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Phomoeuphorbins **A**–**D**, azaphilones from the fungus *Phomopsis euphorbiae*

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ABSTRACT

Four azaphilones, named phomoeuphorbins **A**–**D** (**1**–**4**) were isolated from cultures of *Phomopsis euphorbiae*, an endophytic fungus isolated from *Trewia nudiflora*. Structures of **1**–**4** were established on the basis of spectroscopic analyses, including application of 2D NMR spectroscopic techniques. Phomoeuphorbins A and C exhibited very weak inhibitory activities against HIV replication in C8166 cells *in vitro*.

1. Introduction

Endophytes, microorganisms that reside in the tissues of living plants, are relatively unstudied as potential sources of novel natural products for potential exploitation in medicine, agriculture and industry (Tan and Zou, 2001; Strobel and Daisy, 2003; Strobel et al., 2004). Due to gene recombination during evolution, endophytic microorganisms have often developed biochemical abilities to produce compounds either similar or identical to those produced by their host plants (Zhang et al., 2006). *Trewia nudiflora* L. (Euphorbiaceae), which is mainly distributed in India, Malaysia and the south of China, is a source of many bioactive compounds, for example, the maytansinoids (Powell et al., 1981, 1982, 1983). Therefore, in order to potentially find novel bioactive natural products, this plant was selected for the presence of possible endophytes.

During the course of a bioactive survey of the endophytes from *T. nudiflora*, the fungus *Phomopsis euphorbiae* was isolated, identified (see Section 3) and selected for further investigation. Up to now, many kinds of bioactive compounds have been isolated from various *Phomopsis* sp. (Prachya et al., 2007; Vatcharin et al., 2008). In this article, we report four new compounds with the proposed names phomoeuphorbins **A**–**D** (**1**–**4**), as well as their very weak anti-HIV activities for **1** and **3** (see here Fig. 1).

2. Results and discussion

The fungal strain was cultivated on PDA plates for 20 days at 26 °C. An ethyl acetate–methanol–formic acid (80:15:5) extract of the culture was partitioned between ethyl acetate and water. The ethyl acetate extract was subjected to RP-18 gel, Sephadex LH-20, silica gel and silica gel H chromatographic purification steps to yield four new compounds **(1–4)**.

2.1. Structural determination

Phomoeuphorbin **A (1)** was obtained as a colorless amorphous solid. Its molecular formula was determined to be $C_{15}H_{16}O_6$ by negative ion HR-ESI-MS (calc. for $[M-H]^-$: 291.0868; found: 291.0872), indicating 8° of unsaturation. The IR spectrum showed absorption bands at 3429, 1690 and 1667 cm^{-1}, implying hydroxyl, and conjugated keto carbonyl groups which was supported by the ^{13}C signal at δ 202.8 in the ^{13}C NMR spectrum, and by a carboxylic carbonyl as indicated by the resonance at δ 175.1. The ^{13}C NMR and DEPT spectra further established the presence of one methyl group [δ_C 19.2(q)], one methylene [δ_C 33.6(t)], two oxymethine [δ_C 76.2(d), 74.2(d)], one oxygen-linked quaternary carbon [δ_C 78.9(s)] and as well eight olefinic carbons between δ 115.3 and 149.9 [δ_C 115.3(s), 117.9(d), 124.6(d), 130.9(d), 139.2(d), 144.4(d), 149.3(d), 149.9(s)]. The above functional groups represented 6° of unsaturation, thus, the remaining two degrees of unsaturation should be due to two rings in the molecule.

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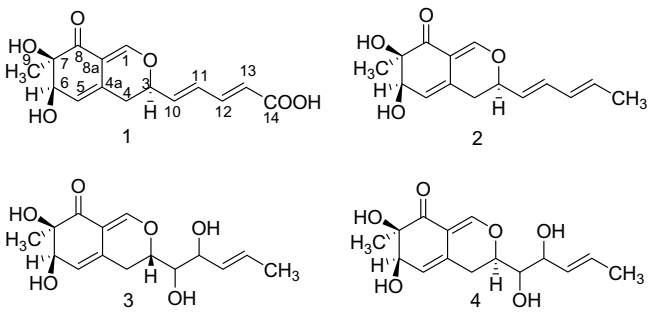

Fig. 1. Structures of compounds **1**–**4**.

