

Article

Three New Oblongolides from *Phomopsis* sp. XZ-01, an Endophytic Fungus from *Camptotheca acuminata*

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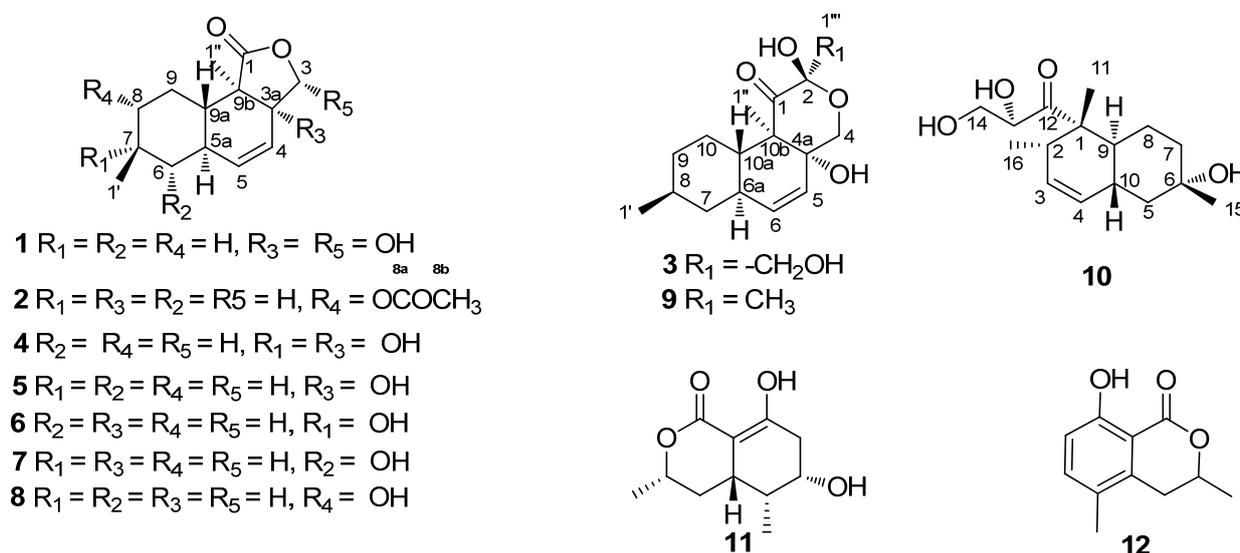
Abstract: Four new metabolites, including three new oblongolides named C1, P1, and X1 (**1-3**) and 6-hydroxyphomodiol (**10**), along with eight known compounds – oblongolides B (**4**), C (**5**), D (**6**), O (**7**), P (**8**) and U (**9**), (3*R*,4*aR*,5*S*,6*R*)-6-hydroxy-5-methylramulosin (**11**), and (3*R*)-5-methylmellein (**12**) – were isolated from the endophytic fungal strain *Phomopsis* sp. XZ-01 of *Camptotheca acuminata*. Their structures were elucidated by spectroscopic analyses, including ¹H- and ¹³C-NMR, 2D NMR (HSQC, HMBC, ¹H-¹H COSY and NOESY) and HR-FT-MS. Cytotoxic activities of these compounds were evaluated. Some of them showed weak selective activities.

Keywords: *Camptotheca acuminata*; endophytic fungus; *Phomopsis* sp. XZ-01; oblongolides; new metabolites

1. Introduction

Endophytes, especially those found in medicinal plants, have drawn a lot of attention for the past few years as a rich and reliable source of bioactive and chemically novel compounds with huge medicinal and agricultural potential [1]. In the course of our exploration for bioactive or new chemical entities from the endophytic fungus of *Camptotheca acuminata* Decne (Cornaceae), numerous new compounds were obtained [2,3]. Continuous research on the secondary metabolisms of another endophytic fungus of *Camptotheca acuminata* (*Phomopsis* sp. XZ-01), led to the discovery of three new oblongolides C1 (**1**), P1 (**2**), and X1 (**3**), oblongolides B (**4**) [4], C (**5**) [4], D (**6**) [4], O (**7**) [3], P (**8**) [3] and U (**9**) [3], the new phomodiol 6-hydroxyphomodiol (**10**), (3*R*,4*aR*,5*S*,6*R*)-6-hydroxy-5-methylramulosin (**11**) [5], and (3*R*)-5-methylmellein (**12**) [6]. In this paper, we report the isolation and structural elucidation of compounds **1-12** (Figure 1) and their anticancer activities.

