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Aspernolides A and B, butenolides from a marine-derived fungus *Aspergillus terreus*

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ABSTRACT

Two aromatic butenolides, aspernolides A and B along with the known metabolites, butyrolactone I, terrein and physcion were isolated from the fermentation broth of a soft coral derived fungus *Aspergillus terreus*. The structures of these metabolites were assigned on the basis of detailed spectroscopic analysis. The absolute stereochemistry of aspernolides A (**1**) and B (**2**) was established by their preparation from the known butyrolactone I. Biogenetically aspernolides A and B must be derived from butyrolactone I, a well known specific inhibitor of cyclin dependent kinase (cdk) from *A. terreus*. When tested, aspernolide A exhibited mild cytotoxicity against cancer cell lines.

1. Introduction

Marine-derived microbes, fungi in particular have long been recognized as potential source of structurally novel and biologically potent metabolites (Faulkner, 2000; Bugni and Ireland, 2003; Saleem et al., 2007). Fungi belonging to *Aspergillus* genera are one of the major contributors to the secondary metabolites of fungal origin. *Aspergillus terreus* is a ubiquitous fungus in our environment and although *Aspergillus* sp. are normally considered terrestrial species, the genus is tolerant to high salt concentrations. In recent years, we sought to draw marine microbial diversity into the arena of drug discovery. The present investigation is an outcome of such a study on the fungus *A. terreus* associated with a soft coral *Sinularia kavarattiensis*.

Terrestrial isolates of *A. terreus* are well known for the production of butenolides. Cytotoxic butyrolactones I–IV, biogenetically derived from tyrosine (Rao et al., 2000; Kiriyama et al., 1977; Nitta et al., 1983) and non prenylated-decarboxylated butenolides, xenofuranones A (**4**) and B (**5**) (Morishima et al., 1994), biogenetically produced from phenyl alanine are known to be derived from *A. terreus*. Xenofuranones A (**4**) and B (**5**) are also known as metabolites of bacterium *Xenorhabdus szentitmaii* (Brachmann et al., 2006). There is a recent report on the identification of **3** and its sulfated derivatives (**6** and **7**) (Niu et al., 2008) from a strain *A. terreus* (HKI0499).

This report focuses on the isolation and structure elucidation of new butenolides, aspernolides A (**1**) and B (**2**) and other known metabolites from culture medium of fungus *A. terreus*. Although, **1** has been described in the literature (Kiriyama et al., 1977) as a reaction product in the structure elucidation of **3**, neither its NMR data is reported nor it is known to be a natural product. Moreover this is the first report describing the isolation of **3** and other related butenolides from marine-derived fungus.

2. Results and discussion

Fungus, *A. terreus* was isolated as an epiphyte from a soft coral *Sinularia kavarattiensis* collected from the coast of Mandapam, Tamil Nadu, India. This fungus was grown on potato dextrose broth prepared in seawater. New secondary metabolites, aspernolides A (**1**) and B (**2**) were identified from the chloroform and ethyl acetate extracts of the culture broth respectively. These butenolides along with their plausible biogenetic precursor **3** and the known metabolites physcion and terrein were purified using repeated silica gel and Sephadex LH-20 gel filtration chromatography.

Aspernolide A (**1**) was obtained as white sticky solid. The molecular formula $C_{24}H_{24}O_7$ of **1** was determined by HRESITOFMS which showed pseudomolecular ion peaks $[M + Na]^+$ at 447.1433 (calcd. 447.1420 for $C_{24}H_{24}O_7Na$) and $[2M + Na]^+$ at 871.2959 (calcd. 871.2942 for $C_{48}H_{48}O_{14}Na$). The IR spectrum showed the presence of ester/lactone carbonyl at 1731 and 1738 cm^{-1}, phenolic OHs were evident at 3330 cm^{-1} and the presence of an absorption at 1660 cm^{-1} was suggestive of aromaticity in the molecule.

