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Antifungal dibenzofuran bis(bibenzyl)s from the liverwort *Asterella angusta*

Jianbo Qu [a], Chunfeng Xie [a], Huaifang Guo [a], Wentao Yu [b], Hongxiang Lou [a,*]

[a] *School of Pharmaceutical Sciences, Shandong University, No. 44 West Wenhua Road, Jinan 250012, PR China*
[b] *State Key Laboratory of Crystal Materials, Shandong University, No. 27 Shanda Nanlu, Jinan 250100, PR China*

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Abstract

Bioactivity-guided separation of an antifungal extract from the liverwort *Asterella angusta* afforded four bis(bibenzyl)s, asterelin A (**1**), asterelin B (**2**), 11-*O*-demethyl marchantin I (**3**), and dihydroptychantol A (**4**), together with six known ones. Their structures were established by extensive spectroscopic analysis (1D and 2D-NMR, MS), and that of **2** was confirmed by X-ray crystallographic diffraction analysis. Compounds **1** and **2** are the first examples of dibenzofuran bis(bibenzyl)s. The antifungal activity of the isolated bis(bibenzyl)s against the common clinical pathogenic fungus *Candida albicans* was evaluated using both the thin-layer chromatography bioautographic assay and the broth microdilution method. They showed moderate antifungal activities with minimal inhibitory concentration (MIC) values ranging from 16 μg/ml to 512 μg/ml.

Keywords: Liverworts; *Asterella angusta*; Aytoniaceae; Bis(bibenzyl)s; Antifungal; Asterelin A; Asterelin B; *Candida albicans*

1. Introduction

A variety of bis(bibenzyl)s have been isolated from liverworts belonging to the orders Jungermanniales, Metzgeriales, Marchantiales, and Monocoleales (Asakawa, 1995, 2001), as well as acyclic bis(bibenzyl)s from ferns (Oiso et al., 2001; Wu et al., 2005). These natural products reportedly have diverse biological activities; these include cytotoxic (Lorimer et al., 1993; Scher et al., 2002), antimicrobial and antifungal (Baek et al., 2004; Niu et al., 2006; Scher et al., 2004), thrombin, 5-lipoxygenase, cyclooxygenase and DNA polymerase β inhibitory (Nagashima et al., 1996; Schwartner et al., 1995; Yoshida et al., 1996), anthelmintic (Lorimer et al., 1996), muscle-relaxing (Taira et al., 1994), as well as antioxidative (Schwartner et al., 1996) properties. As characteristic constituents of liverworts, the bis(bibenzyl)s are mainly responsible for their antimicrobial and antifungal effects. For example, Asakawa (1988, 1999) reported the antimicrobial and antifungal activities of marchantin A, a representative bis(bibenzyl) in liverworts, against fungi from the *Candida*, *Aspergillus*, *Staphylococcus*, *Cryptococcus*, and *Trichophyton* species. Our previous studies have also indicated that the aromatic compounds from liverworts, especially the bis(bibenzyl)s, showed significant antifungal activity against the sensitive and fluconazole-resistant strains of *Candida albicans* (Leng et al., 2007; Niu et al., 2006). They also facilitated the accumulation of fluconazole in cells of fluconazole-resistant *C. albicans*, when used in combination with this reagent (Leng et al., 2007; Sun et al., 2004, 2005).

The genus *Asterella* contains approximately 80 species, but only a few have been studied phytochemically (Asakawa and Heidelberger, 1982; Paliwal et al., 1991; Siddiqui et al., 1993; Neves et al., 1998). Herein, we report the identification of antifungal compounds from the liverwort *Asterella angusta* (Steph.) Pandé et al. (Aytoniaceae), collected

* Corresponding author. Tel.: +86 531 88382012; fax: +86 531 88382019.
E-mail address: louhongxiang@sdu.edu.cn (H. Lou).

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from Mount Gui-cheng, Sichuan Province, China. Thin-layer chromatography (TLC) bioautographic assay-guided fractionation of the diethyl ether extract of *A. angusta* furnished four new bis(bibenzyl)s, asterelin A (**1**), asterelin B (**2**), 11-*O*-demethyl marchantin I (**3**), and dihydroptychantol A (**4**), together with six known ones, marchantin H (**5**), marchantin M (**6**), marchantin P (**7**), perrottetin E (**8**), plagiochin E (**9**), and riccardin B (**10**). All of these displayed moderate antifungal activity against the common clinical pathogenic fungus *C. allbicans*, with minimal inhibitory concentration (MIC) values ranging from 16 μg/ml to 512 μg/ml.

