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VIRIDOBRUNNINES A AND B, ANTIMICROBIAL PHENOXAZINONE ALKALOIDS FROM A SOIL ASSOCIATED *STREPTOMYCES* SP.

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Abstract – Chemical investigation of a *Streptomyces* sp. strain designated A1302 isolated from soil sample led to the discovery of two new phenoxazinone alkaloids, viridobrunnines A and B (**1** - **2**), along with two known analogues exfoliazone (**3**) and chandrananimycin D (**4**). Their structures were established by means of spectroscopic methods. The antibacterial and antifungal activity of isolated compounds was assayed. Viridobrunnine B (**2**) exhibited potent antibacterial activity against *Bacillus subtilis* with inhibition zone from 13 mm to 15 mm.

Phenoxazinones, namely phenoxazin-3-one, are olefinically rearranged derivatives of phenoxazines which contain a central oxazine ring fused to two benzene rings.[1,2] Compounds containing the phenoxazinone core are generally yellow to purple colored substance, accordingly, some of them e.g. resazurin, resorufin and orceins are used as dyes.[3,4] Benzene-fused phenoxazinone derivatives were also synthesized as fluorescent probes for labeling biomolecules.[5] Naturally occurring phenoxazines and phenoxazinones are frequently encountered as metabolites of diverse organisms such as lichen, fungi and actinomycetes.[4,6,7] Among of them, actinomycin D is the most well-studied compound and has been used as anticancer drug in clinic for several decades.[8] Actinomycin D and other phenoxazinone alkaloids such as exfoliazone,[9] glucosylquestiomycin,[10] pitucamycin,[7] Phx-3,[11] and 2-amino-phenoxazinone[12] etc produced by actinomycetes also displayed important antibacterial or antifungal properties. With the aim of discovering new antimicrobial agents from extraordinary eco-environment microorganism, we isolated a *Streptomyces* sp. designated A1302 strain from the anthill of an unidentified ant. The EtOAc extract of A1302 fermentation broth showed potent antibacterial activity in disc diffusion assay. Subsequent chemical investigations of EtOAc extract of fermentation broth including mycelia of A1302 strain led to the identification of four phenoxazinone alkaloids including two new compounds viridobrunnine A (**1**) and viridobrunnine B (**2**) (Figure 1).

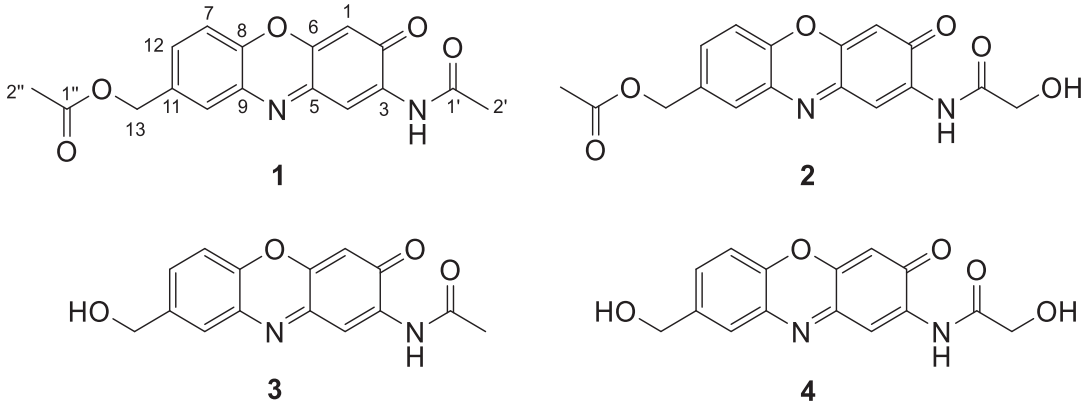


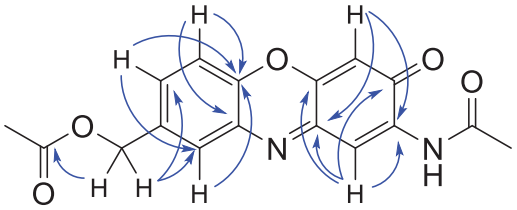
Figure 1. Structures of compounds **1** - **4**

