

## Butenolide and Furandione from an Endophytic *Aspergillus terreus*

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**A new butenolide, aspernolide D (1), and furandione, asperterone (2), together with four known butenolides, butyrolactones I–IV and aspernolide B, were obtained from cultures of the endophytic fungus *Aspergillus terreus*, isolated from the flowering plant *Mammea siamensis*. The structures of these compounds were elucidated by analysis of NMR spectroscopic and mass spectrometric data.**

**Key words** butenolide; furandione; *Aspergillus terreus*

Endophytic fungi have proved to be a rich source of bioactive secondary metabolites,<sup>1,2)</sup> and recently several novel bioactive substances have been isolated from these microorganisms.<sup>3–5)</sup> *Aspergillus terreus* has been isolated from both marine and terrestrial sources and is well known for the production of butenolides. In the course of our search for biologically active metabolites from endophytic fungi from Thai medicinal plants, a subculture of an isolate of *A. terreus*, obtained from the leaves of *Mammea siamensis*, was cultivated in medium containing corn steep liquor. We report here the isolation and structural elucidation of a new butenolide, aspernolide D (**1**), and furandione, asperterone (**2**), together with four known butenolides, butyrolactones I–IV<sup>6,7)</sup> and aspernolide B,<sup>8)</sup> and the evaluation of compounds **1** and **2** for their antibacterial activity against both Gram-positive and Gram-negative bacteria. To the best of our knowledge, this is the first report of butyrolactone III from a natural source.

### Results and Discussion

The EtOAc crude extract of the culture broth of *A. terreus* RCBC 1002 was successively subjected to Sephadex LH20 and silica gel column chromatography to afford butyrolactones I and II as major constituents. Compounds **1** and **2** (Fig. 1), as well as butyrolactones III, IV, and aspernolide B, were isolated as minor metabolites according to TLC analyses.

Aspernolide D (**1**) was isolated as a light yellow gum and its molecular formula was assigned to be C<sub>24</sub>H<sub>26</sub>O<sub>9</sub> based on high resolution-electrospray ionization (HR-ESI)-MS analysis (*m/z* 481.1472 [M+Na]<sup>+</sup>, Calcd 481.1475), indicating 12 degrees of unsaturation. IR absorptions at 3443 and 1697 cm<sup>-1</sup> implied the presence of hydroxyl and carbonyl groups, respectively. The <sup>1</sup>H-NMR spectrum (Table 1) showed the signals of two methyls at δ<sub>H</sub> 1.12 and 1.27, a

methoxy group at δ<sub>H</sub> 3.67, and of an A<sub>2</sub>B<sub>2</sub> system at δ<sub>H</sub> 6.90 (d, *J*=8.8 Hz) and 7.60 (d, *J*=8.8 Hz), indicating the presence of a *para*-disubstituted benzene ring. Another three aromatic signals at δ<sub>H</sub> 6.47 (d, *J*=8.4 Hz), 6.54 (d, *J*=8.4 Hz), and 6.64 (s) were suggestive of the existence of an additional unsymmetrical trisubstituted benzene ring in the molecule. Analysis of the <sup>13</sup>C-NMR spectrum and the information from 2D NMR studies (<sup>1</sup>H–<sup>1</sup>H correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond connectivity (HMBC)) revealed the presence of 10 aromatic carbon resonances for two benzene moieties, an ester carbonyl (δ<sub>C</sub> 169.4), tetrasubstituted olefinic carbons (δ<sub>C</sub> 128.2, 137.4), two methylenes [δ<sub>H</sub> 2.97 m, δ<sub>C</sub> 30.5; δ<sub>H</sub> 3.47 (d, *J*=14.4 Hz), 3.54 (d, *J*=14.4 Hz), δ<sub>C</sub> 38.8], two oxygenated quaternary carbons (δ<sub>C</sub> 72.4, 86.0), an oxygenated methine [δ<sub>H</sub> 4.49 (t, *J*=8.4 Hz), δ<sub>C</sub> 89.1], and a carbomethoxy group (δ<sub>H</sub> 3.76 s, δ<sub>C</sub> 53.6, 169.8). The <sup>1</sup>H- and <sup>13</sup>C-NMR data of **1** were closely related to those of butyrolactone I, but with differences evident in the region of side chain unit (C-7'' to C-11''). Observed HMBC correlations from Me-10'' to C-8'' and C-9'', from Me-11'' to C-8'' and C-9'', and from H<sub>2</sub>-7'' to C-8'' were indicative of the presence of a dihydroxyl group at C-8'' and C-9'' in **1** instead of the Δ<sup>8'',9''</sup> double bond for butyrolactone I. The absolute stereochemistry at C-8'' of **1** was assigned by application of the modified Mosher's method.<sup>9,10)</sup> The difference in chemical shift values (Δδ=δ<sub>S</sub>–δ<sub>R</sub>) for its α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) esters, **1a** and **1b**, indicated the *S*-configuration at C-8'' (Fig. 2).