2. Results and discussion

2.1. Structure elucidation

An antifungal activity-guided separation of the components in the diethyl ether extract of the air-dried *A. angusta* against *C. albicans*, using the TLC bioautographic assay, resulted in isolation of a series of bis(bibenzyl)s (**1**–**10**) as its active principles.

Compound **1** was obtained as a white powder. Its HREIMS gave a molecular ion peak at *m/z* 422.1514, in accordance with a molecular formula of $C_{28}H_{22}O_4$ (calcd. 422.1518). The IR spectrum showed the presence of hydroxyl groups (3422 cm^{-1}) and aromatic rings (1611 and 1503 cm^{-1}). The ^1H and ^{13}C NMR spectroscopic data of **1** (Table 1) displayed four benzylic methylenes and 24 benzene ring carbon atoms, including twelve aromatic protons, which indicated **1** was a bis(bibenzyl) (Asakawa et al., 2000). The coupling patterns of proton resonances in the range from δ_H 5.33 to 7.47 established the presence of four independent aromatic rings. The four signals at δ_H 6.61 (*dd*, $J = 2.6$, 8.3 Hz, H-2), 7.47 (*dd*, $J = 2.2$, 8.3 Hz, H-3), 5.98 (*dd*, $J = 2.2$, 8.2 Hz, H-5), and 6.29 (*dd*, $J = 2.6$, 8.2 Hz, H-6) could be assigned to a 1,4-disubstituted benzene ring (ring A) from their coupling patterns using ^1H–^1H COSY, while the two resonances at δ_H 6.83 (*d*, $J = 2.1$ Hz, H-10) and 6.87 (*d*, $J = 2.1$ Hz, H-12) suggested the presence of a 1,2,3,5-tetrasubstituted benzene ring (ring B). The signals at δ_H 5.33 (*d*, $J = 1.7$ Hz, H-3′), 6.84 (*dd*, $J = 1.7$, 8.4 Hz, H-5′), and 6.84 (*d*, $J = 8.4$ Hz, H-6′), together with resonances at δ_H 6.28 (*dd*, $J = 1.4$, 7.9 Hz, H-10′), 7.14 (*d*, $J = 7.9$ Hz, H-11′), and 7.26 (*d*, $J = 1.4$ Hz, H-14′) indicated the presence of two 1,2,4-trisubstituted benzene rings (rings C and D, respectively). The linkages of rings A and B *via* fragment CH_2(7)–CH_2(8) and rings C and D *via* CH_2 (7′)–CH_2 (8′) were confirmed, respectively, by the following long-range correlations (Fig. 1): H-3 and H-5 with C-7, H-10 with C-8; H-3′ and H-5′ with C-7′, H-10′ and H-14′ with C-8′ by use of HMBC. The highfield-shifted characteristic resonance for H-3′ (δ_H 5.33) suggested a biphenyl ether linkage between C-1 and C-2′ (Tori et al., 1985). The presence of a biphenyl linkage between C-14 and C-12′ was determined

Table 1
^1H and ^{13}C NMR spectroscopic data for compounds **1** and **2** in acetone-d_6[a]

