http://dx.doi.org/10.5012/bkcs.2014.35.10.3059

Butyrolactones Derivatives from the Fermentation Products of an Endophytic Fungus *Aspergillus versicolor*

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Received June 13, 2014, Accepted July 7, 2014

Two new butyrolactones, asperphenol A (**1**) and B (**2**), together with four known butyrolactones (**3**-**6**) were isolated from the fermentation products of an endophytic fungus *Aspergillus versicolor.* Their structures were elucidated by spectroscopic methods including extensive 1D- and 2D-NMR techniques. Compounds **1**-**6** were also tested for their anti-tobacco mosaic virus (anti-TMV) activities. The results showed that compound **2** exhibited high anti-TMV activity with inhibition rate of 46.7%. The other compounds also exhibited potential anti-TMV activities with inhibition rates in the range of 21.8-28.4%.

Key Words : Butyrolactones, *Aspergillus versicolor*, Structure elucidation, Anti-TMV activity

Introduction

The genus *Aspergillus* (Moniliaceae), with over 180 species, has attracted considerable attention as a rich source of alkaloids, terpenoids, xanthones, and polyketides, some of which showed antifungal, antibacterial, antifouling, and cytotoxic activities.[1-3] Butyrolactones were mainly found as metabolites from fungi and high plants in nature.[4] They appeal to medicinal chemists because of their pronounced pharmacological effects including antibacterial,[5,6] anti-tumor,[7,8] anti-inflammatory,[9,10] anti-virus,[11,12] and the like.

In previous studies, some butyrolactones were isolated from the fermentation products of fungus *Aspergillus*.[13-16] With the aim of searching for new bioactive metabolites from the fermentation products of microbe, an endophytic *Aspergillus versicolor* were isolated from the rhizome of *Paris polyphylla* var. *yunnanensis*, collected in Shizhong, Yunnan province, P. R. China, and the chemical constituents of it fermentation products were investigated. As a result, two new (**1**-**2**) and four known (**4**-**6**) butyrolactones were isolated. The structures of new compounds were elucidated on the basis of a comprehensive analysis of the ^{1}H and ^{13}C NMR. In addition, the anti-tobacco mosaic virus (anti-TMV) active of compounds **1**-**6** was evaluated. The details of the isolation, structure elucidation, and anti-tobacco mosaic virus active of the compounds isolated are reported in this article.

Results and Discussion

The fermented substrate was extracted with 70% aqueous acetone. The extract was subjected repeatedly to column chromatography on silica gel, RP-18, and semi-preparative RP-HPLC separation to afford compounds **1**-**6**. The structures of **1**-**6** were shown in Figure 1, and the ^{1}H and ^{13}C NMR data of the compounds **1** and **2** were listed in Table 1. By comparing with the literature, the known compounds

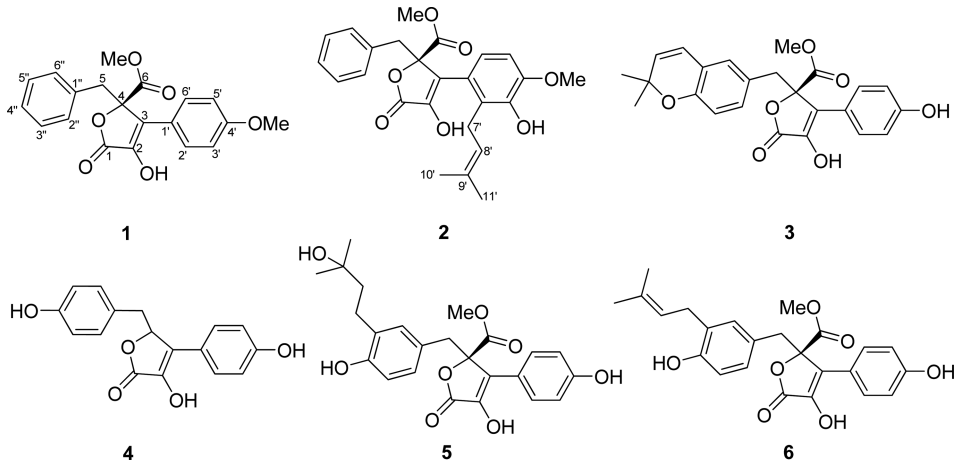

Figure 1. The structures of compounds **1**-**6**.