Figure 1. Structures of compounds **1-12**.

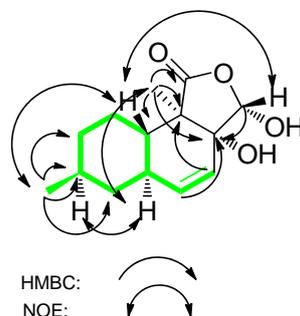


2. Results and Discussion

We obtained oblongolide C1 (**1**) as white needles and determined it to have the molecular formula $C_{14}H_{20}O_4$ by HR-FT-MS. The ^{13}C -NMR, DEPT and HSQC spectra of compound **1** showed 14 carbon signals: two methyl groups, three methylene groups, three methine groups, one hemiacetal methine (δ_C 100.6), a disubstituted olefin (δ_C 137.8 and 124.2), an oxygenated quaternary carbon (δ_C 78.7), a lactone carbonyl (δ_C 176.6), and a quaternary carbon. The 1H - 1H COSY correlations between H-4 and H-5, H-5 and H-5a, H-5a and H-6, H-5a and H-9a, H-6 and H-7, H-7 and H-1', H-8 and H-7, H-8 and H-9, H-9a and H-9 established the structure of a 9-carbon moiety (Figure 2, in green). Key HMBC correlations from H-1'' to C-1, C-3a, C-9a and C-9b, from H-5 to C-3a, from H-4 to C-9b, and from H-3 to C-3a established the planar structure of **1**. The relative configuration of **1** was deduced on the basis of NOESY spectroscopic data. The NOE correlations between H-7 and H-5a and between H-5a and H-1'' established the α -orientations of H-5a, H-7 and H-1''. NOESY cross-peaks from H-3 to H-9a and from H-9a to H-1' indicated the β -orientations of H-3, H-9a and H-1'. A comparison of the 1H and ^{13}C -NMR spectra of **1** with that of oblongolide C indicated that **1** was the 3 α -hydroxy derivative of oblongolide C [4].

Therefore, we determined the structure of **1** to be 3 α -hydroxyoblongolide C and it was named as oblongolide C1 for consistency with the literature [4].

Figure 2. Key HMBC and NOE Correlations of compound **1**.



Oblongolide P1 (**2**) was isolated as a white powder. The molecular formula $C_{16}H_{22}O_4$ was deduced from HR-FT-MS and ^{13}C -NMR. NMR data of **2** were similar to those of **1**, except that the hemiacetal methine [δ_H 5.69 (1H, d, $J = 11.5$ Hz) and δ_C 100.6, CH-3], quaternary carbon (δ_C 78.7, C-3a) and methylene [δ_H 1.82 (1H, m), δ_H 0.91 (1H, m) and δ_C 34.6, CH-8] in **1** were replaced by oxymethylene [δ_H 4.44 (1H, t, $J = 8.6$ Hz), δ_H 3.85 (1H, dd, $J = 10.9, 8.9$ Hz) and δ_C 70.1, CH₂-3], methine [δ_H 2.78 (1H, m) and δ_C 44.6, CH-3a], oxymethine [δ_H 4.54 (1H, dt, $J = 10.8, 4.4$ Hz) and δ_C 77.1, CH-8], and there was an acetyl group in **2**. Key HMBC correlations from H-8 to C-8a, C-1' and C-9a, from H-1' to C-6, C-7 and C-8, from H-1'' to C-1, C-3a, C-9a and C-9b indicated the planar structure of **2**. We determined the relative configuration of **2** by analysis of the NOESY spectrum. The NOE correlations between H-8 and H-1', between H-8 and H-9a, between H-8 and H-9 β , and between H-1' and H-6 β established the β -orientations of H-1', H-8 and H-9a. The NOE correlations between H-3a and H-1'', between H-3 α and H-3a and between H-1'' and H-5a indicated the α -orientations of H-1'', H-3a and H-5a. A comparison of the 1H - and ^{13}C -NMR data of **2** with those of oblongolide P [3] revealed that these two compounds had similar structures, except that an acetyl group was attached to the C-8 hydroxyl group in **2**. Therefore, we determined **2** to be 8-acetyloblongolide P and named it oblongolide P1.

