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Bioactive phenazines from an earwig-associated *Streptomyces* sp.

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[ABSTRACT] Three new phenazine-type compounds, named phenazines SA–SC (**1**–**3**), together with four new natural products (**4**–**7**), were isolated from the fermentation broth of an earwig-associated *Streptomyces* sp. NA04227. The structures of these compounds were determined by extensive analyses of NMR, high resolution mass spectroscopic data, as well as single-crystal X-ray diffraction measurement. Sequencing and analysis of the genome data allowed us to identify the gene cluster (*spz*) and propose a biosynthetic pathway for these phenazine-type compounds. Additionally, compounds **1**–**5** exhibited moderate inhibitory activity against acetylcholinesterase (AChE), and compound **3** showed antimicrobial activities against *Micrococcus luteus*.

[KEY WORDS] Earwig-associated actinomycete; Phenazine; Biosynthetic pathway; Antimicrobial activity; Acetylcholinesterase inhibitory activity

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Introduction

Phenazines constitute a large family of nitrogen-containing natural products featuring a typical pyrazine ring (1, 4-diazabenzene) with two annulated benzenes and different types of side chains [1]. The first phenazine-type natural products, pyocyanin and chlororaphin, were isolated from severe purulent wounds of patients in the 19th century [2]. Subsequently, more than 150 phenazine-type natural products have been isolated from Gram-positive (e.g. *Streptomyces*) [3] and Gram-negative bacteria (e.g. *Pseudomonas*) [4], or from archaeal *Methanosarcina* species [5]. Phenazines display a broad range of biological functions including antibacterial [6], cytotoxic [7], and antimalarial activities [8]. In our continuing efforts to discover novel/bioactive natural products from microorganisms living in special niches [9-10], previously we investigated secondary metabolites from an earwig-associated actinomycete *Streptomyces* sp. NA04227 which led to the discovery of aurachin SS, a new aurachin-type antibiotic together with two known compounds [11]. Besides aurachin SS, HPLC analysis of its culture extract showed the presence of a series of unidentified metabolites with long UV wavelength absorption. After large-scale fermentation, extraction with ethyl acetate and repeated combinatorial chromatographic purification, seven phenazine-type compounds (**1**–**7**) were isolated, in which **1**–**3** were identified as new compounds on the basis of HRESIMS, NMR, as well as single-crystal X-ray diffraction analysis, while **4**–**7** were firstly isolated as natural products [12-13]. Herein, we report the isolation, structure elucidation and bioactivities of these phenazine compounds (Fig.

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1) from the fermentation broth of *Streptomyces* sp. NA04227, as well as their plausible biosynthetic pathway based on the bioinformatic analysis.

Results and Discussion

Compound **1** was isolated as a brown powder. The molecular formula was determined to be $C_{19}H_{20}N_2O_2$ on the basis of HR-ESI-MS data ($[M + H]^+$, *m/z* 309.1604, Calcd. for $[C_{19}H_{20}N_2O_2H]^+$, 309.1603), indicating eleven degrees of unsaturation. The ^1H, ^{13}C and HSQC NMR spectra of **1** (Table 1) revealed the presence of two methyls, two methoxyls, one methylene, and six olefinic/aromatic protons. The ^1H-^1H COSY correlations of H-2 (d, δ_H 7.14) and H-3 (d, δ_H 7.85), together with the HMBC correlations of H-2 with C-4 (δ_C 132.3) and C-12 (δ_C 136.9) and of H-3 with C-1 (δ_C 153.5) and C-5 (δ_C 141.3), indicated the presence of a 1, 2, 3, 4-tetrasubstituted benzene ring (ring A in **1**). The ^1H-^1H COSY correlations of H-8 (d, δ_H 7.24, J = 8.2 Hz)/H-9 (t, δ_H 7.82, J = 8.2 Hz)/H-10 (d, δ_H 7.81, J = 8.2 Hz), and HMBC correlations of H-8 and H-10 with C-6 (δ_C 135.7), and of H-9 with C-7 (δ_C 155.7) and C-11 (δ_C 142.6) revealed a 1, 2, 3-trisubstituted benzene ring (ring C in **1**). Furthermore, the ^{13}C NMR spectrum also suggested that six quaternary carbons are not identified, along with the ^1H-^1H COSY and HMBC experiment of **1** displayed a typical pattern of phenazine skeleton. Two putative methoxyl groups resonance at δ_H