Table 1. ^{1}H and ^{13}C NMR Data of compounds **1** and **2** (δ in ppm, 500 and 125 MHz)

No.	Compound **1**[a]		Compound **2**[b]	
	δ_C (m)	δ_H (m, *J*, Hz)	δ_C (m)	δ_H (m, *J*, Hz)
1	169.3 s		169.1 s	
2	137.7 s		138.3 s	
3	128.1 s		128.6 s	
4	85.6 s		85.0 s	
5	39.3 t	3.53, 3.62 d (14.5)	37.9 t	3.35, 3.43 d (14.6)
6	169.9 s		170.0 s	
1′	121.8 s		123.2 s	
2′	130.6 d	7.61 d (8.8)	134.5 s	
3′	116.0 d	6.91 d (8.8)	143.9 s	
4′	160.3 s		150.0 s	
5′	116.0 d	6.91 d (8.8)	112.9 d	6.96 d (8.5)
6′	130.6 d	7.61 d (8.8)	119.2 d	7.62 d (8.5)
7′			27.3 t	3.60, d (6.8)
8′			124.1 d	5.19, t (6.8)
9′			131.4 s	
10′			18.1 q	1.81 s
11′			25.0 q	1.62 s
1″	132.8 s		132.4 s	
2″,6″	130.4 d	6.84 d (8.0)	130.5 d	6.86 d (8.0)
3″,5″	127.9 d	7.13 dd (7.6, 8.0)	127.8 d	7.15 dd (7.6, 8.0)
4″	127.2 d	7.10 d (7.6)	127.2 s	7.18 d (7.6)
4′-OMe	55.9 q	3.82 s	56.0 q	3.82 s
6-OMe	53.5 q	3.77 s	53.1 q	3.79 s
3′-OH				12.47 s

[a]in $CDCl_3$. [b]in $(CD_3)_2CO$

were identified as aspernolide E (**3**),[15] 4-(4-hydroxyphenyl)-5-(4-hydroxyphenylmethyl)-2-hydroxyfurane-2-one (**4**),[16] aspernolide B (**5**),[14] and butyrolactone I (**6**).[17]

Asperphenol A (compound **1**) was obtained as white solid. The molecular formula $C_{20}H_{18}O_6$ of **1** was determined by HRESIMS which showed pseudomolecular ion peaks $[M+Na]^+$ at 377.1006 (calcd. 377.1001). The IR spectrum showed the presence of ester/lactone carbonyl at 1740 and 1730 cm^{-1}, hydroxyl were evident at 3342 cm^{-1}, and the presence of the absorptions at 1610, 1521, and 1496 cm^{-1} was suggestive of aromaticity in the molecule. The ^{1}H NMR signals revealed the presence of a monosubstituted benzene moiety (δ_H 6.84 d, J = 8.0, 2H; 7.13, dd, J = 7.6, 8.0, 2H; 7.10, d, J = 7.6, 1H), a 1,4-disubstituted benzene moiety (δ_H 7.61, d, J = 8.8, 2H; 6.91, d, J = 8.8, 2H), a methylene protons (δ_H 3.53, 3.62, d each, J = 14.5), and two methoxy proton (δ_H 3.79 s and 3.82 s). Its ^{13}C NMR showed the presence of a monosubstituted benzene moiety [δ_C 132.8 s, 130.4 d (2C), 127.9 d (2C) 127.2 d], a 1,4-disubstituted benzene moiety [δ_C 121.8 s, 130.6 d (2C), 116.0 d (2C) 160.3 s] moiety, a methoxycarbonyl group (δ_C 169.9 s, 53.5 q), a ester carbonyl (δ_C 169.3 s), a pair of olefenic carbon (δ_C 137.7 s and 128.1 s), a methylene carbon (δ_C 39.3 t), and a quaternary carbon (δ_C 85.6 s). The molecular formula $C_{20}H_{18}NaO_6$ requires 12 degrees of unsaturation. The presence of two aromatic rings accounts for eight, while two carbonyls and one olefenic