Table 1. 1H - and ^{13}C -NMR spectroscopic data of compounds **1** and **2** (**1** and **2** at 600 MHz, $CDCl_3$, chemical shift values are in ppm relative to TMS; multiplicity and J values (in Hz) are presented in parentheses).

No.	1		2	
	δ_H	δ_C	δ_H	δ_C
1		176.6		179.4
3 α			4.44 (t, 8.6)	70.1
3 β	5.69 (d, 11.5)	100.6	3.85 (dd, 8.9, 10.9)	77.1
3a		78.7	2.78 (m)	44.6
4	5.53 (dd, 10.2, 2.8)	124.2	5.62 (dd, 12.8, 2.5)	122.2
5	5.79 (d, 9.9)	137.8	5.65 (d, 12.8)	133.0
5a	2.03 (m)	36.3	1.97 (m)	35.4
6 α	0.84 (q, 12.4)	41.0	1.00 (m)	39.0

Table 1. *Cont.*

6 β	1.90 (m)	41.0	1.94 (m)	39.0
7	1.50 (m)	32.7	1.68 (m)	37.4
8 α	0.91 (m)	34.6		
8 β	1.82 (m)	34.6	4.54 (dt, 4.4, 10.8)	77.1
9 α	1.34 (dd, 12.4, 3.1)	25.6	1.38 (q, 12.2)	31.1
9 β	1.79 (m)	25.6	2.10 (m)	31.1
9a	1.49 (m)	44.1	1.50 (m)	37.3
9b		51.5		42.8
1'	0.93 (d, 6.6)	22.2	0.94 (d, 6.5)	18.0
1''	1.18 (s)	9.5	1.16 (s)	16.1
8a				170.5
8b			2.06 (s)	21.1

Oblongolide X1 (**3**) was obtained as white oil. Its molecular formula, C₁₆H₂₄O₅, was deduced on the basis of HR-FT-MS and ¹³C-NMR data. A comparison of the NMR data of **3** with those of known compound oblongolide X [7] indicated that **3** was a hydroxy-derivative of the latter. The HMBC correlations from H-1''' to C-1 and C-2 located the hydroxyl substitution at C-2. The NOE correlations between H-10a and H-1', between H-6a and H-8 and between H-6a and H-1'' determined the relative configuration of **3**. Therefore, we determined **3** to be 1'''-hydroxyoblongolide X and named it oblongolide X1.

Table 2. ¹H- and ¹³C-NMR spectroscopic data of compound **3** (**3** at 600 MHz, CDCl₃, chemical shift values are in ppm relative to TMS; multiplicity and *J* values (in Hz) are presented in parentheses).

No.	3	
	δ_{H}	δ_{C}
1	-	207.0
2	-	94.9
4 α	3.57 (d, 12.4)	66.1
4 β	4.63 (d, 12.4)	66.1
4a		78.2
5	5.36 (dd, 10.1, 2.8)	126.9
6	5.68 (dd, 10.1, 1.6)	136.2
6a	1.93 (m)	37.9
7 α	1.86 (m)	41.1
7 β	0.89 (m)	41.1
8	1.49 (m)	33.0
9 α	1.77 (m)	34.8
9 β	1.03 (m)	34.8
10 α	1.26 (m)	26.8
10 β	1.23 (m)	26.8

Table 2. *Cont.*

10a	2.33 (ddd, 11.5, 10.6 3.0)	43.8
10b		55.4
1'	0.93 (d, 6.5)	22.3
1□	1.09 (s)	10.4
1□	3.61 (d, 11.9)	65.2
1□ β	3.95 (d, 11.9)	65.2