The ^1H NMR signals of the A_2B_2 system at δ_H 7.56, *d*, 2H, $J = 8.7$ Hz and 6.86, *d*, 2H, $J = 8.7$ Hz revealed the presence of para di-substituted benzene moiety. Two aromatic signals 6.53, *s*, 1H and 6.47, *s*, 2H (two doublets merged into a singlet) were indicative of the presence of additional unsymmetrical trisubstituted benzene ring in the molecule. Its ^{13}C NMR showed the presence

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of 10 aromatic signals for two aromatic rings, two ester carbonyls δ_c 169.3 *s*, and 169.6 *s*, olefenic carbon signals δ_c 137.2 *s* and 128.8 *s*, three sp^3 CH_2 *s* δ_c 22.1 *t*, 32.5 *t* and 38.6 *t*, a carbomethoxy δ_c 53.4 *q* and two oxygenated quaternaries δ_c 86.1 *s* and 74.2 *s*. The molecular formula $C_{24}H_{24}O_7$ requires 13 degrees of unsaturation. The presence of two aromatic rings accounts for eight while two carbonyls and two olefenic carbons account for another three, which makes a total of eleven degrees of unsaturation. Therefore **1** must posses two aliphatic rings in addition to two aromatic rings.

A detailed comparison of the NMR data of **1** with that of butyrolactone I (**3**) (Rao et al., 2000), confirmed a common hydroxyphenylpyruvate dimer type of network in the molecule. The significant difference observed in the NMR spectra of **1** as compared to that of **3** was the absence of an olefinic proton signal δ_H 5.0, *t*, 1H, two olefinic carbon signals δ_c 121.0 *d* and 130.7 *s* and the presence of three methylenes and two oxygenated sp^3 quaternaries compared to two methylenes and one oxygenated sp^3 quaternary in **3** in both ^{13}C and DEPT NMR spectra. This data was indicative of the presence of a dihydropyran ring fused to a trisubstituted benzene ring in place of the open prenyl chain present in **3**. HMBC was in good agreement with the structure **1** (Fig. 1). Key HMBC correlations from H-2″ to C-3″ and C-7″, from H-7″ to C-2″, C-3″ and C-4″ and from H-8″ to C-9″ and C-10″ (11″) established a dihydropyran ring fused through the C3″–C4″ bond of a benzene ring. HMBC correlations from H-2″ and H-6″ to C-6 and from H-6 to C-1″, C-6″, C-2″, C-4, C-5 and C-3 were evidence of the benzodihydropyranmethylene moiety linked to a lactone ring at C-4. Furthermore HMBC correlation from H-2′(6′) to C-3 established *para* di-substituted phenolic moiety at C-3. Out of two carbonyls, C-5 and C-1, δ_c 169.6 was assigned to C-5 on the basis of its HMBC correlation to the protons H-6 and H_3–5OMe.

Aspernolide B (**2**) (Rf, 0.51), more polar than **1** (Rf, 0.81) was obtained as a light brown syrup $[\alpha]_D$ + 48.27 (c 0.29, MeOH). The IR spectrum showed the presence of – OHs at 3330 cm^{-1}, ester/lactone carbonyls overlapping peaks at 1732 and 1747 cm^{-1} and 1610 and 1519 cm^{-1} for aromatic rings. Although chemical shift variations were present in the ^1H and ^{13}C NMR of **2**, they were similar to those of **1**. Significant variations in the chemical shifts were observed for the ring carrying the iso-pentyl chain wherein C-1″, C-3″, C-7″, C-8″ C-10″ and C-11″ were considerably deshielded to resonate at δ_c 128.1 ($\Delta\delta$ 4.6 ppm), 124.0 ($\Delta\delta$ 3.7 ppm), 24.2 ($\Delta\delta$ 2.1 ppm), 43.2 ($\Delta\delta$ 10.7 ppm), 28.4 ($\Delta\delta$ 1.8 ppm) and 28.5 ($\Delta\delta$ 1.9 ppm) while C-5″ and C-9″ were considerably shielded to resonate at δ_c 114.6 ($\Delta\delta$ 2.0 ppm) and 70.8 ($\Delta\delta$ 3.4 ppm) as compared to **1**, suggesting a change on the aromatic ring carrying iso-pentyl chain. Compound **2** was well distinguished from ESI–MS spectrum which showed pseudomolecular ions $[M + H]^+$ at 443.1699 (calcd. 443.1706 for $C_{24}H_{27}O_8$) and $[M + Na]^+$ at 465.1516 (calcd. 465.1525 for $C_{24}H_{26}O_8Na$) suggesting a molecular weight of 442 for the compound **2**, which was 18 units more than that of **1**. Based on these observations it was evident that **2** has open chain hydroxylated prenyl chain ortho to a phenolic –OH(C-4″).