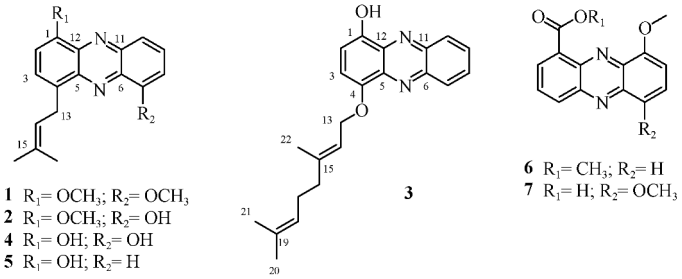

Fig. 1 Structures of compounds 1–7

Table 1 ^1H and ^{13}C NMR data for compounds 1–3

Position	**1**[c]		**2**[d]		**3**[d]	
	δ_C[a]	δ_H (J in Hz)[a]	δ_C[a]	δ_H (J in Hz)[a]	δ_C[b]	δ_H (J in Hz)[b]
1	153.5		153.5		151.2	
2	107.1	7.14, d (7.8)	106.8	7.03, d (7.8)	109.6	7.25, d (7.4)
3	128.1	7.58, d (7.8)	128.6	7.56, d (7.8)	108.3	7.09, d (7.4)
4	132.3		131.6		154.4	
5	141.3		140.5		132.0	
6	135.7		133.5		137.8	
7	155.7		151.5		120.8	7.94, d (8.8)
8	107.2	7.24, d (8.2)	109.1	7.25, d (8.9)	130.9	7.24, d (8.8)
9	121.2	7.82, d (8.2)	131.3	7.75, t (8.9)	131.3	7.73, td (8.6, 3.2)
10	130.2	7.81, d (8.2)	120.5	7.93, d (8.9)	120.4	7.73, td (8.6, 3.2)
11	142.6		142.1		137.9	
12	136.9		137.9		134.5	
13	28.7	4.02, d (7.5)	29.3	4.00, d (7.2)	66.8	4.94, d (6.1)
14	123.3	5.51, t (7.5)	122.5	5.48, t (7.2)	119.3	5.64, t (6.1)
15	132.1		133.2		140.9	
16	25.1	1.74, s	25.8	1.79, s	26.2	2.10, m
17	17.1	1.87, s	18.0	1.84, s	39.6	2.08, m
18	56.4	4.07, s	56.4	4.15, s	123.8	5.09, t (6.1)
19	56.3	4.13, s			131.9	
20					25.8	1.67, s
21					17.7	1.60, s
22					16.9	1.83, s

[a] Measured at 600 MHz (^1H) and 150 MHz (^{13}C); [b] Measured at 400 MHz (^1H) and 100 MHz (^{13}C); [c] Aquired in acetone-d_6; [d] Acquired in $CDCl_3$

4.07/δ_C 56.4 and δ_H 4.13/δ_C 56.3 were connected to the phenazine skeleton based on HMBC correlations (Fig. 2), however, their relative position on the A and C rings cannot be established. The remaining ^{13}C NMR signals at δ_C 28.7, 123.3, 132.1, 25.1 and 17.1 comprised a characteristic isopentenyl group, which located at the opposite position of CH_3-19 (δ_H 4.13, δ_C 56.3), based on HMBC correlations of H-13 (δ_H 4.02) with C-3 (δ_C 128.1) and C-5 (Fig. 2). Since the relative location of two methoxyl groups was difficult to be fully assigned by NMR interpretation, we attempted to crystalize **1** for single-crystal X-ray diffraction analysis. Fortunately, a high quality single crystal of **1** was obtained from MeOH/H_2O 3 : 1 (*V*/*V*) (Fig. 3), leading to the complete determination of structure for **1**.