Compound **10** had the molecular formula $C_{16}H_{26}O_4$, as established by HR-FT-MS and ^{13}C -NMR spectra. 1H - and ^{13}C -NMR data of **10** were similar to those of phomodiol [8], except that the methine signal [δ_H 1.46 (1H, m), CH-6] was replaced by a quaternary carbon (δ_C 70.0, C-6). Key HMBC correlations from H-15 to C-5, C-6 and C-7, from H-11 to C-1, C-2, C-9 and C-12, from H-16 to C-1, C-2 and C-3, from H-4 to C-2, C-5 and C-9 and from H-10 to C-3, C-6 and C-8 indicated the planar structure of **10**. The relative configuration of **10** was deduced on the basis of NOESY spectroscopic data. The NOE correlations between H-10 and H-11, between H-2 and H-11, between H-13 and H-11, between H-15 and H-10 and between H-9 and H-16 indicated β -orientation of the hydroxyl group (6-OH) and the α -orientation of the side chain attached to C-1. Therefore, the structure of **10** was determined. We named it 6-hydroxyphomodiol [8].

Table 3. 1H - and ^{13}C -NMR spectroscopic data of compound **10** (600 MHz, in $CDCl_3$, chemical shift values are in ppm relative to TMS; multiplicity and J values (in Hz) are presented in parentheses).

No.	10	
	δ_H	δ_C
1	–	5.15
2	2.18 (m)	39.5
3	5.58 (ddd, 9.9, 4.9, 2.6)	130.2
4	5.36 (d, 10.0)	129.0
5 α	1.28 (m)	45.5
5 β	1.75 (m)	45.5
6	–	70.0
7 α	1.56 (dt, 13.6, 4.4)	39.4
7 β	1.69 (dd, 14.1, 3.0)	39.4
8 α	1.09 (brs)	22.8
8 β	1.32 (m)	22.8
9	1.79 (m)	40.5
10	2.22 (m)	33.0
11	1.35 (s)	16.7
12	–	214.0
13	4.52 (brs)	75.7
14	4.03 (dd, 11.8, 3.6), 3.79 (dd, 11.7, 4.7)	63.3
15	1.27 (s)	31.6
16	0.84 (d, 7.0)	18.7

Besides the nine oblongolides, including three new ones, we isolated two more polyketides. We determined **11** to be (3*R*,4*aR*,5*S*,6*R*)-6-hydroxy-5-methylramulosin (**11**) [5] by a comparison of NMR data. This compound was previously isolated from a marine-derived fungus which was derived from the green alga *Codium fragile* [5]. The spectroscopic data of **12** were identical to those of the known compound (3*R*)-5-methylmellein, first isolated as the main phytotoxic metabolite of *Fusicoccum amygdale* [6].

Cytotoxicity

The results of cytotoxic tests of compounds **1-12** are shown in Table 4. They exhibited no significant activity against the three tested cancer cell lines.

Table 4. Biological Activities of Compounds **1-12**.

Compound	Inhibitory rate (%)		
	HeLa	A549	HepG2
Oblongolide C1 (1)	-	-	18.01 ± 0.86
Oblongolide P1 (2)	-	-	28.59 ± 1.04
Oblongolide X1 (3)	-	-	27.89 ± 1.2
Oblongolide B (4)	-	-	-
Oblongolide C (5)	-	14.92 ± 0.86	-
Oblongolide D (6)	22.9 ± 0.78	13.82 ± 1.01	-
Oblongolide O (7)	-	-	-
Oblongolide P (8)	-	-	-
Oblongolide U (9)	-	18.76 ± 0.56	16.89 ± 1.01
6-Hydroxyphomodiol (10)	-	-	23.86 ± 1.21
(3 <i>R</i> ,4 <i>aR</i> ,5 <i>S</i> ,6 <i>R</i>)-6-Hydroxy-5-methylramulosin (11)	-	-	-
(3 <i>R</i>)-5-Methylmellein (12)	-	-	-

