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PHENYL DERIVATED BUTYROLACTONES FROM THE FERMENTATION PRODUCTS OF AN ENDOPHYTIC FUNGUS *ASPERGILLUS TERREUS*

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Abstract – Two new phenyl derivated butyrolactones, terrephenols A and B (**1** and **2**), together with four known butyrolactones (**3**-**6**) were isolated from the fermentation products of a fungus *Aspergillus terreus*. Their structures were elucidated by spectroscopic methods, including extensive 1D- and 2D- NMR techniques. The anti-tobacco mosaic virus (anti-TMV) activities of **1**-**6** were evaluated. Compounds **1** and **2** showed high anti-TMV activities with inhibition rates of 35.2% and 31.0 %, and **3**-**6** showed modest anti-TMV activity with inhibition rates of 15.4-24.6%, respectively. Compounds **1** and **2** were also tested for their cytotoxicities. **1** showed high cytotoxicity against A549 and MCF7 cell with IC_{50} values of 4.2 and 3.6 μM, and **2** showed high cytotoxicity against A549 and MCF7 cell with IC_{50} values of 3.9 and 4.8 μM, respectively.

In recent years, numerous metabolites possessing potent bioactivities have been isolated from strains of bacteria and fungi collected from diverse environments.[1,2] Fungi belonging to *Aspergillus* genera are one of the major contributors to the secondary metabolites of fungal origin.[3] *Aspergillus terreus*, is a fungus (mold) found worldwide in soil, decomposing vegetation and dust. It is commonly used in industry to produce important organic acids, such as itaconic acid and *cis*-aconitic acid as well as enzymes, like xylanase.[4] In addition, some metabolites produced by *A. versicolor* have been received more and more attentions from medicinal chemists because they exhibited various biological activities.[3-7]

Butyrolactones are a class of lactones with a four-carbon heterocyclic ring structure, and these

compounds were mainly found as metabolites from fungi and high plants in nature.[8] They pronounced potential pharmacological effects including antibacterial,[9,10] cytotoxicity,[11,12] anti-inflammatory,[13,14] anti-virus,[10,15] and the like. With the aim of multipurpose utilization endophytic fungus and identify bioactive natural products, the phytochemical investigation on fermentation products of the endophytic fungus *Aspergillus terreus* was carried out. As a result, two new (**1**-**2**), and four known (**4**-**6**) phenyl derivated butyrolactones were isolated. The structures of new compounds were elucidated on the basis of a comprehensive analysis of the ^{1}H, ^{13}C and 2D NMR spectra. In addition, the anti-tobacco mosaic virus (anti-TMV) activities of **1**-**6**, and the cytotoxicities of compounds **1** and **2** were evaluated. The details of the isolation, structure elucidation and biological activities of the compounds are reported in this article.