Based on the reported feeding experiments for establishing the biosynthesis of xenofuranones A (**4**) and B (**5**) together with compound **3** (Brachmann et al., 2006; Nitta et al., 1983) and isolation of **1** and **2** from *A. terreus,* it is apparent that the structures of **1** and **2** are an extension of the biosynthesis of **3**, which is derived from p-hydroxyphenylpyruvate. The enzyme-catalysed cyclization or

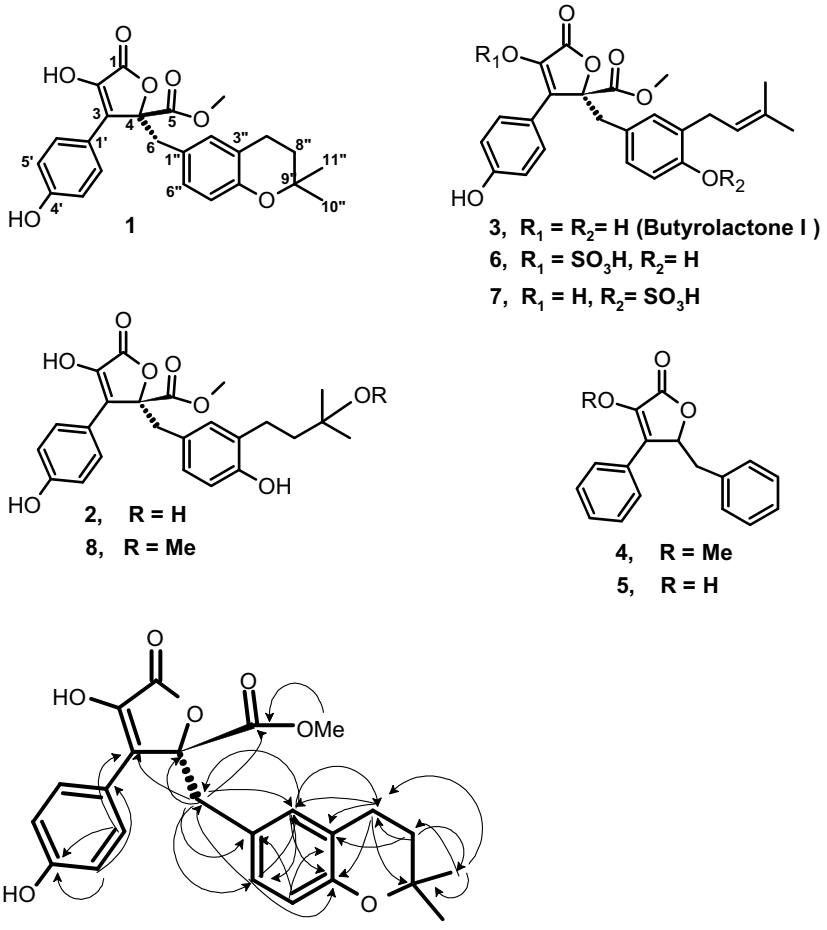
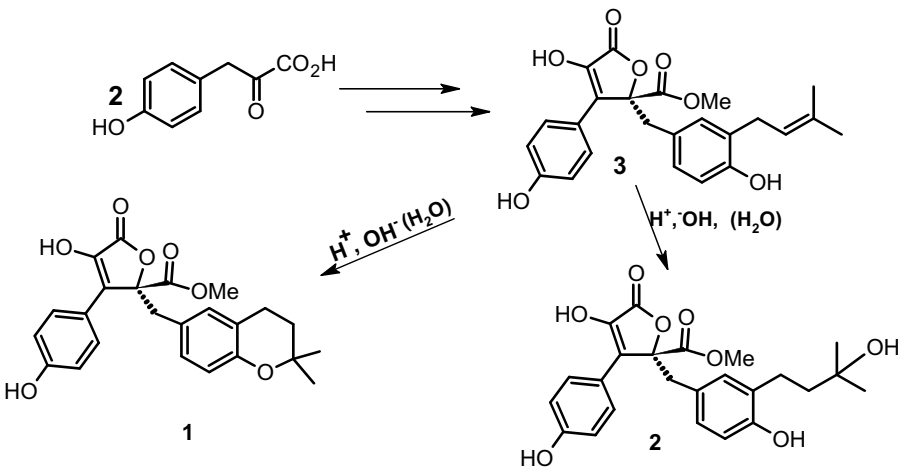


Fig. 1. HMBC correlation for 1.