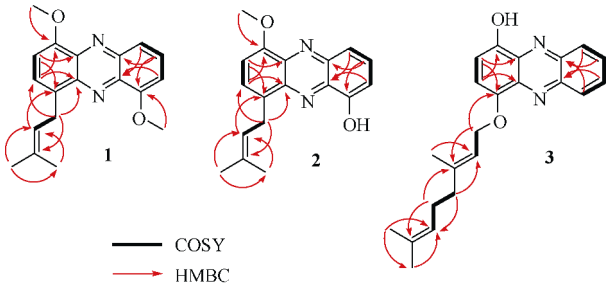

Fig. 2 The Key 2D NMR correlations of 1–3

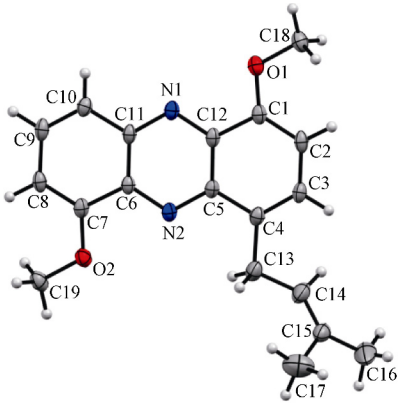

Fig. 3 X-ray crystal structure of compound 1

Compound **2** was obtained as yellow powder and had a molecular formula of $C_{18}H_{18}N_2O_2$ ([M + H]$^+$, *m/z* 295.1449, Calcd. for $[C_{18}H_{18}N_2O_2H]^+$, 295.1447), according to their HR-ESI-MS data. The ^1H and ^{13}C NMR data of **2** resembled those of **1**, indicating **2** possessed the same phenazine skeleton as **1**. The major difference between **2** and **1** observed in the NMR spectra was the absence of one oxygenated methyl signal at C-7 (δ_C 151.5) in **2**. In the phenazine skeleton, HMBC correlations of H-2 (δ_H 7.03) with C-12 (δ_C 137.9), of H-3 (δ_H 7.56) and H_2-13 (δ_H 4.00) with C-5 (δ_C 140.5), of H-8 (δ_H 7.25) and H-10 (δ_H 7.93) with C-6 (δ_C 133.5), and of H-9 (δ_H 7.75) with C-11 (δ_C 142.1) allowed the assignment of the C-12, C-5, C-6 and C-11, respectively. Furtherly combined with detailed 2D NMR data analysis (Table 1, Fig. 2), the structure of compound **2** was elucidated as shown in Fig. 1.

Compound **3** was isolated as red powder with molecular ion peaks at *m/z* 371.1733 [M + Na]$^+$ and Calcd. for $[C_{22}H_{24}N_2O_2Na]^+$, 371.1735. The additional proton chemical shifts at δ_H 2.10 (H-16), δ_H 2.08 (H-17), δ_H 5.09 (H-18), δ_H 1.67 (H-20), δ_H 1.60 (H-21) and the ^1H-^1H COSY correlations between H-16/H-17/H-18 indicated that the isopentenyl group in **5** is replaced by a geranyl group. In the HMBC spectrum, characteristic J^3 HMBC correlations of H-2 (δ_H 7.25) with C-12 (δ_C 134.5), of H-3 (δ_H 7.09) with C-5 (δ_C 132.0), of H-7 (δ_H 7.94) and H-9 (δ_H 7.73) with C-11 (δ_C 137.9), and of H-8 (δ_H 7.24) and H-10 (δ_H 7.73) with C-6 (δ_C 137.8) were observed. The downfield chemical shifts for C-4 (δ_c 154.4) and C-13 (δ_c 66.8) also suggested that the phenazine skeleton and geranyl group could be connected through an oxygen bridge, which was also confirmed by the HMBC correlation of H_2-13 (δ_H 4.94) with C-4 and the NOESY correlation of H_2-13 with H-3. Thus, the structure of **3** was established as shown.