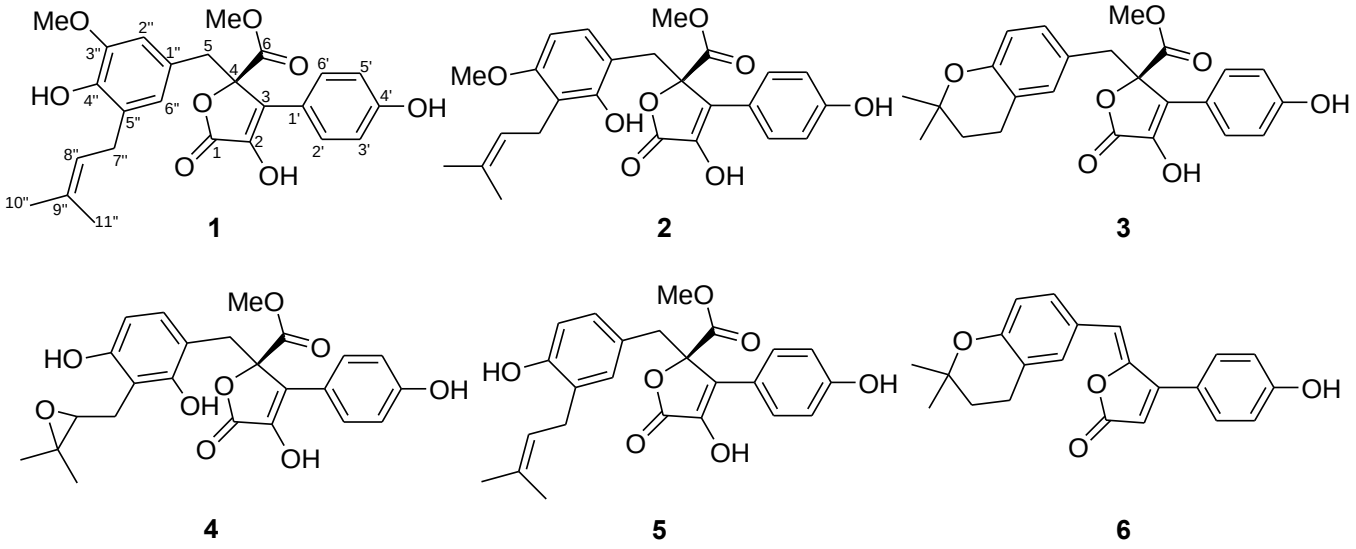

Figure 1. The structures of compounds **1** - **6**

A 70% aq. acetone extract prepared from fermentation products of the endophytic fungus *Aspergillus terreus* was subjected repeatedly to column chromatography on Silic gel, Sephadex LH-20, RP-18 and Preparative HPLC to afford compounds **1**-**6**, including two new phenyl derivated butyrolactones, terrephenols A and B (**1** and **2**), together with four known butyrolactones (**3**-**6**), aspernolide A (**3**),[7] butyrolactone III (**4**),[13] butyrolactone I (**5**),[13] and rubrolide S (**6**).[16] The structures of the compounds **1**-**6** were as shown in Figure 1, and the ^{1}H and ^{13}C NMR data of **1** and **2** were listed in Table 1.

Compound **1** was obtained as pale yellow gum. The molecular formula was determined to be $C_{25}H_{26}O_8$ by high resolution-electrospray ionization-mass spectra (HR-ESIMS), *m/z* 477.1520 $[M+Na]^+$ (calcd 477.1525 for $C_{25}H_{26}NaO_8$). The IR spectrum showed broad and intense absorption bands for hydroxy (3462), ester/lactone carbonyl (1740, 1725) and aromatic rings (1612, 1538, 1482). The ^{1}H NMR signals revealed the presence of a 1,4-disubstituted benzene moiety δ_H [7.68 d (8.8) 2H and 6.79 d (8.8) 2H], a 1,3,4,5-tetrasubstituted benzene moiety δ_H [6.68 d (2.2) and 6.90 d (2.2)], a prenyl group δ_H [3.20 d (6.7) 2H, 5.13 t (6.7) 1H, 1.55 s 3H, and 1.69 s 3H], a methylene protons δ_H [3.33, 3.42 d (14.6)], two methoxy

proton δ_H (3.69 s and 3.79 s), and two phenolic hydroxy protons (11.32 s and 11.67 s). Its ^{13}C NMR showed the presence of 1,4-disubstituted benzene moiety δ_C [121.4 s, 130.4 d (2C), 116.8 d (2C), 157.6 s], a 1,3,4,5-tetrasubstituted benzene moiety δ_C (133.1 s, 114.0 d, 151.1 s, 143.2 s, 131.2 s, 124.0 d), a prenyl group (27.0 t, 120.1 d, 131.9 s, 17.8 q, 25.7 q), one methoxycarbonyl group (δ_C 170.0 s, 52.6 q), one ester carbonyl δ_C (169.1 s), a pair of olefinic carbon signals δ_C (140.0 s and 127.0 s), one methylene carbon δ_C (40.0 t), and one oxidated quaternary carbon δ_C (85.5 s).

