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

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Received: 17 March 2011; in revised form: 1 April 2011 / Accepted: 2 April 2011 / Published: 19 April 2011

**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), (3R,4aR,5S,6R)-6-hydroxy-5-methylramulosin (11), and (3R)-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 \textit{Camptotheca acuminata} Decne (Cornaceae), numerous new compounds were obtained [2,3]. Continuous research on the secondary metabolisms of another endophytic fungus of \textit{Camptotheca acuminata} (\textit{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), (3R,4aR,5S,6R)-6-hydroxy-5-methyl-ramulosin (11) [5], and (3R)-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.](image-url)

2. Results and Discussion

We obtained oblongolide C1 (1) as white needles and determined it to have the molecular formula C\textsubscript{14}H\textsubscript{20}O\textsubscript{4} by HR-FT-MS. The \textsuperscript{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 (\(\delta_C\) 100.6), a disubstituted olefin (\(\delta_C\) 137.8 and 124.2), an oxygenated quaternary carbon (\(\delta_C\) 78.7), a lactone carbonyl (\(\delta_C\) 176.6), and a quaternary carbon. The \(^1\)H-\(^1\)H 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\(^{\prime}\), H-8 and H-7, H-8 and H-9 established the structure of a 9-carbon moiety (Figure 2, in green). Key HMBC correlations from H-1\(^{\prime\prime}\) 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\(^{\prime}\) established the \(\alpha\)-orientations of H-5a, H-7 and H-1\(^{\prime}\). NOESY cross-peaks from H-3 to H-9a and from H-9a to H-1\(^{\prime}\) indicated the \(\beta\)-orientations of H-3, H-9a and H-1\(^{\prime}\). A comparison of the \(^1\)H and \textsuperscript{13}C-NMR spectra of 1 with that of oblongolide C indicated that 1 was the 3\(\alpha\)-hydroxy derivative of oblongolide C [4].
Therefore, we determined the structure of 1 to be 3α-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, CH_{2}-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-9β, and between H-1′ and H-6β established the β-orientations of H-1′, H-8 and H-9a. The NOE correlations between H-3a and H-1″, between H-3α 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 $^{1}$H- 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-acetylobolngolide P and named it oblongolide P1.

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

| No. | δ_H  | δ_C  | δ_H  | δ_C  |
|-----|------|------|------|------|
| 1   | 176.6|      | 179.4|      |
| 3α  | 5.69 (d, 11.5) | 100.6| 5.62 (dd, 12.8, 2.5) | 122.2|
| 3β  | 78.7 | 2.78 (m) | 137.8| 5.65 (d, 12.8) | 133.0|
| 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| 1.97 (m) | 35.4|
| 5a  | 2.03 (m) | 36.3| 1.00 (m) | 39.0|
| 6α  | 0.84 (q, 12.4) | 41.0|      |      |
Oblongolide X1 (3) was obtained as white oil. Its molecular formula, C_{16}H_{24}O_{5}, was deduced on the basis of HR-FT-MS and $^{13}$C-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. $^1$H- and $^{13}$C-NMR spectroscopic data of compound 3 (3 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. | $\delta_H$ | $\delta_C$ |
|-----|------------|------------|
| 1   | -          | 207.0      |
| 2   | -          | 94.9       |
| 4$\alpha$ | 3.57 (d, 12.4) | 66.1 |
| 4$\beta$ | 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$\alpha$ | 1.86 (m) | 41.1       |
| 7$\beta$ | 0.89 (m) | 41.1       |
| 8   | 1.49 (m)   | 33.0       |
| 9$\alpha$ | 1.77 (m) | 34.8       |
| 9$\beta$ | 1.03 (m) | 34.8       |
| 10$\alpha$ | 1.26 (m) | 26.8       |
| 10$\beta$ | 1.23 (m) | 26.8       |

Table 1. Cont.
Compound 10 had the molecular formula C_{16}H_{26}O_{4}, as established by HR-FT-MS and $^{13}$C-NMR spectra. $^1$H- and $^{13}$C-NMR data of 10 were similar to those of phomodiol [8], except that the methine signal [$\delta_H$ 1.46 (1H, m), CH-6] was replaced by a quaternary carbon ($\delta_C$ 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 $\beta$-orientation of the hydroxyl group (6-OH) and the $\alpha$-orientation of the side chain attached to C-1. Therefore, the structure of 10 was determined. We named it 6-hydroxyphomodiol [8].

Table 2. Cont.

| No. | $\delta_H$          | $\delta_C$ |
|-----|---------------------|------------|
| 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 $\beta$ | 3.95 (d, 11.9) | 65.2       |

Table 3. $^1$H- 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. | $\delta_H$          | $\delta_C$ |
|-----|---------------------|------------|
| 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$\alpha$ | 1.28 (m)  | 45.5       |
| 5$\beta$ | 1.75 (m)  | 45.5       |
| 6   | –                   | 70.0       |
| 7$\alpha$ | 1.56 (dt, 13.6, 4.4) | 39.4       |
| 7$\beta$ | 1.69 (dd, 14.1, 3.0) | 39.4       |
| 8$\alpha$ | 1.09 (burs) | 22.8       |
| 8$\beta$ | 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 (burs)         | 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 \((3R,4aR,5S,6R)-6\)-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 \((3R)-5\)-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.

| 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 ± 1.2 |
| \((3R,4aR,5S,6R)-6\)-Hydroxy-5-methylramulosin (11) | - | - | - |
| \((3R)-5\)-Methylmellein (12) | - | - | - |

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

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 five 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 afford 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; [α]₂₀° −22.6 (c 0.0072, MeOH). IR (KBr) νmax 2919, 2359, 1219, 772, 668 cm⁻¹. ⋅H- 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; [α]₂₀° −72.2(c 0.0025, MeOH). IR (KBr) νmax 3344, 2922, 1588, 1383, 772 cm⁻¹. ⋅H- 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; [α]₂₀° −21.7(c 0.0056, MeOH). IR (KBr) νmax 3422, 1583, 773, 685 cm⁻¹. ⋅H- 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; [α]₂₀° + 43.3(c 0.002, MeOH). IR (KBr) νmax 2365, 1223, 771 cm⁻¹. ⋅H- 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 × 10³/100 µL medium in 96-well microtitter plate and treated with the compounds at the concentration of 20 µg/mL. Viable cells were incubated with MTT (5 mg/mL) for 4 h and formazan precipitate was dissolved in 100 µL 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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