The structure of **1** was connected by the functional fragments based on 2D NMR spectroscopic analysis especially using HMBC (see here Fig. 2). A long-range correlation between H-12 [δ_H 7.24(*dd*)] and C-14 [δ_C 171.5(*s*)] and between H-13 [δ_H 5.94(*d*)] and C-14, placed the carboxyl group [δ_C 171.5(*s*)] adjacent to a double bond C-12 and 13 [δ_C 124.6(*d*), 144.4(*d*)]. This double bond was also connected to another double bond containing C-10 [δ_C 139.2(*d*)] and C-11 [δ_C 130.9(*d*)], since the long-range correlation between H-10 [δ_H 6.13 (*dd*)] and C-12 and between H-11 [δ_H 6.50(*dd*)] and C-12. C-10 was attached to C-3 [δ_C 76.2(*d*)], which was supported by the correlation between H-3 [δ_H 4.94(*m*)] and C-10 and between H-3 and C-11. Moreover, C-3 should be linked to an oxygen atom based on its chemical shift at δ_C 76.2(*d*). Because of the long-range correlation between H-4 [δ_H 2.92(*dd*); 2.68(*dd*)] and C-3 and between H-4 and C-10, C-4 [δ_C 33.6(*t*)] was connected to C-3. The C-4 moiety was also linked to C-4a [δ_C 149.9(*s*)] of the double bond containing C-4a and C-5 [δ_C 117.9(*d*)], as evidenced by the long-range correlation between H-4/C-4a, and H-4/C-5 and between H-3/C-4a. The double bond was also connected to the tertiary carbon C-6 [δ_C 74.2(*d*)], which was supported by the correlation between H-6 [δ_H 4.37(*d*)] and C-4a. Since this was a long-range correlation between H-6 [δ_H 4.37(*d*)] and C-7 [δ_C 78.9(*s*)] and H-5 [δ_H 5.55(*s*)] to C-7, C-7 was connected to C-6. The connection of C-9 [δ_C 19.2(*q*)] to C-7 was supported by the long-range correlation between H-9 [δ_H 1.11(*s*)] and C-7 and between H-9 and C-6. C-7 was also connected to the keto carbonyl [C-8, δ_C 202.8(*s*)], as indicated from the long-range correlation between H-9 and C-8. The long-range correlation between H-5 and C-8a [δ_C 115.3(*s*)] and between H-4 and C-8a also indicates that C-8a was connected to C-4a. Thus, the double bond containing C-8a and C-1 [δ_C 149.3(*d*)], which was supported by the correlation between H-1 [δ_H 6.97(*s*)] and C-4a and between H-1 and C-8a, was further connected to C-8, since the double bond have polarized chemical shifts, characteristic of a conjugated keto system. The enol-ether linkage between C-1 and C-3 was confirmed by a long-range correlation between H-1 and C-3 and between H-3 and C-1.

The relative stereochemistry of phomoeuphorbin **A** (**1**) was determined from the coupling constants and NOE analyses (see here Fig. 3). Since the coupling constants between H-10/H-11 and between H-12/H-13 were 15.4 and 15.0 HZ, respectively, the double bonds in the diene side chain should both be in a *trans*-configuration. The positive NOE between H-6 and H-9 indicated that these two groups of protons are, therefore, on the same side of the ring. Correlations between H-3 with H-4α and H-4β suggested a H-3 equatorial. According to the above data, structure **1** was elucidated as (2*E*,4*E*)-5-[(3*R*,6*R*,7*R*)-6,7-dihydroxy-7-methyl-8-oxo-4,6,7,8-tetrahydro-3*H*-2-benzopyran-3-yl] penta-2,4-dienoic acid.