Position	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
1		157.36		157.70
2	6.61 *dd* (2.6, 8.3)	121.56	6.59 *dd* (1.8, 8.3)	121.49
3	7.47 *dd* (2.2, 8.3)	131.98	7.44 *dd* (1.8, 8.3)	131.92
4		136.31		135.89
5	5.98 *dd* (2.2, 8.2)	130.78	5.96 *dd* (1.8, 8.2)	130.81
6	6.29 *dd* (2.6, 8.2)	119.99	6.27 *dd* (1.8 , 8.2)	119.99
7	2.73 *m*	39.32	2.72 *m*	39.28
	3.23*m*		3.22*m*	
8	3.47 *m*	35.13	3.44 *m*	35.14
	3.34 *m*		3.32 *m*	
9		138.55		138.58
10	6.83 *d* (2.1)	113.07	6.85 *d* (1.9)	113.07
11		157.96		157.94
12	6.87 *d* (2.1)	96.81	6.87 *d* (1.9)	96.79
13		158.36		158.37
14		117.35		117.32
1′		147.79		150.83
2′		146.38		147.96
3′	5.33 *d* (1.7)	122.22	5.41 *d* (1.7)	122.95
4′		133.30		134.58
5′	6.84 *dd* (1.7, 8.4)	125.43	6.93 *dd* (1.7, 8.4)	125.19
6′	6.84 *d* (8.4)	116.67	6.97 *d* (8.4)	113.36
7′	2.98 *m*	38.09	3.01 *m*	37.98
	2.77 *m*		2.79 *m*	
8′	3.13 *m*	38.46	3.13 *m*	38.41
	2.58 *m*		2.63 *m*	
9′		139.38		139.38
10′	6.28 *dd* (1.4, 7.9)	124.78	6.26 *dd* (1.4, 7.9)	124.67
11′	7.14 *d* (7.9)	121.42	7.14 *d* (7.9)	121.49
12′		122.93		123.07
13′		157.11		157.08
14′	7.26 *d* (1.4)	110.73	7.26 *d* (1.4)	110.72
OCH_3			3.83 *s*	56.26

[a] Recorded at 600 and 150 MHz for ^1H and ^{13}C, respectively. All assignments are based on HMQC and HMBC experiments. *J* values in Hz are in parentheses.

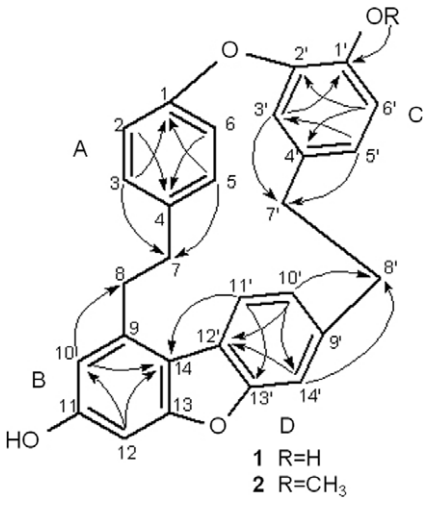
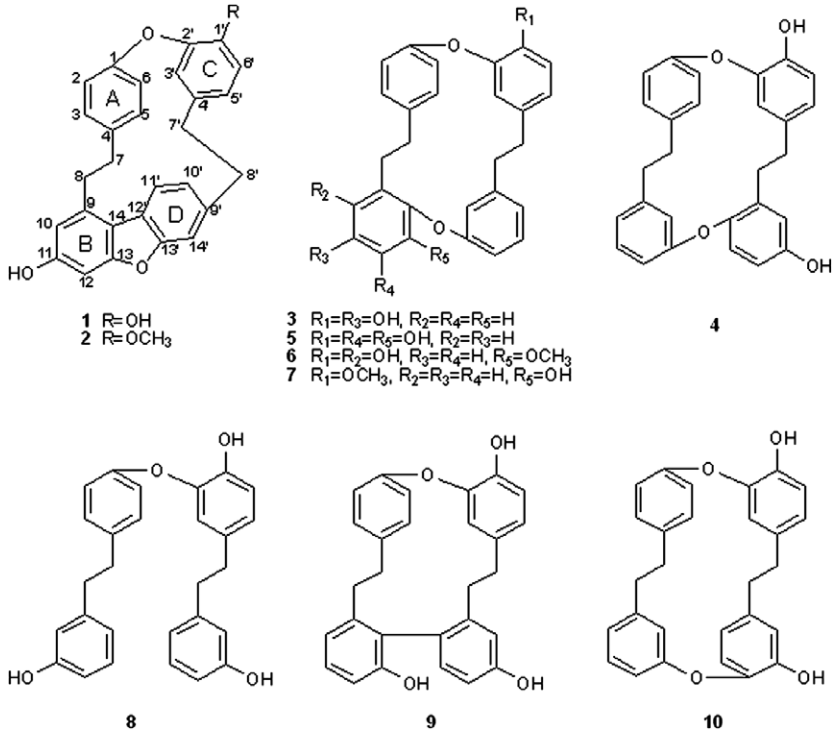


Fig. 1. Key long-range correlations in HMBC spectrum of compounds **1** and **2**.