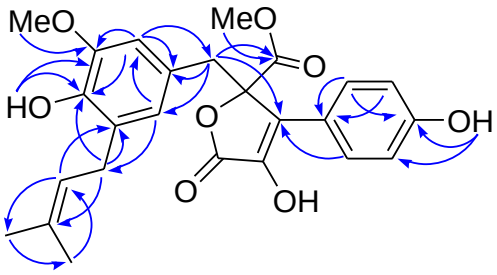

Figure 2. Key HMBC ( ) correlations of (**1**)

The molecular formula $C_{25}H_{26}O_8$ requires 13 degrees of unsaturation. The presence of two aromatic rings accounts for eight while two carbonyls and two olefinic carbons account for another four, which makes a total of twelve degrees of unsaturation. Therefore, **1** must posses one aliphatic ring in addition to two aromatic rings. The typical carbon signals δ_C (169.1 s, 140.0 s, 127.0 s, 85.5 s, 40.0 t, 170.0 s) indicated that **1** should be a phenyl derivated butyrolactone.[10,13] A detailed comparison of the NMR data of **1** with these of known compound, butyrolactone I (**5**) [13] revealed that the only difference due to the appearance of an addition methoxy group (δ_C 55.9 q, δ_H 3.79 s) in **1**. The HMBC correlation (Figure 2) of methoxy proton (δ_H 3.79) with C-3″ (δ_C 151.1) suggested the

Table 1. ^{1}H and ^{13}C NMR Data of compounds **1** and **2** (δ in ppm, in $(CD_3)_2CO$, 500 MHz)

No.	Compound **1**		Compound **2**	
	δ_C (m)	δ_H (m, *J*, Hz)	δ_C (m)	δ_H (m, *J*, Hz)
1	169.1 s		169.2 s	
2	140.0 s		139.0 s	
3	127.0 s		126.8 s	
4	85.5 s		85.1 s	
5	40.0 t	3.33, 3.42 d (14.6)	39.0 t	3.47, 3.52 d (14.4)
6	170.0 s		170.0 s	
1′	121.4 s		122.1 s	
2′,6′	130.4 d	7.68 d (8.8)	130.9 d	7.61 d (8.8)
3′,5′	116.8 d	6.79 d (8.8)	116.0 d	6.81 d (8.8)
4′	157.6 s		157.5 s	
1″	133.1 s		123.9 s	
2″	114.0 d	6.68 d (2.2)	152.1 s	
3″	151.1 s		118.2 s	
4″	143.2 s		156.6 s	
5″	131.2 s		106.4 d	6.43 d (8.2)
6″	124.0 d	6.90 d (2.2)	125.0 d	6.94 d (8.2)
7″	27.0 t	3.20 d (6.7)	27.3 t	3.28 d (6.8)
8″	120.1 d	5.13 t (6.7)	119.9 d	5.14 t (6.8)
9″	131.9 s		132.0 s	
10″	17.8 q	1.55 s	17.1 q	1.53 s
11″	25.7 q	1.69 s	25.1 q	1.70 s
6-OMe	52.6 q	3.69 s	53.0 q	3.70 s
3″-OMe	55.9 q	3.79 s		
4″-OMe			55.9 q	3.82 s
4′-OH		11.32 s		11.30 s
2′-OH				11.58 s
3″-OH		11.67 s		