Scheme 1. Biogenetic pathway of 1 and 2.

addition of water across the double bond of the prenyl chain of **3** results in the formation of **1** and **2**, respectively (Scheme 1). The last step in the biogenetic scheme was mimicked using mild acid catalysis to confirm the structure of **1**.

On heating with 1% aqueous sulphuric acid, **3** was converted to **1** as well as small amount of **2** (Scheme 2). Only 50% conversion was observed. When the same reaction was carried out using 2% conc. HCl in methanol, complete conversion of **3** was observed as indicated by TLC (90:10, $CHCl_3$/MeOH). On chromatographic separation, 75% of **1** was obtained along with minor amounts of **2** and a new product **8**. Compound **8** displayed an extra methoxy signal δ_H 3.15, *s*, δ_C 49.0 *q* in its NMR spectra compared with **2**. In the rest of the NMR spectra it was seen that C-7", C-8", C-10" and C-11″ were shielded and observed at δ_c 23.2($\Delta\delta$ 1.0), 40.0 ($\Delta\delta$ 3.2), 24.0 ($\Delta\delta$ 4.4), and 24.4($\Delta\delta$ 4.1) while only methoxylated quaternary carbon C-9″ was deshielded to 75.4 ($\Delta\delta$ 4.6) compared to **2**. Pseudomolecular ion peaks $[M + H]^+$ at 457.1851 and $[M + Na]^+$ at 479.1669 observed in ESI–MS indicated molecular weight of 456 for the compound. On the basis of these observations, the structure was assigned as shown in **8**. Compounds **2** and **8** are formed by Markovnikoff's addition of water and methanol respectively, across the double bond of the prenyl chain.

During the structure elucidation of **3**, compound **1** has been reported as the product of its reaction with ethanolic HCl (Kiriyama et al., 1977). In the present investigation, the same reaction in methanolic HCl yielded besides **1**, two additional products **2** and **8**. Co-metabolite **3** used in the above reaction was determined to be 4*R* configured by comparison of specific rotation data $[\alpha]_D$ + 84.32° with the previously reported result (Kiriyama et al., 1977). The absolute configurations of **1** and **2,** therefore could be deduced to be also 4*R* based on the biosynthetic grounds and similarity of the specific rotations $[\alpha]_D$ + 88.73° for **1** and +48.27° for **2**. Further confirmation of the absolute stereochemistry of **1** and **2** results from the fact that the natural **1** and **2** and those obtained as products of the acid-catalyzed reaction had identical spectral data and specific rotations $[\alpha]_D$ + 88.73° for **1** and +48.27° for **2**. Therefore, we conclude that the compounds **1** and **2** also have 4*R*-configuration.

Butyrolactone I (**3**), a metabolite of *A. terreus* var. *africans* IFO 8355 discovered in 1977, seems to be a common metabolite of this fungus (Kiriyama et al., 1977; Rao et al., 2000; Niu et al., 2008; Schimmel and Parsons, 1999). To our best knowledge, this is the first report of **3** and its derivatives from marine-derived fungus. Aspernolides B (**2**) and C (**3**) were unstable and were converted to **1** on long standing. Aspernolide C (**1**) when tested against five cell lines displayed weak cytotoxicity against H460, ACHN, Calu, Panc1 and HCT116 cell lines (IC_{50} > 88, >103, >147, >130, >121 μM, respectively).