Strain *Streptomyces* sp. A1302 was isolated from an anthill soil sample collected in campus of Shandong University at Weihai, China. 16S rRNA nucleotide sequence analysis indicated that A1302 strain is a member of *Streptomyces* genus.[13] Its RNA nucleotide sequence (deposited to GenBank with accession number KT240119) is similar to those of *S. viridobrunneus* (T) (AJ781372) and *S. showdoensis* (AY999741) in GenBank, though, with only 96.4% similarity. Therefore, A1302 strain is likely to be a new *Streptomyces* species needed to further classification confirmation.

Viridobrunnine A (**1**) was obtained as light-brown powder. Its IR spectrum showed absorption bands of ester group (1742 cm^{-1}), conjugated carbonyl group (1694 cm^{-1}) and benzene skeleton (1618, 1573 and 1472 cm^{-1}). The sharp intense IR absorption at 3283 cm^{-1} indicated the presence of one N-H bond. The absorptions at 1642 and 1512 cm^{-1} were assigned to the carbonyl of acylamino group. The quasi molecular ion peak at *m/z* 327.0978 ($[M+H]^+$, calcd. $C_{17}H_{15}N_2O_5$: 327.0981) in positive-ion HRESIMS spectrum indicates the molecular formula to be $C_{17}H_{14}N_2O_5$. The ^1H NMR spectrum of **1** exhibited the protons resonances of a trisubstituted aromatic ring at δ_H 7.42 (1H, d, J = 8.5 Hz), 7.56 (1H, dd, J = 8.5, 2.0 Hz) and 7.89 (1H, d, J = 2.0 Hz), another two aromatic protons at δ_H 6.48 (1H, s) and 8.44 (1H, s), an oxygenated methylene at δ_H 5.22 (2H, s) and two methyl protons of acetyl group at δ_H 2.16 (3H, s) and 2.30 (3H, s) (Table 1). The broad signal centered at δ_H 8.58 (H, brs) was assigned to the amino proton in accordance with the IR analysis. Apart from the signals of two acetyl groups, there are thirteen carbon signals including twelve aromatic carbons and an oxygenated methylene carbon left in the ^{13}C NMR spectrum (Table 1). These data were nearly identical to that of aminophenoxazinone alkaloid exfoliazone (**3**) except for the presence of one more acetyl group.[9] The HMBC correlations from the aromatic protons H-1, H-4, H-7, H-10 and H-12 to the carbons in aromatic rings, and methylene protons to C-10, C-11 and C-12 further confirmed the existence of phenoxazinone skeleton (Figure 2). Compared to the structure of exfoliazone (**3**), the other acetyl group was confirmed to be located at C-13 by the HMBC correlation of

H-13 to C-1". Hence, compound **1** was established as acetylexfoliazone, and gave it a trivial name viridobrunnine A.

Viridobrunnine B (**2**) has a molecular formula of $C_{17}H_{14}N_2O_6$ determined by its quasi molecular ion peak at *m/z* 343.0925 ($[M + H]^+$, calcd. for $C_{17}H_{15}N_2O_6$ 343.0930) in HRESIMS spectrum. The ^1H and ^{13}C NMR data of compound **2** were very similar to that of **1** (Table 1). The significant difference was the replacement of methyl resonances of acetyl group at δ_H 2.30 (3H, s) and δ_C 25.1 (C-2') in compound **1** by those of an oxygenated methylene at δ_H 4.28 (2H, s) and δ_C 63.1(C-2') in **2**. The NOESY correlation between amino proton at δ_H 9.38 (1H, brs) and methylene protons at δ_H 4.28 (2H, s) indicated that the oxygenated acetyl group was attached to amino group. Accordingly, compound **2** was identified as acetylated chandrananimycin D,[14] and named as viridobrunnine B.