3. Experimental

3.1. General

Optical rotations were measured with a Perkin-Elmer 341 automatic polarimeter in methanol. IR spectra were recorded on a Nicolet AVATAR 330FT spectrometer. NMR spectra were taken on a Bruker Avance III-600 NMR spectrometer with TMS as an internal standard. HR-FT-MS data were acquired by using En Apex ultra 7.0 FT-MS. TLC was carried out using glass-precoated silica gel GF254 (Qingdao) and visualized under UV light or by spraying with vanillin (contains H₂SO₄) ethanol reagent. Sephadex LH-20 (40-70 μm, Amersham Pharmacia Biotech AB, Uppsala, Sweden), silica gel (200-300mesh, Qingdao Marine Chemical, Inc., Qingdao, China), and lichroprep reversed-phase RP-18 silica gel (40-63 μm, Merck, Darmstadt, Germany) were used for column chromatography (CC).

3.2. Fungal Material

The fungus (XZ-01) was isolated from current-year twigs (8-12 × 1-2 cm, length × diameter) of *Camptotheca acuminata* collected from the Jiangshi Natural Reserve, Shaowu, Fujian, China. It was identified as a non-sporulating fungus by traditional morphology. A BLAST search result showed that the internal transcribed spaces (ITS) sequence of XZ-01 was highly homologous (98% percent similarity) to that of a *Phomopsis* species (BCC 9789 [GU086404]), indicating that XZ-01 belongs to this genus.

3.3. Fermentation and Extraction

XZ-01 was cultivated on potato dextrose agar at 28 °C. The agar blocks were chopped and transferred into Erlenmeyer flasks (10 × 3 L), each containing 1 L of potato dextrose broth (PDB), and then fermented at 28 °C on a rotary shaker (150 rpm) for 7d. The culture was filtered to separate broth and mycelia. The culture broth was extracted with EtOAc (6 × 10 L) for six times. The combined organic layer was concentrated under vacuum to afford 3.2 g of residue.

3.4. Isolation and Spectral Data

The crude extract was separated into fifteen fractions (1-15) by column chromatography on RP-18 silica gel, eluted by methanol/H₂O (0:100, 30:70, 50:50, 70:30, and 100:0). Fraction 3 (100 mg) was subjected to silica gel CC (step gradient, elution with 0-10% MeOH in CHCl₃) to afford eleven fractions (3-1-3-11). Fractions 3-11 (4.9 mg) were further separated by silica gel CC (step gradient, elution with 22.2-33.3% EtOAc in hexane) to yield **4** (2.3 mg). Fraction 5 (92.1 mg) was separated by Sephadex LH-20 (elution with 100% methanol) to give three subfractions (fraction 5-1–5-3). Fraction 5-2 (23.6 mg) was purified by silica gel CC (step gradient, 7.7-50% EtOAc in hexane) to produce fraction 5-2-1. Fraction 5-2-1 (3.7 mg) was separated by silica gel (eluted with 50% CHCl₃ in petroleum ether) to afford **11** (2mg). Fraction 6 (225.8 mg) was fractionated by Sephadex LH-20 CC (elution with 100% MeOH) to provide nine fractions (6-1–6-9). Fraction 6-1 (28.8 mg) was further purified by silica gel CC (step gradient, 0-17% MeOH in CHCl₃) to furnish **6** (11.5 mg), **8** (2.6 mg) and **10** (6.4 mg). Fraction 7 (247.1 mg) was subjected to Sephadex LH-20 CC (elution with 100% MeOH) to give 5 fractions (7-1–7-5). Fraction 7-4 (36.1 mg) was purified by silica gel CC (elution with CHCl₃) to yield **7** (3.1 mg). Fraction 10 (109 mg) was fractionated by Sephadex LH-20 CC (elution with 100% MeOH) to provide two fractions (10-1–10-2). Fraction 10-1 (72 mg) was further purified by silica gel CC (step gradient, elution with 0-10% MeOH in CHCl₃) to give two subfractions (10-1-1 and 10-1-2). Fraction 10-1-2 (11.7 mg) was separated by silica gel CC (elution with 100% CHCl₃) to yield **3** (3.8 mg). Fraction 11 (318.3 mg) was separated by Sephadex LH-20 (elution with 100% MeOH) to provide five fraction (11-1–11-5). Fraction 11-5 (23.9 mg) was further purified by silica gel CC (elution with 10% CHCl₃ in petroleum ether) to afford **12** (22.8 mg). Fraction 11-3 (99 mg) was separated by silica gel CC (step gradient, elution with 0-10% MeOH in CHCl₃) to give **5** (34 mg) and **9** (2.3 mg). Fraction 12 (117 mg) was fractionated by Sephadex LH-20 CC (elution with 100% MeOH) to provide three fractions (12-1–12-3). Fraction 12-1 (12.8 mg) was further separated by silica gel CC (elution with 33.3% CHCl₃ in petroleum ether) to yield **2** (7.4 mg). Fraction 9 (232 mg) was