by the correlation between H-12 and C-14 in the HMBC spectrum (Fig. 1). Accordingly, the riccardin skeleton of **1** was established (Asakawa et al., 2000). Comparison of the ^{13}C NMR spectrum with those of riccardins A and B (Asakawa et al., 2000) led to the observation of an ~10 ppm upfield shift for the C-11′ resonance (δ_C 121.42), and an ~6 ppm upfield shift for the C-14′ signal (δ_C 110.73), which indicated the presence of another biphenyl ether linkage between C-13 and C-13′ (a dibenzofunan linkage) (Alvarez et al., 2001). This assignment was further supported by the upfield shift of the C-12 resonance (δ_C 96.81, about 5 ppm) due to the presence of a dibenzofuran linkage (Tanaka et al., 2000; Gollapudi et al., 1994) and of OCH_3 with C-1′ (δ_C 150.83) in the HMBC spectrum (Fig. 1). The structure of **2** was, therefore, 13-hydroxy-1′-methoxyasterelin (asterelin B), which was further confirmed by X-ray diffraction analysis of a single-crystal (Fig. 2).

Both **1** and **2** contained a novel dibenzofuran linkage. The stereochemical structure of **2** also suggested the presence of a magnetically anisotropic effect in ring A, which resulted in non-equivalence of the NMR signals between CH-2 and CH-6, and between CH-3 and CH-5. Neither compounds were artefacts, as an HPLC-UV analysis confirmed their presence in the cold diethyl ether extract of *A. angusta* (Supplementary data).



from the molecular formula as well. The structure of this new bis(bibenzyl) **1** was thus determined and is the first example of a dibenzofuran bis(bibenzyl), for which we propose the general name asterelin to reflect the origin of the plant material. Compound **1** was therefore named 13,1′-dihydroxyasterelin (asterelin A).

Compound **2** was obtained as colorless needles and its molecular formula was established to be $C_{29}H_{24}O_4$ by HREIMS with a molecular ion at m/z 436.1655 $[M]^+$ (calcd. 436.1675). The IR (3420, 1609, and 1504 cm^{-1}), ^1H and ^{13}C NMR spectra (Table 1) also indicated the presence of a bis(bibenzyl) structure, which was very similar to that of **1**. The only differences in the ^1H and ^{13}C NMR spectra were the additional resonances at δ_H 3.83 (3H, *s*) and δ_C 56.26 when compared with **1**, which suggested the presence of an additional aromatic *O*-methyl group in **2**. The location of this methoxyl group was deduced to be at C-1′ from the long-range correlation

Compound **3** was obtained as a white powder. Its molecular formula $C_{28}H_{24}O_4$ was determined by HREIMS at m/z 424.1712 $[M]^+$ (calcd. 424.1718). **3** was also established to have a bis(bibenzyl) structure belonging to the marchantin family, as indicated by its characteristic NMR spectra (Table 2) (Asakawa et al., 2000). The ^1H and ^{13}C NMR spectroscopic data (Table 2) were consistent with four benzylic methylene signals [δ_H 2.80–3.01, 8H; δ_C 35.94, 29.40, 33.62, and 34.93], four aromatic protons at δ_H 6.57 (*d*, $J = 8.4$ Hz, H-2 and H-6) and 7.02 (*d*, $J = 8.4$ Hz, H-3 and H-5) on a 1,4-disubstituted benzene ring (ring A), three aromatic protons at δ_H 6.93 (*d*, $J = 2.9$ Hz, H-10), 6.61 (*dd*, $J = 2.9$, 8.7 Hz, H-12), and 6.67 (*d*, $J = 8.7$ Hz, H-13) together with resonances at δ_H 5.96 (*d*, $J = 1.7$ Hz, H-3′), 6.74 (*dd*, $J = 1.7$, 8.2 Hz, H-5′), and 6.80 (*d*, $J = 8.2$ Hz, H-6′) on two 1,2,4-trisubstituted benzene rings (rings B and C), as well as four aromatic protons at δ_H 6.55 (*m*, H-10′), 6.71 (*dd*, $J = 2.2$, 8.1 Hz, H-12′), 7.09 (*t*,