1

Figure 2. The key HMBC correlations for **1**

Table 1. ^1H, ^{13}C NMR data of compounds **1** and **2** ($CDCl_3$, δ in ppm, TMS)[a]

No.	1		2	
	δ_H	δ_C	δ_H	δ_C
1	6.48 (s)	104.4	6.40 (s)	104.6
2	-	179.9	-	179.8
3	-	137.3	-	136.7
4	8.44 (s)	114.0	8.40 (s)	114.7
5	-	149.4	-	149.5
6	-	149.4	-	149.4
7	7.42 (d, J = 8.5 Hz)	116.5	7.34 (d, J = 8.5 Hz)	116.5
8	-	143.0	-	143.1
9	-	134.0	-	134.1
10	7.89 (d, J = 2.0 Hz)	129.6	7.80 (d, J = 2.0 Hz)	129.7
11	-	134.1	-	134.2
12	7.56 (dd, J = 8.5, 2.0 Hz)	131.6	7.47 (dd, J = 8.5, 2.0 Hz)	131.7
13	5.22 (s)	65.2	5.13 (s)	65.2
1'	-	169.4	-	170.8
2'	2.30 (s)	25.1	4.82 (s)	63.1
1"	-	170.9	-	170.8
2"	2.16 (s)	21.1	2.07 (s)	21.1
-NH	8.58 (brs)	-	9.38 (brs)	-
-OH	-	-	2.52 (brs)	-

[a] Measured at 500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR.

The antibacterial and antifungal activity of compounds **1** - **4** were determined in a primary screen by the disc diffusion assay against three bacteria *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*, and three fungi *Aspergillus niger*, *Aspergillus flavus* and *Mucor mucedo*. Viridobrunnine B (**2**) showed moderate antibacterial activity against *B. subtilis* (Table 2). Both viridobrunnine A (**1**) and exfoliazone (**3**) exhibited minor antibacterial activity. None of tested compounds showed any antifungal activities against three targeted strains.

Table 2. Antimicrobial activity of compounds **1** - **4**.[a]

	1	**2**	**3**	**4**	streptomycin	nysfungin
Escherichia coli	-	-	-	-	+++	
Staphylococcus aureus	-	-	-	+	+++	
Bacillus subtilis	-	++	-	+	+++	
Aspergillus niger	-	-	-	-		+++
Aspergillus flavus	-	-	-	-		+++
Mucor mucedo	-	-	-	-		+++

[a] zone diameter of growth inhibition: < 10 mm (-), 10-12 mm (+), 13-15 mm (++) and 16-20 mm (+++).

EXPERIMENTAL

General Procedures. Silica gel (300-400 mesh) used for column chromatography (CC) and silica GF_{254} for thin layer chromatography were purchased from Qingdao Marine Chemical Factory in China. Silica gel C-18 (20-50 μm) used for low pressure CC and precoated plates (C-18) were purchased from Merck in Germany. IR spectrum was recorded with a Nicolet Avatar-360-ESP spectrophotometer at room temperature in KBr. UV spectrum was measured by Agilent 8453E UV-visible spectroscopy system. ^{1}H, ^{13}C NMR (DEPT) and 2D NMR were recorded on a Bruker AVANCE 500 spectrometer. HR-ESI-MS measured by LTQ-Orbitrap XL spectrometer.