As the co-metabolite, butyrolactone I was determined to have the 4*R*-configuration by comparison of specific rotation data, [α]<sub>D</sub> +84.32, with the previously reported result.<sup>6)</sup> Therefore the stereochemistry at C-4 of **1** could also be deduced to be 4*R* based on biosynthetic grounds and similarity

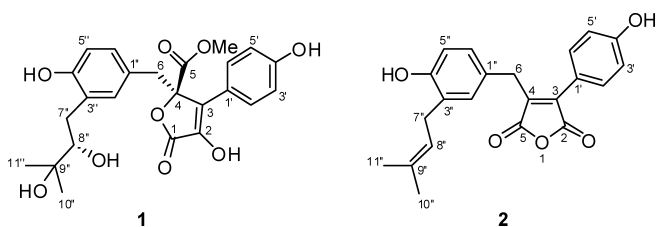


Fig. 1. Structures of Compounds **1** and **2**

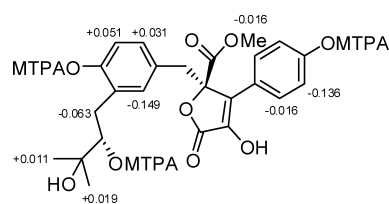


Fig. 2. Δδ Values (δ<sub>S</sub>–δ<sub>R</sub>) of the MTPA Esters **1a** and **1b**

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of the specific rotation,  $[\alpha]_D +40$ . In addition, compound **1** was treated with  $\text{NaIO}_4$  to yield the corresponding aldehyde to remove another chiral center at C-8'', but one of the products obtained was butyrolactone IV,<sup>7)</sup> not the desired aldehyde. However, this helped to confirm the 4*R*-configuration at C-4 of **1**. The most plausible biosynthetic pathway for the formation of compound **1** from butyrolactone I is the epoxidation of the  $\Delta^{7,8''}$  double bond to give another co-metabolite, butyrolactone III, followed by ring-opening with water, as shown in Chart 1.

Asperterone (**2**) was isolated as a yellow gum. The molecular formula  $\text{C}_{22}\text{H}_{20}\text{O}_5$  was established on the basis of the  $[\text{M}+\text{Na}]^+$  peak at  $m/z$  387.1210 (Calcd 387.1208) in the HR-ESI-MS, implying 13 degrees of unsaturation. The NMR spectrum of **2** (Table 1) displayed a similar signal pattern to those of butyrolactone I for a *para*-disubstituted benzene ring, a trisubstituted benzene ring with an isopentenyl unit at