Phomoeuphorbin **B (2)** was assigned the molecular formula of $C_{15}H_{18}O_4$, as deduced from the positive ion HR-ESI-MS (calc. for $[M+Na]^+$: 285.1102; found: 285.1107). Comparison of the spectroscopic data for **2** with those of **1** suggested that they were quite similar, except for the moiety at C-14. Observation of the presence of a methyl group [δ_C 18.1(*q*)] and the absence of a carboxyl group [δ_C 171.5(*s*)] in the ^{13}C NMR spectrum of **2** showed that at the C-14 position a methyl group was evident for **2** instead of a carboxyl group at the same position in **1**. The other parts and chiral carbons of **2** were identical to those of **1**, again being supported by analysis of its 1D and 2D NMR spectra. Therefore, structure **2** was elucidated as (3*R*,6*R*,7*R*)-6,7-dihydroxy-7-metyl-3-[(1*E*,3*E*)-penta-1,3-dien-1-yl]-3,4,6,7-tetrahydro-8*H*-2-benzopyran-8-one.

Phomoeuphorbin **C** (**3**) was assigned the molecular formula $C_{15}H_{20}O_6$, as deduced from the positive ion HR-ESI-MS (calcd. for $[M+Na]^+$:319.1157; found: 319.1167). Comparison of the spectroscopic data of **3** with those of **1** established that they were quite similar, except for the absence of an olefinic group at C-10 and C-11, instead of two hydroxyl groups at C-10 and C-11 positions as for **3** [C-10, δ_C 75.8(*d*); C-11 δ_C 72.6(*d*)] in the ^{13}C NMR spectrum. Moreover, H-3 in **3** might be placed in an axial position, since H-3 only has a correlation with H-4β, in its ROESY spectrum, unlike **1**. The other parts and chiral carbons of **3** were identical to those of **1**, as indicated by analysis of the 1D and 2D NMR spectra. According to the above data, structure **3** was elucidated as (2*E*)-5-[(3*S*,6*R*,7*R*)-6,7-dihydroxy-7-metyl-8-oxo-4,6,7,8-tetrahydro-3*H*-2-benzopyran-3-yl]-4,5-dihydroxypent-2-enoic acid.

Table 1
^1H and ^{13}C NMR spectroscopic data of compounds **1**–**4** (in CD3OD; δ in ppm, *J* in Hz)[a]

No.	1		2		3		4	
	δH	δC	δH	δC	δH	δC	δH	δC
1	6.97*s*	149.3	6.97*s*	148.7	7.05*s*	151.6	7.00*s*	150.8
3	4.94*m*	76.2	4.83*m*	76.4	4.34*m*	76.7	4.19*m*	77.6
4	2.92*dd*(17.1;4.9)	33.6	2.90*dd*(14.8;4.8)	33.7	2.98*dd*(16.7;12.5)	31.8	2.88*dd*(16.7;8.8)	29.7
	2.68*dd*(17.1;6.8)		2.67*dd*(14.8;6.8)		2.61*dd*(16.9;3.4)		2.80*dd*(16.7;4.2)	
4a		149.4		149.4		151.8		151.1
5	5.55*s*	117.9	5.54*s*	116.8	5.53*s*	116.7	5.56*s*	117.3
6	4.37*s*	74.2	4.41*s*	73.8	4.35*s*	74.1	4.37*s*	74.1
7		78.9		78.3		77.9		78.2
8		202.8		201.3		202.2		202.5
8a		115.3		114.5		115.2		115.2
9	1.11*s*	19.2	1.09*s*	19.3	1.19*s*	19.5	1.22 *s*	19.4
10	6.13*dd*(15.4;4.9)	139.2	5.64*dd*(15.4;6.1)	127.8	3.38*dd*(8.0;2.6)	75.8	3.74*t*(5.6)	75.0
11	6.50*dd*(15.4;11.1)	130.9	6.33*dd*(15.4;10.5)	133.7	4.14*t*(8.0)	72.6	4.11*t*(6.0)	74.0
12	7.24*dd*(15.0;11.2)	144.4	5.79dd(15.1;10.5)	131.6	5.62*dd*(16.8;7.1)	132.7	5.64*dd*(15.4;7.2)	131.3
13	5.94*d*(15.0)	124.6	6.10*m*	131.5	5.80*m*	129.5	5.80*s*	129.9
14		171.5	1.72*d*(6.9)	18.1	1.73*d*(6.3)	18.1	1.75*d*(6.0)	18.1

[a] Assignments were based on DEPT, HMQC and HMBC experiments.