3. Conclusion

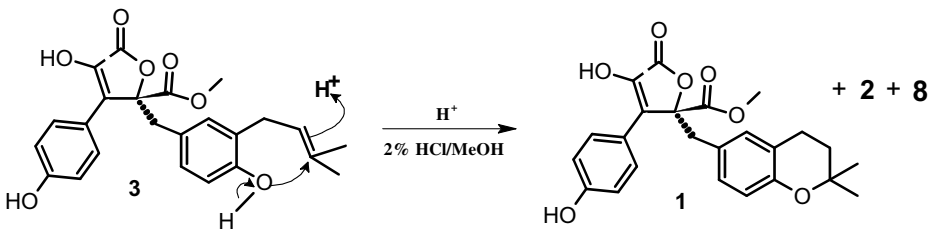
A. terreus is known to be a producer of the cholesterol lowering agent lovastatin (mevinoline) (Alberts et al., 1980) and many other important secondary metabolites such as terreineol (Macedo et al., 2004), terreulactone A (Kim et al., 2002), terrein, terreic acid and aspulvinones. The list of metabolites also includes butyrolactone I (**3**), a specific inhibitor of cdk1/cyclin B and cdk2/cyclin A (Fischer and Lane, 2000) as potent as roscovitine, a drug currently undergoing phase IIb clinical evaluation.[1] Compound **3** exhibits antiproliferative activity against colon and pancreatic carcinoma, human lung cancer (Nishio et al., 1996) and prostatic cancer (Suzuki et al., 1999). Computer aided molecular modeling using automated docking methods and molecular dynamics simulations studies (Brana et al., 2004) have proven the importance of the alkenyl (prenyl) side chain in the molecule and explains why these butenolides lacking the alkenyl side chain do not maintain antitumor activity. After considering the potential of **3** as a cdk inhibitor it can be suggested that these marine natural products **1** and **2** or synthetically modified natural products such as **8,** which retain the alkenyl side chain, could be tested in future to explore as potential cdk inhibitors.

4. Experimental

4.1. General experimental procedures

Sephadex LH-20 (Pharmacia) and silica gel (60–120 mesh, Qualigens) were used for column chromatography. Culture media Czapek agar and potato dextrose broth were procured from Himedia Ltd., Mumbai. Solvents of laboratory reagent grade used for column chromatography were purchased from a local supplier and were distilled prior to use. Petroleum ether of boiling range 60–80 °C was used for column chromatography. Precoated kiesegel 60 F_{254} TLC plates were used for analytical TLC. A mixture of methanol and chloroform (10:90, v/v) was used as mobile phase for TLC analysis. Compounds were visualized as intense rose coloured spots on spraying with methanolic sulphuric acid

[1] www.cyclacel.com/cyc/investors/news/pressreleases/2006/2006-06-29/.



Scheme 2. Conversion of butyrolactone I (3) to aspernolide A (1).

(95:5, v/v) followed by heating at 120 °C. UV and IR spectra were recorded on Shimadzu UV-2401 PC and Shimadzu FTIR-8201 PC spectrometers. Optical rotations were measured on optical polarimeter ADP220 (Bellingham & Stanley Ltd.). NMR (^{1}H, ^{13}C, DEPT, HSQC and HMBC) data were obtained on Bruker Avance 300 and Bruker Avance 500 spectrometer with TMS as internal standard. EI-MS and HRESITOFMS were recorded on Shimadzu 2010 and QSTARXL MS/MS, Applied Biosystems, Switzerland.

4.2. Fungal isolation, identification and cultivation

Soft coral *Sinularia kavarattiensis* was collected by scuba diving at a depth of 8–10 m from the coast of Mandapam, Tamil Nadu, India in May 2004. After washing the soft coral with sterile sea water, fungus *A. terreus* was isolated as an epiphyte using Czapek agar containing (g/l) $NaNO_3$ (2.0), $MgSO_4{\cdot}7H_2O$ (5.0) $FeSO_4{\cdot}7H_2O$ (0.1), KH_2PO_4 (1.0), KCl (0.5), agar (3.0) sucrose (30.0) at pH 5.5 prepared in seawater supplemented with penicillin benzyl sodium salt (0.02) to avoid any bacterial growth. After 6–7 days sand brown, velvety colonies were observed. The strain was identified as *A. terreus* from the morphological features of conidiophores by Dr. Sanjay K. Singh, mycologist, Agharkar Research Institute, Pune, India. A voucher specimen of the fungus is deposited at National Institute of Oceanography, Dona Paula, Goa, India. Stock cultures of the fungus, maintained at −20 °C preserved with 20% glycerol was used to inoculate 500 ml of seed medium in an Erlenmeyer flask (4 L) containing 24 g/l of potato dextrose broth in seawater. It was then cultured at 27 ± 2 °C on a rotary shaker at 200 rpm. The flask was incubated for 72 h and used as a first stage inoculum. The same medium (1 L) was made in 10 Erlenmayer flasks (4 L) and inoculated with 5% of first stage inoculum. The flasks were incubated for 21 days at 27 ± 2 °C on a rotary shaker at 200 rpm for 10 h/day.