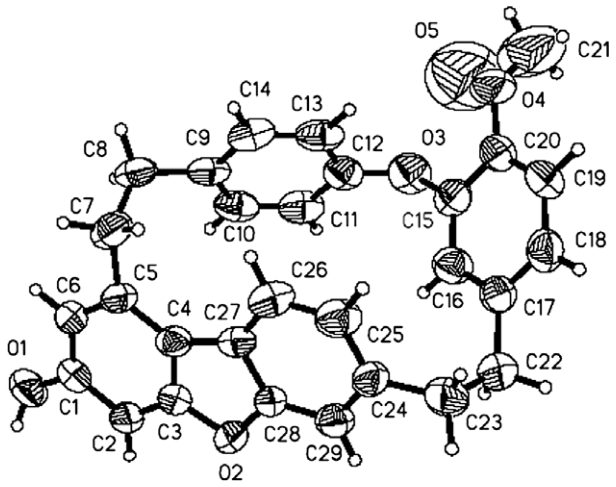


Fig. 2. Single crystal X-ray structure of **2**.

Table 2
^{1}H and ^{13}C NMR spectroscopic data for compounds **3** and **4** in acetone-d_6[a]

Position	**3**		**4**	
	δ_H	δ_C	δ_H	δ_C
1		154.22		153.35
2	6.57 *d* (8.4)	119.56	6.86 *d* (8.4)	121.06
3	7.02 *d* (8.4)	129.42	7.01 *d* (8.4)	130.24
4		136.94		137.05
5	7.02 *d* (8.4)	129.42	7.01 *d* (8.4)	130.24
6	6.57 *d* (8.4)	119.56	6.86 *d* (8.4)	137.05
7	3.01 *m* (2H)	35.94	3.00 *m* (2H)	36.07
8	3.01 *m* (2H)	29.40	3.00 *m* (2H)	37.14
9		133.16		142.51
10	6.93 *d* (2.9)	116.74	6.98 *br d* (8.3)	121.15
11		152.80	7.13 *t* (8.3)	128.77
12	6.61 *dd* (2.9, 8.7)	113.11	6.20 *br d* (8.3)	109.48
13	6.67 *d* (8.7)	118.31		158.39
14		146.64	6.32 *m*	118.28
1′		145.05		148.02
2′		145.58		144.78
3′	5.96 *d* (1.7)	116.92	6.13 *d* (1.4)	114.60
4′		132.08		133.22
5′	6.74 *dd* (1.7, 8.2)	122.93	6.71 *dd* (1.4, 8.3)	121.19
6′	6.80 *d* (8.2)	115.67	6.80 *d* (8.3)	115.30
7′	2.80 *m*	33.62	2.55 *m*	36.81
	2.89 *m*		2.55 *m*	
8′	2.80 *m*	34.93	2.41 *m*	34.22
	2.89 *m*		2.41 *m*	
9′		142.49		135.47
10′	6.55 *m*	117.23		144.35
11′		156.96	6.78 *d* (8.4)	121.92
12′	6.71 *dd* (2.2, 8.1)	114.97	6.71 *dd* (2.8, 8.4)	113.77
13′	7.09 *t* (8.1)	128.70		154.08
14′	6.60 *d* (8.1)	122.38	6.82 *d* (2.8)	116.49

[a] Recorded at 600 and 150 MHz for ^{1}H and ^{13}C, respectively. All assignments are based on HMQC and HMBC experiments. *J* values in Hz are in parentheses.

$J = 8.1$ Hz, H-13′), and 6.60 (*d*, $J = 8.1$ Hz, H-14′) on a 1,3-disubstituted benzene ring (ring D). In the HMBC spectrum, the long-range correlations of H-3 and H-5 with C-7, and H-10 with C-8, suggested the connection of rings A and B *via* CH_2 (7)–CH_2 (8). Analogously, rings C and D were linked *via* CH_2 (7′)–CH_2 (8′) because of the presence of the long-range correlations between H-3′(H-5′) and C-7′, H-14′ and C-8′. The characteristic resonance at δ_H 5.96 (H-3) suggested that C-1 (δ_C 154.22) and C-2′ (δ_C 145.58) were linked by an ether bridge (Tori et al., 1985). The hydroxyl proton at δ_H 8.14 (*s*) correlated with C-9, C-10, and C-11, and δ_H 7.77 (*s*) correlated with C-1′, C-2′, and C-6′ in the HMBC, which indicated that these two OH groups were positioned at C-10 and C-1′, respectively. The structure of **3** was determined to be 14,1′-dihydroxymarchantin, which was named 11-*O*-demethyl marchantin I (Asakawa et al., 2000).