position of methoxy group at C-3″. The prenyl group located at C-5″ was supported by the HMBC correlations of H-7″ (δ_H 3.20) with C-4″ (δ_C 143.2), C-5″ (δ_C 131.2), and C-6″ (δ_C 124.0), and of H-6″ (δ_H 6.90) with C-7″ (δ_C 27.0). Finally, two hydroxy groups were assigned to C-4′ and C-4″ on the basis of HMBC correlations between the hydroxy proton (δ_H 11.32) and C-3′,5′ (δ_C 116.8), and C-4′ (δ_C 157.6), as well as those between the other hydroxy proton (δ_H 11.67) and C-3″ (δ_C 151.1), C-4″ (δ_C 143.2), and C-5″ (δ_C 131.2). A methoxy group at C-3″ and prenyl group at C-5″ were also suppoted by the ROESY correlations of methoxy proton (δ_H 3.79) with C-2″ (δ_C 114.0) and H-7″ (δ_H 3.20) with C-6″ (δ_C 124.0), repectively. In addition, compound **1** showed the Cotton effects at 226 and 315 nm in CD spectrum, and the optical rotation valve of +68.4, respectively, which were similar to those of known compound,[12,13,17] indicating the presence of *R* configuration at C-4 in **1**. The structure of **1** is therefore determined, and gives the trivail name of terrephenol A.

Compound **2,** terrephenol B was assigned the molecular formula $C_{25}H_{26}O_8$ by its HRESIMS at *m/z* 477.1532 $[M+Na]^+$. The ^1H and ^{13}C NMR spectra of **2** are similar to those of compound **1** at the positions of C-1 ~ C-6 and C-1′ ~ C-6′. The difference in the positions of C-1″ ~ C-6″ suggested that the constituent positions at this aromatic ring should be varied. The further analysis its HMBC spectra suggested the position of phenolic hydroxy group at C-2″, the prenyl group at C-3″, and the methoxy group at C-4″. The typical protons signals [δ_H 6.43 d (8.2) and 6.94 d (8.2)] supported the 1,2,3,4-tetrasubstituted for this aromatic ring. Furthermore, a methoxy group at C-4″ was also supported by the ROESY correlation of methoxy proton (δ_H 3.82) with C-3″ (δ_C 118.2). Accordingly, the structure of **2** was determined as shown.

Since some butyrolactones are known to exhibit potential anti-virus activities,[10,15] compounds **1-6** were tested for their anti-TMV activities. The anti-TMV activities were tested using the half-leaf method.[18,19] Ningnanmycin (a commercial product for plant disease in China), was used as a positive control. Their antiviral inhibition rates at the concentration of 20 μM were listed in Table 2. The results revealed that **1** and **2** showed high anti-TMV activities with inhibition rate of 35.2% and 31.0 %, which is almost equivalent to that of ningnanmycin (30.2%). Compounds **3-6** showed modest anti-TMV activity with inhibition rate of 15.4-24.6%, respectively.

Table 2. TMV Infection Inhibition Activities of **1-6**

Compounds	Inhibition rate (%)	Compounds	Inhibition rate (%)
1	35.2 ± 3.3	**5**	21.8 ± 2.4
2	31.0 ± 3.0	**6**	15.4 ± 2.6
3	24.6 ± 3.2	ningnanmycin	30.2 ± 2.8
4	20.8 ± 2.5		

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

Since some butyrolactones are known to exhibit potential cytotoxicity,[11-13] the cytotoxicities of compounds **1** and **2** against five tumor cell lines (NB4, A549, SHSY5Y, PC3, and MCF7), with taxol as the positive control were tested using a previously reported procedure.[18,19] The results revealed that compound **1** showed high cytotoxicity against A549 and MCF7 cell with IC_{50} values of 4.2 and 3.6 μM, and **2** showed

high cytotoxicity against A549 and MCF7 cell with IC_{50} values of 3.9 and 4.8 μM, respectively.

EXPERIMENTAL

General. Optical rotations were measured in a Horiba SEPA-300 polarimeter. UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. ECD spectra were measured on a JASCO J-810 spectropolarimeter. IR spectra were obtained in KBr disc on a Bio-Rad Wininfmred spectrophotometer. ESI-MS were measured on a VG Auto Spec-3000 MS spectrometer. ^{1}H, ^{13}C and 2D NMR spectra were recorded on Bruker 500 instrument with TMS as internal standard. Column chromatography was performed on silica gel (200-300 mesh), or on silica gel H (10 ~ 40 μm, Qingdao Marine Chemical Inc., China). Second separate was used an Agilent 1100 HPLC equipped with ZORBAX-C_{18} (21.2 mm × 250 mm, 7.0 μm) column and DAD detector.