4.3. Extraction and isolation of metabolites

Twenty-one days old fermentation broth (10 L) was separated from fungal mat and concentrated to a volume of 1 L under reduced pressure. The broth was extracted first with chloroform (200 ml X 4) followed by ethyl acetate (200 ml X 4). The chloroform and ethyl acetate layers were separately concentrated under reduced pressure to yield chloroform extract (470 mg) and ethyl acetate extract (430 mg). The ethyl acetate extract was chromatographed over Sephadex LH-20 using MeOH–$CHCl_3$ (1:1) to yield pure crystalline compound, terrein (92 mg) (Dunn et al., 1975) and a fraction containing an intense rose coloured spot. This fraction was flash chromatographed over silica gel using gradient elution of MeOH–$CHCl_3$ (5:95–20:80) to yield **2** (7.2 mg; Rf, 0.51). The chloroform extract was repeatedly chromatographed over Sephadex LH-20 using MeOH–$CHCl_3$ (1:1) and 100% MeOH, which yielded a pure yellow coloured compound, physcion (17 mg) (Bachmann et al., 1979). Other fractions giving two prominent rose coloured spots on TLC were further purified on a silica gel column using gradient elution with MeOH–$CHCl_3$ (0:100–20:80) to afford **3** (32.0 mg; Rf, 0.64) and another nearly pure compound which was purified over Sephadex LH-20 using MeOH–$CHCl_3$–Pet. ether (40:40:20) to yield **1** (26.3 mg; Rf, 0.81).

4.3.1. Aspernolide A (*1*)

White sticky solid; $[\alpha]_D^{28} + 88.73^\circ$ (c 0.58, $CHCl_3$); UV (MeOH) λ_{max} nm : 303, 240; IR (NaCl) ν_{max} cm^{-1} 3340, 3024, 2970, 2935, 1737, 1732, 1610, 1519, 1498, 1436, 1386, 1261, 1182, 1122, 1068, 1037, 948, 839, 754; For NMR data see Table 1; HRESITOFMS $[M + Na]^+$ *m/z* 447.1433 (calcd. 447.1420 for $C_{24}H_{24}O_7Na$), $[2M + Na]^+$ *m/z* 871.2959 (calcd. 871.2942 for $C_{48}H_{48}O_{14}Na$); EIMS *m/z*(%): M^+ 424(2.7), 380(37.8), 348(35.1), 320(6.8), 293(13.5), 265(8.1), 237(10.8), 218(5.4), 205(6.8) 189(6.8), 175(100), 157(8.1), 145(10.8), 131(16.2), 119(13.5), 107(5.4), 91(24.3), 77(3.5), 69(12.1), 44(37.8).

4.3.2. Aspernolide B (*2*)

Light brown syrup; $[\alpha]_D^{28} + 48.27^\circ$ (c 0.29, MeOH); UV (MeOH) λ_{max} nm : 303, 240; IR (NaCl) ν_{max} cm^{-1} 3380, 3024, 2975, 2933, 1745, 1610, 1519, 1442, 1386, 1182, 1070, 1037, 838, 762; For ^{1}H and ^{13}C NMR spectroscopic data see Table 2; HRESITOFMS: $[M + H]^+$ *m/z* 443.1699 (calcd. 443.1706 for $C_{24}H_{27}O_8$), $[M + Na]^+$ *m/z* 465.1516 (calcd. 465.1525 for $C_{24}H_{26}O_8Na$); EIMS *m/z*(%): $[M–CO_2]^+$ 398(13), 380(70), 348(100), 333(18), 320(15), 293(40), 205(26), 249(10), 237(23), 218(17), 205(25), 188(16), 175(77), 145(13), 131(41), 119(23), 107(15), 91(35), 77(17), 69(20), 59(33), 43(23), 41(11).

Table 1
NMR spectroscopic data of aspernolide A (**1**) (500 MHz, $CDCl_3$).

Carbon No.	δ_C, mult.	δ_H, mult., *J*(Hz)	HMBC
1	169.3 *s*		
2	137.2 *s*		
3	128.8 *s*		
4	86.1 *s*		
5	169.6 *s*		
6	38.6 *t*	3.39, *d* (15.0), 3.59, *d*, (15.0)	C4, C1″, C6″, C3, C2″, C5
1′	122.2 *s*		
2′(6′)	129.5 *d*	7.56, *d* (8.7)	C4′, C3
3′(5′)	115.9 *d*	6.86, *d* (8.7)	C1′, C4′
4′	156.4 *s*		
1″	123.5 *s*		
2″	131.4 *d*	6.53, *s*	C7″, C6, C6″, C4″
3″	120.3 *s*		
4″	152.9 *s*		
5″	116.6 *d*	6.47, *s*	C1″, C3″
6″	129.0 *d*	6.47, *s*	C6, C2″, C4″
7″	22.1 *t*	2.53 *m*	C8″, C9″, C2″, C3″, C4″
8″	32.5 *t*	1.66, *t* (6.5)	C3″, C7”, C9”, C10″(11″)
9″	74.2 *s*		
10″(11″)	26.6 *q*	1.21, *s*	C7″, C8″, C9″
5-OMe	53.4 *q*	3.72, *s*	C5