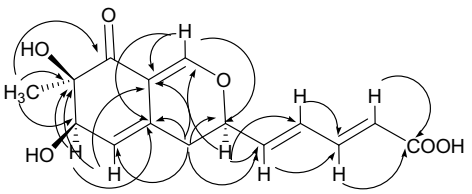

Fig. 2. Long-range correlations (HMBC) of compound **1**.

Phomoeuphorbin **D** (**4**) was assigned the molecular formula $C_{15}H_{20}O_6$, as deduced from the negative ion HR-ESI-MS (calc. for $[M-H]^-$: 295.1181; found: 295.1176). Compound **4** was very similar to **3** except H-3 should be equatorial as indicated by careful examination of its ROESY spectrum, in which both H-4α and H-4β showed correlations with H-3. Therefore, structure **4** was elucidated as (2*E*)-5-[(3*R*,6*R*,7*R*)-6,7-dihydroxy-7-metyl-8-oxo-4,6,7,8-tetrahydro-3*H*-2-benzopyran-3-yl]-4,5-dihydroxypent-2-enoic acid.

2.2. Anti-HIV activity

Phomoeuphorbin **A** (**1**) and phomoeuphorbin **C** (**3**) were tested for in vitro inhibitory effects against HIV replication in C8166 cells. The data are listed in Table 2. Phomoeuphorbin **A** (**1**) and phomoeuphorbin **C** (**3**) both exerted minimal cytotoxicity against C8166 cells (CC_{50} > 200 μg/mL) and each showed anti-HIV activity with EC_{50} = 79 μg/mL and 71 μg/mL. The therapeutic index >2.5 of phomoeuphorbin **A** (**1**) and >2.5 of phomoeuphorbin **C** (**3**) were considered unfavorable for their future development.

3. Concluding remarks

Azaphilones are widespread in *Hypoxylon* sp., and they show many types of bioactivity (Quang et al., 2006). In this article, phomoeuphorbins **A**–**D**, isolated from the fungus *P. euphorbiae*, had substituents in a lower oxidation state than normally noted for this type of compounds; the very weak anti-HIV activities of these azaphilones are also first reported, although quite unfavourable.

4. Experimental

4.1. General experimental procedure

Optical rotations were carried out using a Jasco DIP-370 digital polarimeter, whereas UV spectra were obtained with a Shimadzu UV-2401PC spectrometer. IR spectra were measured in a Bio-Rad FTS-135 spectrometer with KBr pellets, and MS were recorded on a VG Auto Spec-3000 spectrometer. 1D and 2D NMR spectra were measured on either a Bruker AM-400 or a Bruker DRX-500 instrument with TMS as internal standard. Column chromatography (CC) was performed on silica gel (200–300 mesh; Qingdao Marine Chemical Inc., Qingdao, People's Republic of China), silica gel H (10–40 μm; Qingdao Marine Chemical Inc.), Lichroprep Rp-18 gel (40–63 μm; Merck, Darmstadt, Germany) and Sephadex LH-20(Amersham Biosciences), respectively. Fractions were monitored by TLC, and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH.

Table 2
Anti-HIV activities of phomoeuphorbins **A** and phomoeuphorbin **C**

	EC_{50} (μg/ml)	CC_{50} (μg/ml)	TI
Phomoeuphorbin A	79	>200	>2.5
Phomoeuphorbin C	71	>200	>2.8
AZT	0.005	643.2	128640

Anti-HIV activity was tested by inhibition assay for cytopathic effects of HIV-1 (IIIB isolate).