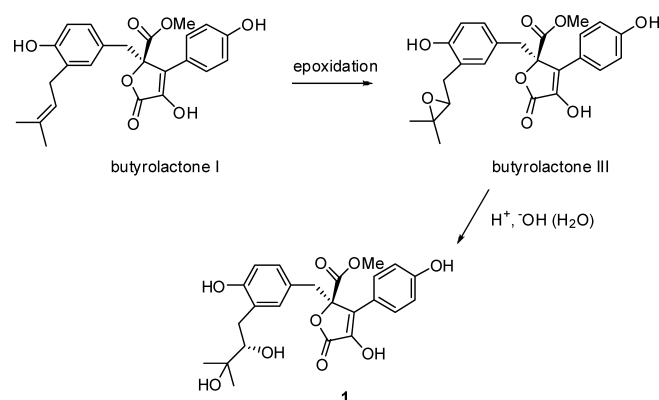


Chart 1. Proposed Biosynthetic Pathway of **1**

C-3'', except for the absence of the carbomethoxy moiety and the presence of an additional ester carbonyl group. The existence of the furan-3-en-2,5-dione at the central core was corroborated by HMBC correlations from H-2' to C-3, from H<sub>2</sub>-6 to both ester carbonyl carbons (C-2, C-5) at  $\delta_C$  165.1 and 166.1, and from H<sub>2</sub>-6 to  $\Delta^{3,4}$  olefinic carbons, which were considerably deshielded to resonate at  $\delta_C$  139.7 and 137.6, respectively. This suggests that each of these olefinic carbons should be connected to the ester carbonyls. In addition, two aromatic rings, an isopentenyl group, two carbonyls, and a double bond account for a total of 12 unsaturation units, and thus the two ester groups must be connected through an oxygen atom to form a furandione ring, consistent with another unsaturation remaining. Therefore the structure of **2** was established as shown. This compound gradually decomposed in  $\text{CDCl}_3$ .

Compounds **1** and **2** were tested for their antibacterial activity against five Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus hominis*, *Staphylococcus epidermidis*, and *Enterococcus faecalis*), and four Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Salmonella typhimurium*). Unfortunately, both compounds were inactive against all bacterial strains tested (minimum inhibitory concentration (MIC) >256  $\mu\text{g}/\text{ml}$ ).

#### Experimental

**General Experimental Procedures** Optical rotations were measured on a Perkin-Elmer 341 polarimeter using a sodium lamp at wavelength of 589 nm, and UV data were recorded on a Shimadzu UV-160 spectrophotometer. IR spectra were recorded on a Bruker vector22 Fourier-transform infrared spectrophotometer. The NMR spectra were recorded on a Varian YH400 spectrometer at 400 MHz for <sup>1</sup>H-NMR and at 100 MHz for <sup>13</sup>C-NMR using tetramethylsilane as the internal standard. HR-ESI-MS were obtained using a Bruker micrOTOF mass spectrometer.

Table 1. NMR Spectroscopic Data (400 MHz for <sup>1</sup>H-NMR and 100 MHz for <sup>13</sup>C-NMR,  $\text{CDCl}_3$ ) for Aspernolide D (**1**) and Asperterone (**2**)

Position	<b>1</b> <sup>a)</sup>			<b>2</b> <sup>b)</sup>		
	$\delta_H$ (J in Hz)	$\delta_C$ , mult	HMBC correlations	$\delta_H$ (J in Hz)	$\delta_C$ , mult	HMBC correlations
1		169.4, qC				
2		137.4, qC			165.1, qC	
3		128.2, qC			139.7, qC	
4		86.0, qC			137.6, qC	
5		169.8, qC			166.1, qC	
6	3.54 d (14.4) 3.47 d (14.4)	38.8, CH <sub>2</sub>	2,3,4,1'',2'',6'' 2,3,4,1'',2'',6''	3.94 s	28.8, CH <sub>2</sub>	2,3,4,5,1'',2'',6''
1'		122.1, qC		118.7, qC		
2' (6')	7.60 d (8.8)	129.5, CH	3,1',3',6'(2')	7.64 d (8.7)	131.1, CH	3,1',4',6'(2')
3' (5')	6.90 d (8.8)	116.1, CH	1',2',4',5'(3')	6.99 d (8.7)	115.3, CH	2',4',5'(3')
4'		156.8, qC			159.5, qC	
1''		124.7, qC			126.5, qC	
2''	6.64 s	126.9, CH	6,6'',7''	7.00 s	129.1, CH	4''
3''		126.7, qC			127.8, qC	
4''		158.7, qC			153.4, qC	
5''	6.46 d (8.4)	108.5, CH	4''	6.75 d (8.2)	114.7, CH	4''
6''	6.54 d (8.4)	130.1, CH	6,2'',4''	6.92 d (8.2)	126.1, CH	6,5''
7''	2.97 m	30.5, CH <sub>2</sub>	4'',2'',3'',8''	3.26 d (7.4)	27.5, CH <sub>2</sub>	2'',3'',4'',8''
8''	4.49 t (8.4)	89.1, CH	9'',10'',11''	5.27 t (7.4)	122.1, CH	9'',10'',11''
9''		72.4, qC			131.2, qC	
10''	1.27 s	25.8, CH <sub>3</sub>	8'',9'',11''	1.69 s	24.5, CH <sub>3</sub>	8'',9'',11''
11''	1.14 s	23.9, CH <sub>3</sub>	8'',9'',10''	1.66 s	16.4, CH <sub>3</sub>	8'',9'',10''
5-OMe	3.76 s	53.6, CH <sub>3</sub>	5			
4'-OH				9.15 s		4',6'
4''-OH				8.20 s		4''