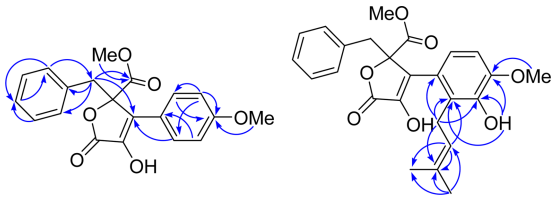

Figure 2. Key HMBC (↷) correlations of (**1**) and (**2**).

carbons account for another three, which makes a total of eleven degrees of unsaturation. Therefore **1** must possessing one aliphatic ring in addition to two aromatic rings. The tropical carbon signals (δ_C 169.3 s, 137.7 s, 128.1 s, 85.6 s, 39.3 t, 169.9 s) indicated that compound **1** has the butyrolactone skeleton.[15-17] A detailed comparison of the NMR data of **1** with these of methyl 3-(4-hydroxyphenyl)-4-oxidanyl-5-oxidanylidene-2-(phenylmethyl) furan-2-carboxylate (**7**) [18] revealed that the only difference due to a hydroxy group at C-4′ in **7** was substituted by a methoxy group in **1**. This difference was supported by the HMBC correlations (Figure 2) from 4′-OMe (δ_H 3.82) to C-4′ (δ_C 160.3). Therefore, the structure of **1** was assigned as shown.

Compound **2** was also isolated as a pale yellow gum, and its molecular formula was determined as $C_{25}H_{26}NaO_7$ through HRESI-MS analysis (pseudomolecular ion $[M+Na]^+$ at *m/z* 461.1571). The ^{1}H and ^{13}C NMR data of **2** were very similar to these of **1** (see Table 1) at the positions of C-1 ~ C-6 and C-1″ ~ C-6″. The maior difference resulted from the the chemical siift varietions on the other aromatic ring (C-1′ ~ C-6′), and the appearance of an additional prenyl group [δ_C 27.3 t, 124.1 d, 131.4 s, 18.1 q, 25.0 q; δ_H 3.60 d (J = 6.8), 5.19 t (J = 6.8), 1.81 s, and 1.62 s] and a phenolic hydroxy group (δ_H 12.47 s). These indicated that the additional prenyl group and phenolic hydroxy group were located at this aromatic ring. Long-range HMBC correlations of H-7′ (δ_H 3.60) to C-1′ (δ_C 123.2), C-2′ (δ_C 134.5), C-3′ (δ_C 143.9) were observed in **2**. This indicated that the prenyl group was attached to C-2′. The HMBC correlations of methoxy protons (δ_H 3.82) with C-4′ (δ_C 150.0) revealed that the methoxy group were located at C-4′. One phenolic hydroxy group located at C-3′ were supported by the HMBC correlations of the phenolic hydroxy proton signal (δ_H 12.47) with C-2′ (δ_C 134.5), C-3′ (δ_C 143.9) and C-4′ (δ_C 150.0). The typical ^{1}H NMR signals of the aromatic protons, H-5 (δ_H 6.96, d, J = 8.5) and H-6 (δ_H 8.06, d, J = 8.5) also supported the above substituent positions. Thus, the structure of **2** was established, and gives the trivail name of asperphenol B.