4.2. Biological materials

The fungal strain was isolated from the petioles of *Trewia nudiflora* L. (Euphorbiaceae), which were collected from greenhouse of Kuming Institute of Botany, People's Republic of China. The petioles were washed in running tap water and cut into 5 mm × 5 mm pieces. These small pieces were surface sterilized successively with 0.01% Tween-20 for 30 s, 1% NaOCl solution for 5 min, sterilized water for 5 min and EtOH–H_2O (3:1, v/v) for 5 min. The surface sterilized pieces were incubated at 26 °C on PDA and cultivated until either a colony or mycelium appeared surrounding the pieces. The hyphal tips of the developing fungal colonies were transferred onto fresh PDA plates. After purifying the isolates several times, the final pure cultures were transferred to PDA slant tubes.

4.3. Fungus identification

P. euphorbiae (Sacc.) Trarerso. was identified by Prof. Yunlong Liu (Yunnan Agriculture University, Kunming, China) through its morphological characteristics with additional confirmation from its 18S rDNA sequences. The latter gave a 98% similarity to those accessible at the BLASTN of *Phomopsis* sp. The sequence of the strain has been deposited to the GenBank, as EU520302. A living culture was perserved in Kunming Institute of Botany, Chinese Academy of Science, Kunming, China (Voucher specimen number: 4TnP1-2).

4.4. Culture conditions and extraction

Agar fermentation was performed with PDA medium (18L) for 20 days. The cultured agar was chopped, diced and extracted four times with ethyl EtOAC–MeOH–HCO_2H (80:15:5, v/v) exhaustively. The combined extracts were evaporated in vacuo at 45 °C to give a crude extract which was suspended in H_2O (0.5 L) and then extracted successively with petroleum ether (0.5 L × 3) and EtOAc(0.5 L × 3).

4.5. Isolation of pure compounds

The EtOAC extract (5.0 g) was subjected to MPLC over RP-18 silica gel (146 g) eluted with a MeOH–H_2O (0–100%) gradient system to afford 12 fractions (YP0–YP11). YP1 (240 mg) was subjected to Sephadex LH-20 (MeOH, 90 g) to afford three main fractions (YP1a–YP1c). YP1a (130 mg) was subjected to on VLC RP-18(20 g), eluted with a MeOH–H_2O (3:17–6:14) gradient system, to afford 2 main fractions (YP1a1–YP1a2). YP1a1 (24 mg) was repeatedly purified on Sephadex LH-20 (40 g), eluted with MeOH, and VLC silica gel H(2.0 g) eluted with $CHCl_3$-Me_2CO (6:1 and 5:1, v/v, respectively) to yield compound **3** (6 mg). YP1a2 (33 mg) was repeatedly purified using Sephadex LH-20 (40 g), eluted with MeOH and VLC silica gel H (2.5 g), eluted with a $CHCl_3$–Me_2CO (10:1–4:1) gradient system to yield compound **4** (5 mg). YP2 (300 mg) was subjected to Sephadex LH-20 (90 g), eluted with MeOH to afford 9 fractions (YP2a–YP2i). YP2g was applied to a VLC RP-18 column (20 g), eluted with MeOH–H_2O (20:80 and 23:77) system, to afford one main fraction (YP2g1) which was repeatedly purified by preparative TLC ($CHCL_3$–MeOH–HCOOH, 100:10:1, v/v) and Sephadex LH-20 (10 g) eluted with MeOH to give compound **1** (5 mg). YP6 (420 mg) was subjected to Sephadex LH-20 (90 g), eluted with MeOH to afford 2 main fractions (YP6a–YP6b). The YP6b fraction was applied to a VLC RP-18 column (20 g) eluted with a MeOH-H_2O (7:13–9:11) gradient system to afford

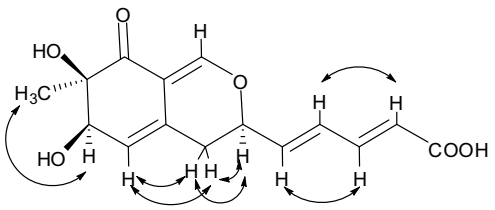

Fig. 3. Key NOE correlations of compound **1**.

three fractions (YP6b1–YP6b3). The YP6b2 fraction was repeatedly purified by Sephadex LH-20 (40 g), eluted with MeOH and silica gel (1.9 g) eluted with petroleum ether–EtOAC (6:1 and 5:1) to afford compound **2** (9 mg).