a) Recorded in  $\text{CDCl}_3$ , b) Recorded in acetone-*d*<sub>6</sub>.

**Fungal Material** The fungus *A. terreus* RCBC1002 was isolated from the leaves of *M. siamensis* collected from Rayong province, Thailand, in May 2008. This fungus was identified as *A. terreus* by one of authors (N.B.) based on sequence (GenBank Accession number GU362937) analysis of the 18S ribosomal DNA and internal transcribed spacer genes.

The isolate was subcultured on potato dextrose agar plates at 25 °C for 7 d. Agar plugs were used to inoculate 1000-ml Erlenmeyer flasks, each containing 200 ml of medium (corn steep liquor 1 g, mannitol 10 g, maltose 10 g, glucose 5 g, monosodium glutamate 10 g,  $\text{KH}_2\text{PO}_4$  0.5 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.3 g, yeast extract 3 g per liter). Flask cultures were incubated at 30 °C under static conditions for 21 d.

**Extraction and Isolation** Ten-liter cultures were filtered, and the culture broth was extracted with EtOAc ( $\times 3$ ). The combined organic layer was concentrated to afford the crude extract (12.60 g). The extract was fractionated by Sephadex LH20 column chromatography eluted with MeOH to yield six fractions (I–VI). Fraction III was chromatographed on a silica gel column eluted with acetone–benzene (1 : 3) to give butyrolactone I (2.76 g), butyrolactone II (1.64 g), aspernolide B (346.0 mg), and 10 subfractions (III-1 to III-10). Subfraction III-1 was subjected to flash silica gel column chromatography (acetone–benzene, 1 : 9) and further purified on a silica gel column (hexane–acetone, 3 : 1) to afford **2** (8.2 mg), whereas purification of subfraction III-7 by flash column chromatography eluted with MeOH– $\text{CHCl}_3$  (1 : 30) gave **1** (16.2 mg) and butyrolactone III (45.5 mg). Fraction III-8 was rechromatographed on a silica gel column (acetone–benzene, 1 : 2) followed by preparative TLC (MeOH– $\text{CH}_2\text{Cl}_2$ , 5 : 95) to yield butyrolactone IV (9.2 mg).

Aspernolide D (**1**): Light yellow gum;  $[\alpha]_D^{20} +40$  ( $c=0.1$ , MeOH); UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 290 (4.14) nm; IR (KBr)  $\nu_{\text{max}}$  3443, 2956, 1697, 1633, 1620, 1513, 1439, 1262, 1108, 939  $\text{cm}^{-1}$ ;  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data ( $\text{CDCl}_3$ ), see Table 1; HR-ESI-MS,  $m/z$  481.1472 (Calcd for  $\text{C}_{24}\text{H}_{26}\text{O}_6\text{Na}$ , 481.1475).