As the whole genome of *Streptomyces* sp. NA04227 was fully sequenced and analyzed by antiSMASH previously [11]. One of the gene clusters was annotated as putative phenazine biosynthetic gene cluster (accession No. MH990330) as it contained the conserved proteins for phenazine biosynthesis. This gene cluster showed identical genetic organization, with 60%–90% sequence identity, to that phenazine gene cluster in *Streptomyces* sp. SpC080624SC-11 (Fig. 4) [13].

This cluster spanned a 17.9 kb contiguous DNA region consisting of 16 open reading frames. Six genes, from *spzB* to *spzG*, exhibited high sequence identity to *phzBCDEFG* for phenazine biosynthesis in *Pseudomonas fluorescens* [14], while the homologous gene of *phzA* was missing in the *spz* gene cluster. An additional set of genes located at downstream of *the spz* cluster, *spzH* to *spzM*, which were tentatively annotated as enzymes for supplying dimethylallyl pyrophosphate and isopentenyl pyrophosphate in mevalonate pathway [15]. Besides the phenazine and mevalonate synthesis gene, additional four genes, *spzO1*, *spzO2*, *spzS* and *spzP*, were annotated as tailoring enzymes. *SpzS* was annotated as a flavin dependent hydroxylase and showed 53% protein sequence identity to *PhzS* [16], which can convert PCA to 1-hydroxyl phenazine (1-HP) by hydrolyzation and decarboxylation. *SpzO1* was a monooxygenase and showed 58% protein sequence identity to *LaPhzNO1*, which can catalyze phenazine N-oxidation in *Lysobacter antibioticus* [17]. Similarly, *SpzP* exhibited 82% protein sequence identity to *Mpz10* and was responsible for prenylations of 1, 6-dihydroxyphenazine (DHP) [13]. The remaining *SpzO2* only showed 39% identity to *ElmH*, which was a monooxygenase catalyzing naphthacenone into naphthacenequinone in elloramycin biosynthesis [18].

Thus, taking all genetic information together, a biosynthetic pathway was proposed for biosynthesis of phenazine SA-SE in NA04227 (Fig. 4). Chorismic acid was used as the starting unit, and converted to HHPDC (Hexahydro-PDC) by *SpzD*, *SpzE*, *SpzF* and *SpzB*, which was identical to the