Compound **4** was obtained as a white powder. Its molecular formula $C_{28}H_{24}O_4$ was established by HREIMS from its molecular ion at *m*/*z* 424.1720 $[M]^+$ (calcd. 424.1718). The ^{1}H and ^{13}C NMR spectroscopic data indicated four benzylic methylenes (δ_H 2.41–3.00, 8H; δ_C 36.07, 37.14, 36.81, and 34.22) and four benzene rings (14 aromatic protons in the range from δ_H 6.13 to 7.13 and 24 aromatic carbon atoms), suggesting a bis(bibenzyl) structure (Asakawa et al., 2000). According to their coupling patterns, four protons [δ_H 6.86 (*d*, $J = 8.4$ Hz, H-2 and H-6) and 7.01 (*d*, $J = 8.4$ Hz, H-3 and H-5)] were on a 1,4-disubstituted benzene ring (ring A), four protons [δ_H 6.98 (*br d*, $J = 8.3$ Hz, H-10), 7.31 (*t*, $J = 8.3$ Hz, H-11), 6.20 (*br d*, $J = 8.3$ Hz, H-12), and 6.32 (*m*, H-14)] belonged to a 1,3-disubstituted benzene ring (ring B), and six protons [δ_H 6.13 (*d*, $J = 1.4$ Hz, H-3′), 6.71 (*dd*, $J = 1.4$, 8.3 Hz, H-5′), 6.80 (*d*, $J = 8.3$ Hz, H-6′); 6.78 (*d*, $J = 8.4$ Hz, H-11′), 6.71 (*dd*, $J = 2.8$, 8.4 Hz, H-12′), and δ_H 6.82 (*d*, $J = 2.8$ Hz, H-14′)] were on two 1,2,4-trisubstituted benzene rings (rings C and D), respectively. From analysis of the HMBC spectrum, the cross-peaks between H-3(H-5) and C-7, H-10(H-14) and C-8 indicated a connection of rings A and B *via* CH_2 (7)–CH_2 (8), while the cross-peaks between H-3′(H-5′) and C-7′, H-14′ and C-8′ implied the linkage of rings C and D *via* a CH_2 (7′)–CH_2 (8′) bridge. The long-range correlations of the phenolic protons (δ_H 8.27 and 7.82, *s*) with carbon atoms C-12, C-13, C-14 and C-1′, C-2′, C-6′, respectively, also indicated there were two OH groups positioned at C-13 and C-1′. Compound **4** was thus assigned to be 1′,13′-dihydroxyisomarchantin, which was named dihydroptychantol A (Asakawa et al., 2000).

The conformational strains of **3** (CAS registry No. 142502-55-4) and **4** (142502-56-5) have been computed previously by Kesseru and Nogradi (1992) by means of the DTMM and MM2 approach, but neither of them have been isolated from a natural source and been synthesized before. Our isolation of **3** and **4** validated Kesseru's calculations.

The known compounds were identified as marchantin H (**5**) (Tori et al., 1985), marchantin M (**6**) (Wei et al., 1995), marchantin P (**7**) (Tori et al., 1994), perrottetin E (**8**) (Toyota et al., 1985), plagiochin E (**9**) (Niu et al., 2006), and riccardin B (**10**) (Asakawa et al., 1983) on the basis of a

Table 3
Minimal inhibitory quantity (MIQ) and minimal inhibitory concentration (MIC) values for bis(bibenzyl)s on *Candida albicans* ATCC10231 using the bioautographic assay and the microdilution method, respectively

Compounds	MIQ (μg)	MIC (μg/ml)
1	2.0	128
2	10.0	512
3	0.4	32
4	0.8	64
5	4.0	256
6	10.0	512
7	15.0	512
8	2.0	128
9	0.25	16
10	0.5	32
Fluconazole[a]	0.01	0.3

[a] Positive control.

comparison of their ^{1}H and ^{13}C NMR spectroscopic data with those reported.

2.2. Antifungal activity

Significant antifungal activity was found for the diethyl ether extract of *A. angusta* at 100 μg/dot by use of direct TLC bioautography. Compounds **1**–**10** all showed antifungal activity against *C. albicans*; their minimal inhibitory quantity (MIQ) and minimal inhibitory concentration (MIC) values are listed in Table 3.