separated by Sephadex LH-20 (elution with 100% MeOH) to give two fractions (9-1–9-2). Fraction 9-2 (38 mg) was purified by silica gel CC (step gradient, 0-12.3% MeOH in CHCl₃) to yield **1** (5.7 mg).

Oblongolide C-1 (1): White needles; $[\alpha]_D^{20}$: -22.6 (c 0.0072, MeOH). IR (KBr) ν_{\max} 2919, 2359, 1219, 772, 668 cm⁻¹. ¹H- and ¹³C-NMR: see Table 1; HR-FT-MS: $m/z = 251.1281$ [M – H]⁻ (calcd. for C₁₄H₁₉O₄, 251.1283, Temperature: 180, Resolution: 125,508).

Oblongolide O-1 (2): White powder; $[\alpha]_D^{20}$: -72.2 (c 0.0025, MeOH). IR (KBr) ν_{\max} 3344, 2922, 1588, 1383, 772 cm⁻¹. ¹H- and ¹³C-NMR: see Table 1; HR-FT-MS: $m/z = 301.1418$ [M + Na]⁺ (calcd. for C₁₆H₂₂O₄Na, 301.1416, Temperature: 180, Resolution: 14,100).

Oblongolide X-1 (3): White oil; $[\alpha]_D^{20}$: -21.7 (c 0.0056, MeOH). IR (KBr) ν_{\max} 3422, 1583, 773, 685 cm⁻¹. ¹H- and ¹³C-NMR: see Table 2; HR-FT-MS: $m/z = 295.1541$ [M – H]⁻ (calcd. for C₁₆H₂₁O₅, 295.1545, Temperature: 180, Resolution: 106,466).

6-Hydroxyphomodiol (10): Transparent oil; $[\alpha]_D^{20}$: $+43.3$ (c 0.002, MeOH). IR (KBr) ν_{\max} 2365, 1223, 771 cm⁻¹. ¹H- and ¹³C-NMR: see Table 3; HR-FT-MS: $m/z = 305.1736$ [M + Na]⁺ (calcd. for C₁₆H₂₆NaO₄, 305.1729, Temperature: 180, Resolution: 36,000).

3.5. Biological Assay

Cancer cell lines were derived from the cell bank of The Chinese Academy of Sciences. Cells were seeded at a density of $5 \times 10^3/100$ μ L medium in 96-well microtiter plate and treated with the compounds at the concentration of 20 μ g/mL. Viable cells were incubated with MTT (5 mg/mL) for 4 h and formazan precipitate was dissolved in 100 μ L DMSO and the absorbance at 490 nm was measured by Multimode Detector DTX880 (Beckman Coulter).

4. Conclusions

Four new compounds, oblongolides C1 (**1**), P1 (**2**), X1 (**3**), 6-hydroxyphomodiol (**10**), together with eight known compounds were isolated from the endophytic fungus *Phomopsis* sp. XZ-01. oblongolides C1 (**1**), P1 (**2**), X1 (**3**), and 6-hydroxyphomodiol (**10**) showed modest selective activities against HepG2 cancer cell lines. Oblongolide C (**5**) exhibited minor selective activity against A549.

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Sample Availability: Samples of the compounds **1-12** are available from the authors.

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