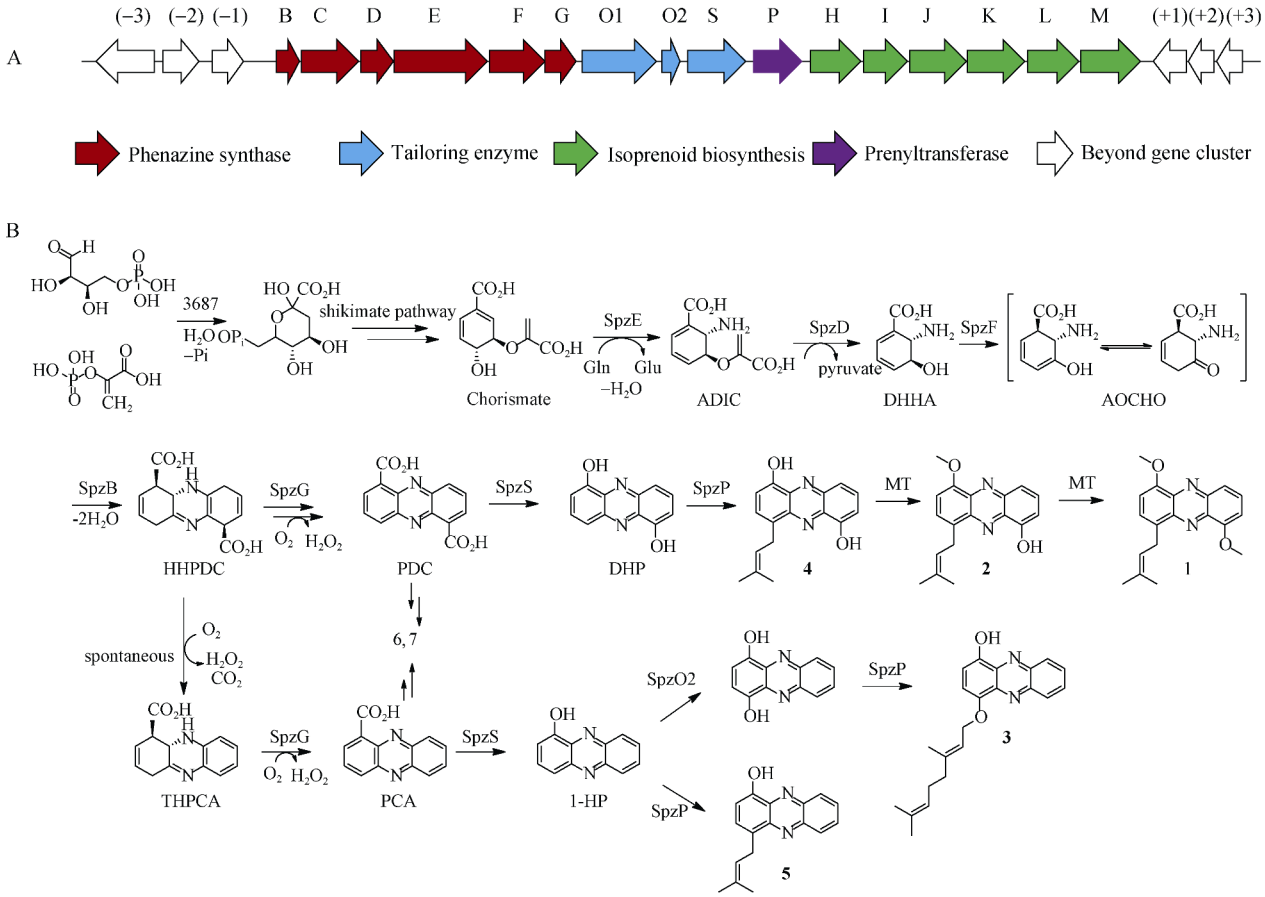

Fig. 4 Biosynthesis of the phenazine compounds in *S.* sp. NA04227. (A) The biosynthetic gene cluster of the phenazine compounds; (B) Plausible biosynthetic pathway of the phenazine compounds

phenazine pathway described before [14]. Subsequently, HHPDC could be directly catalyzed by *SpzG* to generate PDC (phenazine-1, 6-dicarboxylic acid) and further hydrolyzed and decarboxylated by *SpzS* to generate DHP (1, 6-dihydroxyl phenazine). Finally, *SpzP* catalyzed the addition of DMAPP at C-4 to produce **4**, followed by one unidentified methyltransferase, which might locate outside of this cluster, transferring two methyl group step by step to afford **2** and **1**. Alternatively, HHPDC could also go through spontaneous decarboxylation to THPCA (tetrahydrophenazine-1, 6-carboxylic acid) and further be oxidized by *SpzG* and *SpzS* to generate 1-HP (1-hydroxyl phenazine). Similar to **1**, **2**, **4**, 1-HP could firstly and directly go through a prenylation to form **5**. Secondly, 1-HP could also be hydroxylated by *SpzO2* to afford 1, 4-DHP (1, 4-dihydroxyl phenazine) and *SpzP* might also accept GPP instead of DMAPP as substrate to produce **3**, due to the substrate flexibility.

The isolated compounds **1**–**5** were evaluated for their biological activities. As shown in Table 2 and Table 3, only compound **3** showed moderate bioactivity against *Micrococcus luteus* with a MIC value of 4.0 μmol·L^{-1}, indicating the importance of geranyl group for antibacterial activity. Furthermore, all of five compounds showed moderate inhibitory activity against acetylcholinesterase with IC_{50} values ranging from 2.32–3.62 μmol·L^{-1}.