Compound **1**: Amorphous solid; $[\alpha]_D^{25} - 84.0(MeOH; c0.76)$; $UV\lambda_{max}^{MeOH}$nm$(\log\epsilon)$: 323(4.27), 244(4.70)nm; IRν_{max}^{KBr}cm^{-1} : 3429, 2927, 1690, 1667, 1619, 1156, 1104, 999 cm^{-1}. For ^{1}H (MeOH, 100 MHz), and ^{13}C NMR(MeOH, 100 MHz) spectroscopic data, see Table 1; HR-ESI-MS (negative ion) *m*/*z* 291.0872 (calc. for $C_{15}H_{15}O_6$ [M−H]$^-$, 291.0868).

Compound **2**: Amorphous solid; $[\alpha]_D^{25} - 34.1(MeOH; c0.33)$; $UV\lambda_{max}^{MeOH}$nm$(\log\epsilon)$: 326(4.25), 226(4.72)nm;IRν_{max}^{KBr}cm^{-1}: 3425, 2929, 1665, 1617, 1159, 1104, 988 cm^{-1}. For H (MeOH, 400 MHz) and ^{13}C NMR (MeOH, 100 MHz) spectroscopic data, see Table 1; HR-ESI-MS (positive ion) *m*/*z* 285.1107 (calc. for $C_{15}H_{18}O_4Na$ [M+Na]$^+$, 285.1102).

Compound **3**: Amorphous solid; $[\alpha]_D^{25} - 47.5^\circ(MeOH; c0.47)$; $UV\lambda_{max}^{MeOH}$nm$(\log\epsilon)$: 321(4.17), 225(4.11)nm;IRν_{max}^{KBr}cm^{-1}: 3423, 2918, 1665, 1616, 1165, 1107, 972 cm^{-1}. For ^{1}H (MeOH, 500 MHz) and ^{13}C NMR (MeOH, 125 MHz) spectroscopic data, see Table 1; HR-ESI-MS (positive ion) *m*/*z* 319.1167 (calc. for $C_{15}H_{20}O_6Na$ [M+Na]$^+$, 319.1157).

Compound **4**: Amorphous solid; $[\alpha]_D^{25} - 129.6(MeOH; c0.80)$; $UV\lambda_{max}^{MeOH}$nm$(\log\epsilon)$: 323(4.11), 230(4.05)nm;IRν_{max}^{KBr}cm^{-1}: 3440, 2923, 1626, 1164, 1107, 1075 cm^{-1}. For ^{1}H (MeOH, 125 MHz) and ^{13}C NMR (MeOH, 125 MHz) spectroscopic data, see Table 1; HR-ESI-MS (negative ion) *m*/*z* 295.1176 (calc. for $C_{15}H_{19}O_6$ [M−H]$^-$, 295.1181).

4.6. Anti-HIV assay

The cellular toxicity of compounds on C8166 cells was assessed by the MTT colorimetric assay as described previously (Wang et al., 2004). Cell viability (% of control) was calculated by measuring absorbance values. CC_{50} was defined as the concentration of the sample at which the absorbance value was reduced by 50%.

The cytopathic effect was measured by counting the number of syncytia (multinucleated giant cell) in each well under an inverted microscope (Wang et al., 2007). The percentage inhibition of syncytial cell formation was calculated by percentage of syncytial cell numbers in compound treated cultures to that of infected control culture. The concentration of the antiviral sample reducing HIV-1 replication by 50% (EC_{50}) was determined from the dose response curve. The therapeutic index (TI) was calculated from the ratio of CC_{50}/EC_{50} (see here Table 2).

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