Asperterone (**2**): Yellow gum; UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 205 (3.97) nm; IR (KBr)  $\nu_{\text{max}}$  3443, 2926, 1730, 1660, 1604, 1511, 1439, 1238, 1170, 1114  $\text{cm}^{-1}$ ;  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data ( $\text{CDCl}_3$ ), see Table 1; HR-ESI-MS,  $m/z$  387.1210 (Calcd for  $\text{C}_{22}\text{H}_{20}\text{O}_5\text{Na}$ , 387.1208).

**Preparation of MTPA Ester Derivatives of 1** To a solution of **1** (3.0 mg) in pyridine (0.3 ml) was added (*R*)-MTPA Cl (20  $\mu\text{l}$ ).<sup>9,10</sup> The reaction mixture was stirred at room temperature overnight. The mixture was diluted with EtOAc and washed with  $\text{H}_2\text{O}$  and 1 M  $\text{NaHCO}_3$ , and the organic layer was concentrated under reduced pressure. The residue was purified on a short silica gel column (MeOH– $\text{CH}_2\text{Cl}_2$ ) to obtain the (*S*)-MTPA ester **1a** (1.8 mg):  $^1\text{H}$ -NMR data ( $\text{CDCl}_3$ , 400 MHz)  $\delta$ : 7.56 (2H, d,  $J=8.8$  Hz, H-2', H-6'), 6.86 (2H, d,  $J=8.8$  Hz, H-3', H-5'), 6.60 (s, H-2''), 6.50 (1H, d,  $J=8.4$  Hz, H-6''), 6.45 (1H, d,  $J=8.4$  Hz, H-5''), 4.46 (1H, t,  $J=8.4$  Hz, H-8''), 3.72 (s, 5-OMe), 3.50 (1H, d,  $J=14.4$  Hz, H-6a), 3.42 (1H, d,  $J=14.4$  Hz, H-6b), 2.94 (2H, m, H<sub>2</sub>-7''), 1.24 (3H, s, Me-10''), 1.20 (3H, s, Me-11'').

Similarly, the reaction mixture of **1** (3.0 mg), (*S*)-MTPA Cl (20  $\mu\text{l}$ ) and pyridine (0.3 ml) was processed as described above for **1a** to afford **1b** (1.5 mg):  $^1\text{H}$ -NMR data ( $\text{CDCl}_3$ , 400 MHz)  $\delta$ : 7.58 (2H, d,  $J=9.2$  Hz, H-2', H-6'), 6.99 (2H, d,  $J=9.2$  Hz, H-3', H-5'), 6.75 (s, H-2''), 6.47 (1H, d,  $J=8.4$  Hz, H-6''), 6.40 (1H, d,  $J=8.4$  Hz, H-5''), 4.51 (1H, t,  $J=9.6$  Hz, H-8''), 3.74 (s, 5-OMe), 3.62 (1H, d,  $J=14.8$  Hz, H-6a), 3.58 (1H, d,

$J=14.8$  Hz, H-6b), 3.00 (2H, m, H<sub>2</sub>-7''), 1.22 (3H, s, Me-10''), 1.19 (3H, s, Me-11'').

**Biological Assays** A broth microdilution method was used to determine the MIC according to the NCCLS protocol.<sup>11,12</sup> Five reference Gram-positive bacteria, *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *S. hominis* ATCC 27844, *S. epidermidis* ATCC 12228 and *E. faecalis* ATCC 29212; and four Gram-negative bacteria, *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853, *P. vulgaris* ATCC 13315 and *S. typhimurium* ATCC 13311 were used. All tests were performed in Mueller–Hinton broth. Serial doubling dilutions of the compound, prepared in a 96-well microtiter plate, ranged from 0.5 to 256  $\mu\text{g}/\text{ml}$ . The final concentration of each strain was adjusted to  $5 \times 10^5$  colony forming unit (CFU)/ml. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the compound at which the microorganism did not demonstrate visible growth as determined by turbidity.

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