Table 2 The antimicrobial activity of compounds 1–5 (MIC, μmol·L^{-1})

	Micrococcus luteus
1	> 128.0
2	128.0
3	4.0
4	> 128.0
5	> 128.0
Rifampicin	0.1

In conclusion, three new phenazine-type compounds (**1**–**3**) along with four new natural products (**4**–**7**) were isolated and characterized from an earwig-associated *Streptomyces* sp. NA04227. A unified biosynthetic pathway was proposed based on the deduced function of the corresponding gene cluster. Additionally, compounds **1**–**5** exhibited moderate AchE inhibitory activities, and **3** showed antimicrobial activities against *M. luteus*.

Table 3 ***In vitro*** **AChE inhibitory activity of compounds 1–5**

	IC_{50} (μmol·L^{-1})
1	2.91 ± 0.11
2	2.93 ± 0.17
3	2.32 ± 0.25
4	3.36 ± 0.29
5	3.62 ± 0.44
Huperzine	0.05 ± 0.01

Experimental

General experimental procedures

Silica gel (200–300 mesh) for CC (column chromatography) and GF_{254} (10–20 μmol·L^{-1}) for TLC (thin layer chromatography) were purchased from Qingdao Marine Chemical Company, China. Sephadex LH-20 was purchased from GE Biotech, USA. Semi-preparative reverse-phase high performance liquid chromatography (RP-HPLC) was accomplished on an Eclipse XDB-C_{18} column (5 μm, 250 mm × 9.4 mm) from Agilent Technologies Inc. USA (Santa Clara, CA, USA). Mass spectra were acquired on an Agilent 6250 TOF LC-MS instrument equipped with electrospray ionization (ESI) probe operating in positive-ion mode with direct infusion. NMR spectra were acquired on Bruker Avance III 600 MHz or 400 MHz spectrometer with TMS or solvent signals adopted as internal standards: acetone-d_6 (δ_H 2.05; δ_C 29.82), and $CDCl_3$ (δ_H 7.26; δ_C 77.06).

Culture media

ISP4 (Soluble starch 10 g·L^{-1}, $MgSO_4{\cdot}7H_2O$ 2 g·L^{-1}, $(NH_4)_2{\cdot}SO_4$ 2 g·L^{-1}, NaCl 1 g·L^{-1}, KH_2PO_4 1.36 g·L^{-1}, $CaCO_3$ 2 g·L^{-1}, trace elements 1 mL·L^{-1}, Agar powder 15 g·L^{-1}) agar plates were used for strain NA04227 growth and sporulation. The Tryptic Soy Broth (TSB) medium was used to incubate seed cultures, and the fermentation medium was YEME medium (yeast extract 4 g·L^{-1}, glucose 4 g·L^{-1}, malt extract 10 g·L^{-1}).

Cultivation and isolation

The purified strain was cultured in ISP4 agar plates at 28 °C for 6 d. The seed cultures were incubated in TSB medium at 28 °C with 200 r·min^{-1} for 2 d (50 mL media in 250 mL erlenmeyer flask). The seed cultures were used to inoculate in the 300 mL fermentation broth at 28 °C with 150 r·min^{-1} for 8 days [19]. After 8 d cultivation, the filtrate (30 L) were adsorbed by XAD16N resin, washed by water and eluted with methanol. The methanol extract was concentrated in vacuum to obtain 4.6 g of crude paste, which was fractionated by column chromatography (CC) over silica gel using a gradient elution of petroleum ether/EtOAc (*V*/*V*, 100 : 0, 50 : 1, 20 : 1, 10 : 1, 5 : 1, 2 : 1, 1 : 1) to give seven fractions (Frs.1–7). These fractions were then separated by Sephadex LH-20 CC using MeOH as eluents and further purified by semi-preparative HPLC (Agilent Technologies 1260 Infinity II) with CH_3CN (67%). Finally, new compounds **1** (22 mg), **2** (6 mg) and **3** (11 mg) were obtained.