Actinomyces materials. Employing dilution technique, sterile water suspension of soil was cultured on modified Gause's synthetic liquid medium (GAU) at 35 ºC. The actinomycetes colonies were singled out and sub-cultured to give pure morphologies. Genomic DNA was extracted using DNA Isolation kit (Shanghai Sangon Biotech Co., China). PCR amplification of the 16S ribosomal RNA (rRNA) was performed using primers Fd2 (5'-GAGTTTGATCATGGCTCAG-3') and 16Sr (5'-TTGCGGGACTTAACCCAACAT-3') and sequenced by Shanghai Sangon Biotech Co., China. The nucleotide sequence was deposited to GenBank (Accession number KT240119), and the closely related taxa were retrieved from the GenBank database using BLASTN software. Voucher specimen (No. A1302) was deposited at Laboratory of Natural Products Chemistry, Department of Pharmacy, Shandong University at Weihai.

Fermentation, Extraction and Isolation. A1302 strain was inoculated in Erlenmeyer flasks with totally

30 L GAU medium and shaking-cultured for 14 days at 35 ºC. The fermentation broth including mycelia was extracted with equal volume of EtOAc for 3 times. The solvent-removed extract (9.1 g) was isolated by silica gel column chromatography and eluted using petroleum ether/EtOAc (15:1, 1:1, EtOAc) and then with EtOAc/MeOH (15:1, MeOH) to give nine fractions A-I. Fraction E was subjected to a silica column (petroleum ether/EtOAc 5:1) to afford eight subfractions, E1-E8. Subfraction E2 was further separated on silica gel ($CHCl_3$/EtOAc 15:1) and then purified by C-18 reversal column (MeOH/H_2O 4:6) to afford viridobrunnine A (**1**) (2.2 mg). Subfraction E6 was further chromatographed on silica gel ($CHCl_3$/EtOAc 3:1) and then purified by Sephadex LH-20 column to give viridobrunnine B (**2**) (1.8 mg). Fraction F was isolated by silica gel (petroleum ether/EtOAc 1:1) and further purified by C-18 column chromatography (MeOH/H_2O 3:7) to yield exfoliazone (**3**) (4.8 mg) and chandrananimycin D (**4**) (2.9 mg).

Viridobrunnine A (1): Light-brown powder. IR (KBr) ν_{max} cm^{-1}: 3283, 3064, 2924, 2853, 1742, 1694, 1642, 1618, 1573, 1512, 1472, 1438, 1405, 1246. UV λ_{mix} (logε) nm: 243 (3.47), 402 (3.34). ^1H ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data, see Table 1. positive-HR-ESI-MS *m/z*: 327.0978 ([M + H]$^+$, calcd. $C_{17}H_{15}N_2O_5$: 327.0981).

Viridobrunnine B (2): Light-brown powder. IR (KBr) ν_{max} cm^{-1}: 3332, 3080, 2923, 2854, 1741, 1705, 1642, 1621, 1593, 1517, 1455, 1380, 1351, 1239. UV λ_{mix} (logε) nm: 241 (3.23), 397 (2.93). ^1H- ($CDCl_3$, 500 MHz) and ^{13}C-NMR ($CDCl_3$, 125 MHz) data, see Table 1. positive-HR-ESI-MS *m/z*: 343.0925 ([M + H]$^+$, calcd. For $C_{17}H_{15}N_2O_6$: 343.0930).

Antimicrobial activity assay. The antibacterial and antifungal activity of isolated compounds was evaluated by the agar diffusion method.[15] Three bacteria *B. subtilis*, *S. aureus* and *E. coli*, and three fungi *A. niger*, *A. flavus* and *M. mucedo*, were used as target strains. A single colony of each test strain (bacteria) was grown overnight in beef extract peptone liquid medium on a rotary shaker (200 rpm) at 37 ºC. The isolated compounds were dissolved in MeOH in which the paper disks with a diameter of 6 mm were impregnated (bacteria: 60 μg/platelet, fungi: 60 μg/platelet). Streptomycin (10 μg/platelet) and nysfungin (20 μg/platelet) were used as positive control for bacteria and fungi respectively. Paper disks were dried under sterile conditions and put on agar plates inoculated with the above test organisms. After incubation at 35 ºC for 18 h (bacteria) or 28 ºC for 48 h (fungi), the zones of inhibition were measured.

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