Fungal Material. The culture of *Aspergillus terreus* was isolated from the rhizome of *Paris polyphylla* var. *yunnanensis*, collected from Dali, Yunnan, People's Republic of China, in 2012. 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 100 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 fermentation products were extracted four times with 70% acetone (4 × 10 L) at room temperature and filtered. The crude extract (182 g) was applied to silica gel (200–300 mesh) column chromatography, eluting with a $CHCl_3$-Me_2CO gradient system (20:1, 9:1, 8:2, 7:3, 6:4, 5:5), to give six fractions A–F. The further separation of fraction B (9:1, 23.2 g) by silica gel column chromatography, eluted with petroleum ether-EtOAc (9:1, 8:2, 7:3, 6:4, 1:1), yielded mixtures B1–B5. Fraction B2 (8:2, 6.27 g) was subjected to preparative HPLC (68% MeOH, flow rate 12 mL/min) to give **1** (9.2 mg), **2** (14.2 mg), **3** (13.9 mg), **4** (8.4 mg), **5** (35.6 mg) and **6** (18.9 mg).

Anti-TMV Assays. The Anti TMV activities were tested using the half-leaf method,[17] and ningnanmycin, a commercial product for plant disease in China, was used as a positive control.

Cytotoxicity Assay. The cytotoxicity tests for the isolates were performed by against NB4, A549, SHSY5Y, PC3, and MCF7 tumor cell lines by MTT-assay (with taxol as the positive control).[18]

Terrephenol A (1): $C_{25}H_{26}O_8$, Obtained as a yellow gum; $[\alpha]_D^{24.8}$ +68.4 (c 0.20, MeOH); UV (MeOH) λ_{max} (log ε): 220 (4.27), 250 (3.48), 306 (3.78) nm; CD (*c* 0.2, MeOH) $\Delta\varepsilon_{205}$ +19.2, $\Delta\varepsilon_{226}$ -8.22, $\Delta\varepsilon_{315}$ +5.87; IR (KBr) ν_{max} 3462, 3029, 2976, 2892, 1740, 1725, 1612, 1538, 1482, 1437, 1392, 1276, 1147,

1082, 974, 862, 765 cm^{-1}; ^{1}H and ^{13}C NMR (500 and 125 MHz, in $(CD_3)_2CO$) see Table-1; ESIMS (positive ion mode) *m/z* 477 $[M+Na]^+$; HRESIMS (positive ion mode) *m/z* 477.1520 $[M+Na]^+$ (calcd 477.1525 for $C_{25}H_{26}NaO_8$).

Terrephenol B (2): $C_{25}H_{26}O_8$, Obtained as a yellow gum; $[\alpha]_D^{24.6}$ +74.3 (c 0.20, MeOH); UV (MeOH) λ_{max} (log ε): 220 (4.38), 248 (3.42), 305 (3.72) nm; CD (*c* 0.2, MeOH) $\Delta\varepsilon_{207}$ +17.8, $\Delta\varepsilon_{225}$ -6.72, $\Delta\varepsilon_{313}$ +5.26; IR (KBr) ν_{max} 3465, 3027, 2980, 2895, 1738, 1728, 1610, 1535, 1476, 1433, 1386, 1269, 1158, 1079, 970, 857, 769 cm^{-1}; ^{1}H and ^{13}C NMR (500 and 125 MHz, in $(CD_3)_2CO$) see Table-1; ESIMS (positive ion mode) *m/z* 477 $[M+Na]^+$; HRESIMS (positive ion mode) *m/z* 477.1532 $[M+Na]^+$ (calcd 477.1525 for $C_{25}H_{26}NaO_8$).

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