Since some butyrolactones are known to exhibit potential anti-virus activities,[11,12] compounds **1-6** were tested for their anti-TMV activities. The inhibitory activities of compounds **1-6** against TMV replication were tested using the half-leaf method.[19] Ningnanmycin, a commercial product for plant disease in China, was used as a positive control. The antiviral inhibition rates of compounds **1-6** at the concentration of 20 μM were listed Table 2. The results showed that compound **2** exhibited high anti-TMV activity with inhibition rate of 46.7%. The inhibition rate is higher than that of

Table 2. TMV infection inhibition activities of compounds **1-6**

Compounds	Inhibition rates (%)	Compounds	Inhibition rates (%)
1	22.5 ± 3.6	**5**	28.4 ± 3.2
2	46.7 ± 3.0	**6**	24.3 ± 2.8
3	25.2 ± 2.8	ningnamycin	30.6 ± 3.4
4	21.8 ± 2.4		

All results are expressed as mean ± SD; n = 3 for all groups.

positive control. The other compounds also showed potential anti-TMV activities with inhibition rates in the range of 21.8-28.4%, respectively.

Experimental

General Experimental Procedures. Optical rotations were measured with a Horiba SEPA-300 polarimeter. UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. 1D and 2D NMR spectra were recorded on a DRX-400 or 500 NMR spectrometers with TMS as internal standard. Unless otherwise specified, chemical shifts (δ) are expressed in ppm with reference to the solvent signals. HRESIMS was performed on a VG Autospec-3000 spectrometer. Semipreparative HPLC was performed on a Shimadzu LC-8A preparative liquid chromatograph with Zorbax PrepHT GF (21.2 mm × 25 cm) or Venusil MP C_{18} (20 mm × 25 cm) columns. Column chromatography was performed using silica gel (200–300 mesh, Qing-dao Marine Chemical, Inc., Qingdao, People's Republic of China), Lichroprep RP-18 gel (40–63 μm, Merck, Darmstadt, Germany), and MCI gel (75–150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan). The fractions were monitored by TLC, and spots were visualized by heating silica gel plates sprayed with 5% H_2SO_4 in EtOH.

Fungal Material. The culture of *Aspergillus versicolor* was isolated from the rhizome of *Paris polyphylla* var. *yunnanensis* collected from Shizhong, Yunnan province, People's Republic of China, in 2007. The strain was identified by one of authors (Gang Du) based on the analysis of the ITS sequence. It was cultivated at room temperature for 7 days on potato dextrose agar at 28 °C. Agar plugs were inoculated into 250 mL Erlenmeyer flasks each containing 100 mL potato dextrose broth and cultured at 28 °C on a rotary shaker at 180 rpm for five days. Large scale fermentation was carried out in 200 Fernbach flasks (500 mL) each containing 100 g of rice and 120 mL of distilled H_2O. Each flask was inoculated with 5.0 mL of cultured broth and incubated at 25 °C for 45 days.

Extraction and Isolation. The fermented substrate (10.8 kg) was extracted four times with 70% MeOH (4 × 10 L) at room temperature and filtered. The crude extract (287 g) was applied to silica gel (200–300 mesh) column chromatography, eluting with a $CHCl_3$-CH_3OH gradient system (9:1, 8:2, 7:3, 6:4, 5:5), to give six fractions A–E. The further separation of fraction A (9:1, 22.6 g) by silica gel column chromatography, eluted with $CHCl_3$-$(CH_3)_2CO$ (9:1, 8:2, 7:3, 6:4, 1:1), yielded mixtures A1–A5. Fraction A1 (9:1, 8.6 g) was subjected to silica gel column chromatography and preparative HPLC (68% methanol, flow rate 12 mL/min) to give **2** (22.5 mg) and **3** (15.2 mg). Fraction A2 (8:2, 4.6 g) was separated by silica gel column chromatography and preparative HPLC (63% MeOH, flow rate 12 mL/min) to give **5** (11.5 mg) and **6** (14.2 mg). Fraction A3 (7:3, 4.6 g) was separated by silica gel column chromatography and preparative HPLC (58% MeOH, flow rate 12 mL/min) to give **1** (11.5 mg) and **4** (14.2 mg).