Table 2
^{1}H and ^{13}C NMR spectroscopic data of compounds **2** and **3** (300 MHz, $CDCl_3$+ 2drops CD_3OD).

Carbon No.	Aspernolide B (**2**) δ_C, mult.	δ_H, mult., *J* (Hz)	Aspernolide C (**3**) δ_C, mult.	δ_H, mult., *J* (Hz)
1	169.2 *s*		169.3 *s*	
2	137.9 *s*		137.9 *s*	
3	128.3 *s*		128.2 *s*	
4	85.6 *s*		85.7 *s*	
5	170.1 *s*		170.1 *s*	
6	38.3 *t*	3.46, *s*	38.4 *t*	3.43, *s*
1′	121.6 *s*		121.8 *s*	
2′(6′)	129.1 *d*	7.55, *d* (8.7)	129.2 *d*	7.54, *d* (8.7)
3′(5′)	115.6 *d*	6.87, *d* (8.7)	115.0 *d*	6.85, *d* (8.7)
4′	157.5 *s*		157.5 *s*	
1″	128.1 *s*		128.1 *s*	
2″	131.7 *d*	6.41, *d* (1.8)	131.8 *d*	6.40, *d* (1.8)
3″	124.0 *s*		124.0 *s*	
4″	153.2 *s*		153.4 *s*	
5″	114.6 *d*	6.52, *d* (8.7)	114.4 *d*	6.49, *d* (8.1)
6″	128.6 *d*	6.55, *dd* (8.7, 1.8)	128.9 *d*	6.54, *dd* (8.1, 1.8)
7″	24.2 *t*	2.37, *m*	23.2 *t*	2.35, *m*
8″	43.2 *t*	1.53, *t* (7.8)	40.0 *t*	1.54, *t* (7.8)
9″	70.8 *s*		75.4 *s*	
10″	28.4 *q*	1.20, *s*	24.0 *q*	1.12, *s*
11″	28.5 *q*	1.19, *s*	24.4 *q*	1.12, *s*
12″	–		49.0 *q*	3.15, *s*
5-OMe	53.5 *q*	3.76, *s*	53.3 *q*	3.74, *s*

4.3.3. Butyrolactone I (**3**)

White powder; $[\alpha]_D^{28.8} + 84.32^\circ$ (c 0.617, MeOH); and $[\alpha]_D^{28.8} + 87.56^\circ$ (c 0.617, $CHCl_3$); ^{1}H NMR and ^{13}C NMR data were in agreement with the literature (Rao et al., 2000); HRESITOFMS: $[M + H]^+$ *m/z* 424.1516 (calcd. 424.1522 for $C_{24}H_{25}O_7$).

4.3.4. Aspernolide C (**8**)

Light brown syrup; for ^{1}H and ^{13}C NMR spectroscopic data see Table 2; HRESITOFMS: $[M + H]^+$ *m/z* 457.1851 (calcd. 457.1862 for $C_{25}H_{29}O_8$) and $[M + Na]^+$ *m/z* 479.1669 (calcd. 479.1682 for $C_{25}H_{28}O_8Na$).

4.4. Conversion of butyrolactone I (**3**) to aspernolides A (**1**), B (**2**) and C (**8**)

Butyrolactone I (**3**), (76.9 mg) was dissolved in MeOH (10 ml) containing conc. HCl (0.2 ml). The mixture was stirred at rt approximately for 2 h. or until complete conversion of **3** as indicated by TLC. The solvent was removed under vacuum and the resulting residue was separated on a flash Si-gel column using gradient elution of MeOH–$CHCl_3$ (0:100–20:80) to yield in order of increasing polarity **1** (58.6 mg, 75%), **8** (8.8 mg, 10.4%) and **2** (6.9 mg, 8.3%).

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