Phenazine SA (1)

Brown crystal; mp 107–108 °C; UV (CH_2Cl_2) λ_{max} (log ε) 272 (3.94), 364 (2.81) nm; IR (ZnSe) ν_{max} 2955.2, 2922.4, 2850.6, 1712.5, 1463.2, 1377.4, 1270.6, 1153.1 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 1; HR-ESI-MS data $[M + H]^+$, *m/z* 309.1604, Calcd. for $[C_{19}H_{20}N_2O_2H]^+$, 309.1603.

Crystal data for 1

Molecular formula $C_{19}H_{20}N_2O_2$, M_γ =308.37, Monoclinic crystal, a = 15.996 (5) Å, b = 4.9479 (13) Å, c = 22.429 (6) Å, α = 90°, β = 100.32 (3)°, γ = 90°, Z = 4, μ = 0.394 mm^{-1}, F (000) = 656.0 and T = 304 K; Crystal size: 0.15 × 0.1 × 0.05 mm^3, Volume = 1746.5 (9) Å3, 8640 Reflections collected, 3219 independent reflections (R_{int} = 0.0735), the final R indices [I > 2sigma(I)] R_1 = 0.0888, wR_2 = 0.2583, R indices (all data) R_1 = 0.1525, wR_2 = 0.3342. The goodness-of-fit on F^2 was 0.938. Crystallographic data for **1** have been deposited with the Cambridge Crystallographic Data Centre (deposition no. CCDC 1864635). These data can be obtained free of charge *via* www.ccdc.cam.ac.uk/data_request/cif.

Phenazine SB (2)

Yellow powder; mp 105–106 °C; UV (CH_2Cl_2) λ_{max} (log ε) 275 (4.06) nm; IR (ZnSe) ν_{max} 2955.4, 2924.0, 2852.2, 1735.7, 1462.2, 1377.4, 1151.5 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 2; HR-ESI-MS data $[M + H]^+$, *m/z* 295.1449, Calcd. for $[C_{18}H_{18}N_2O_2H]^+$, 295.1447.

Phenazine SE (3)

Red powder; mp 113–114 °C; UV (CH_2Cl_2) λ_{max} (log ε) 273 (4.07) nm, 393 (2.87) nm; IR (ZnSe) ν_{max} 2955.3, 2923.7, 2852.0, 1734.5, 1462.0, 1377.5, 851.6, 738.9 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 2; HR-ESI-MS data $[M + Na]^+$, *m/z* 371.1723, Calcd. for $[C_{22}H_{24}N_2O_2Na]^+$, 371.1735.

Antimicrobial assay

The MIC (minimum inhibition concentration) characters on the antifungal and antibacterial activities, which were determined in serial dilution assays against *Bacillus subtilis* CICC 10283, *Staphylococcus aureus* (MRSA) ATCC 43300, *Streptococcus pyogenes* ATCC 19615, *Staphylococcus aureus* CMCC (B) 26003 and *Escherichia coli* as described previously [20]. The sterilized YPM medium (49 μL) and different concentration of compounds (1 μL) were placed in 96-well microtiter plates and incubated at 37 °C for 24 h. The MIC values were determined as the lowest sample concentration, at which no bacterial growth could be discerned.

Acetylcholinesterase assay

An Ellman's method was applied to test the activity [21]. The phosphate buffer (130 μL, 0.1 mol·L^{-1}, pH 8.0), AchE (20 μL, 0.035 U·mL^{-1}), DTNB (20 μL, 0.333 mmol·L^{-1}), and different concentration of compounds (20 μL) were placed in a 96-well microtiter plates and incubated at 37 °C for 15 min, and the inhibition rates were calculated by the absorbance in 412 nm. Finally, the IC_{50} values were determined by the standard curve line according to changes in concentration and inhibition rate.

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