Anti-TMV Assays. The Anti TMV activities were tested using the half-leaf method, and ningnanmycin, a commercial product for plant disease in China, was used as a positive control.

TMV (U1 strain) was obtained from the Key Laboratory of Tobacco Chemistry, Yunnan Academy of Tobacco Science. The virus was multiplied in *Nicotiana tabacum* cv. K326 and purified as previously described.[20] The concentration of TMV was adjusted to 20 mg/mL as determined by UV absorption. [virus concentration = (A_{260} × dilution ratio)/$E_{1\ cm}^{0.1\%,\ 260\ nm}$]. The purified virus was kept at −20 °C and diluted to 32 μg/mL with 0.01 M PBS before use.

Nicotiana glutinosa plants were cultivated in an insect-free greenhouse. Experiments were conducted when the plants grew to 5- to 6-leaf stage. The tested compounds were dissolved in DMSO and diluted with distilled H_2O to the required concentrations. A solution of equal concentration of DMSO was used as negative control; and ningnanmycin was used as positive control.

For the Half-Leaf Method, the virus was mixed with a solution of the test compound for 30 min before inoculated on the left side of a leaf of *N. glutinosa*, whereas the right side of the leaf was inoculated with a mixture of DMSO and virus as a control. The local lesion numbers were recorded 3-4 days after inoculation. Three leaf blades were used for each compound. The inhibition rates were calculated according to the formula: Inhibition Rate (%) = [(C-T)/C] × 100%, where C is the average number of local lesions in the control and T is the average number of local lesions in the treated leaves.

Asperphenol A (1): $C_{20}H_{18}O_6$, obtained as white amorphous powder; $[\alpha]_D^{24.8}$ +63.2 (c 0.20, MeOH); UV (MeOH) λ_{max} (log ε): 210 (4.18), 236 (3.38), 287 (3.62) nm; IR (KBr) ν_{max} 3342, 3020, 2970, 2938, 1740, 1730, 1610, 1521, 1496, 1435, 1382, 1263, 1180, 1120, 1065, 1022, 921, 876, 763 cm^{-1}; ^1H and ^{13}C NMR (500 and 125 MHz, in $CDCl_3$) see Table 1; ESIMS (positive ion mode) *m/z* 377 $[M+Na]^+$; HRESIMS (positive ion mode) *m/z* 377.1006 $[M+Na]^+$ (calcd 377.1001 for $C_{20}H_{18}NaO_6$).

Asperphenol B (2): $C_{25}H_{26}O_7$, obtained as white amorphous powder; $[\alpha]_D^{24.8}$ +79.8 (c 0.20, MeOH); UV (MeOH) λ_{max} (log ε): 210 (4.32), 246 (3.51), 302 (3.74) nm; IR (KBr) ν_{max} 3467, 3026, 2982, 2921, 1738, 1727, 1610, 1532, 1487, 1430, 1395, 1270, 1176, 1118, 1069, 1025, 947, 859, 774 cm^{-1}; ^1H and ^{13}C NMR see Table 1 (500 and 125 MHz, in $(CD_3)_2CO$); ESIMS (positive ion mode) *m/z* 461 $[M+Na]^+$; HRESIMS (positive ion mode) *m/z* 461.1571 $[M+Na]^+$ (calcd 461.1576 for $C_{25}H_{26}NaO_7$).

Acknowledgments. This research was supported by the National Natural Science Foundation of China (No. 21462051), the excellent Scientific and Technological Team of Yunnan High School (2010CI08), the Yunnan University of Nationalities Green Chemistry and Functional Materials Research for Provincial Innovation Team (2011HC008), and Open Research Fund Program of Key Laboratory of Ethnic Medicine Resource Chemistry (Yunnan University of Nationalities) (2010XY08).

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