251 (−2.0) nm; IR (KBr) $\nu_{max}$ 3375, 2925, 2853, 1722, 1650, 1531, 1433, 1273, 1025, 952 cm$^{-1}$; $^1$H NMR data (500 MHz, in CDCl$_3$) $\delta$H 8.27 (1H, dd, $J$ = 8.7, 1.4 Hz, H-2), 8.00 (1H, m, H-3), 8.36 (1H, dd, $J$ = 8.7, 1.4 Hz, H-4), 7.75 (1H, dd, $J$ = 6.9, 1.4 Hz, H-7), 7.80 (1H, m, H-8), 8.25 (1H, dd, $J$ = 6.9, 1.4 Hz, H-9), 4.13 (3H, s, H-12), 5.43 (1H, d, $J$ = 8.1 Hz, H-1'), 3.31 (1H, m, H-2'), 0.84 (3H, t, $J$ = 6.9 Hz, H-9'); HRESIMS $m/z$ [M + Na]$^+$ 447.1890 (Calcd for C$_{24}$H$_{28}$N$_2$NaO$_5$, 447.1896).

Note: The data with the same labels may be interchanged.

3.4. SRB assay

SRB assay (Yu et al. 2014) was used to evaluate the activity of tested compounds against the proliferation of glioma C6, U87-MG, U251, and SHG-44 cells. Doxorubicin (DOX) was used as a positive control.
3.4.1. Antimicrobial assay

The micro broth dilution method as described in the previous study (Ye et al. 2015) was applied to determine the antibacterial activity of compounds 1–6 against methicillin-resistant *S. aureus* ATCC 43300 and *E. coli* ATCC 25922. Norfloxacin, a broad-spectrum antibiotic against both Gram-positive and Gram-negative bacteria, was used as a positive control.

4. Conclusion

More than 6000 phenazines have been reported during the past century (Laursen & Nielsen 2004). Streptophenazines are 1,6-disubstituted phenazines with a long alkyl chain at C-6 position. So far, only 12 streptophenazine-type phenazines have been identified. In this study, three new streptophenazine-type phenazines, named as (-)-streptophenazines M–O (1, 3, 5), together with three known phenazines of 1-carbomethoxyphenazine (2) and (-)-streptophenazines A (4) and B (6), were isolated from the culture of marine actinomycete *Streptomyces* sp. 182SMLY. The planar structures of these streptophenazines were determined by the analyses of NMR and HRESIMS data, while the absolute configurations of these streptophenazine-type phenazines can be assigned based on their $^3J_{1',2'}$ coupling constant, optical rotation value, and Cotton effect at around 251 nm. (-)-Streptophenazine B exhibited potent activity against the growth of methicillin-resistant *S. aureus*.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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