From a structure–activity point of view, it seems that the free hydroxyl groups present in the structure are necessary for the antifungal activity as has been found in previous studies for bis(bibenzyl)s (Scher et al., 2004; Niu et al., 2006). The MIQ and MIC values of **1** are lower than those of **2**, which indicates that methylation of the OH groups will decrease their antifungal activity.

3. Experimental

3.1. General experimental procedures

Silica gel (200–300 mesh) for column chromatography, and high-performance TLC plates precoated with silica gel GF_{254} were bought from Qingdao Haiyang Chemical Plant (Qingdao, China). Sephadex LH-20 was obtained from Pharmacia Biotek (Denmark). MIC gel (CHP20P, 75–150 μm) was purchased from Mitsubishi Chemical Industries Ltd. (Japan). TLC plates were developed with petroleum spirit/acetone (6:4, v/v). The HPLC system consisted of an Agilent 1100 G1310A isopump, an Agilent 1100 G1322A degasser, and an Agilent 1100 G1314 VU-detector. Column: ZORBAX Eclipse XDB-C18, 4.6 mm × 150 mm, particle size 5 μm; temperature: 25 °C. HPLC condition: mobile phase A, water; mobile phase B, MeOH; linear gradient: 0 min, 60% B, 25 min, 75% B, 60 min, 80% B, 75 min, 95% B; stop time, 90 min; flow-rate: 1.1 ml/min; detection wavelength, 280 nm; injection volume, 15 μl. Melting points (m.p.) were determined with an X-6 micro-melting point apparatus (Beijing Tech Co., Ltd.) and were uncorrected. UV spectra: Shimadzu UV-2450 spectrophotometer. IR spectra: Thermo-Nicolet 670 spectrophotometer. NMR Spectra: Bruker Avance DRX-600 spectrometer operating at 600 (^{1}H) or 150 (^{13}C) MHz in acetone-d_6; δ in ppm reactive to Me_4Si as internal standard. The 2D NMR spectra were recorded with standard pulse programs and acquisition parameters. ESIMS: API 4000 triple-stage quadrupole instrument. HREIMS: VG ZAB-2F mass spectrometer, in m/z (rel. %).

3.2. Plant material

A. angusta (Steph.) Pande et al. was collected from Mount Gui-cheng, Sichuan Province, China and was identified by Professor Qian Gao, Shenyang Institute of Applied Ecology, Chinese Academy of Sciences. A voucher specimen (no. 20040627) has been deposited at the Department of Natural Products Chemistry, School of Pharmaceutical Sciences, Shandong University.

3.3. Microorganisms and media

The test organism used in this study was *C. albicans* ATCC10231, which was purchased from Shandong Provincial Sanitary and Antiepidemic Station. All media were purchased from BioSharp. Fluconazole was kindly provided by the Institute of Biopharmaceuticals of Shandong Province.

3.4. Extraction and isolation

The air-dried and powdered material of *A. angusta* (2.5 kg) was extracted with 6 l of EtOH–H_2O (95:5, v/v) by heating until reflux began and maintaining this for 2.5 h. The process was repeated three times, and the combined extracts were concentrated under reduced pressure to obtain a crude extract (78 g), which was suspended in H_2O (500 ml) and partitioned with Et_2O (3 × 250 ml). TLC bioautographic assays indicated that the Et_2O extract at 100 μg/dot showed significant antifungal activity against *C. albicans*. Evaporation of Et_2O under reduced pressure afforded a dark brown solid (35 g). A portion (32 g) of the Et_2O extract was subjected to open CC over silica gel (500 g) and eluted with a gradient of petroleum spirit/acetone (100:0 to 0:100). A total of 120 fractions were collected and combined on the basis of their TLC profiles to afford seven combined fractions F_1–F_7, of which F_2–F_6 were found to be antifungal against *C. albicans* and visible inhibition zones appeared in the range of $R_f = 0.35$–0.62 on the bioautographic TLC plates. F_2 (1.5 g) was subjected to chromatography of Sephadex LH-20 ($CHCl_3$/MeOH, 1:1, v/v), then fractionated by repeated CC over silica gel (petroleum spirit/acetone gradient 100:0 to 70:30) to afford compound **7** (15 mg). F_6 (3.1 g) was purified by identical procedures as F_2 and yielded compounds **5** (10 mg), **8**

(10 mg), and **9** (50 mg). F_3 (5.0 g) was subjected to an MCI gel column using MeOH–H_2O (9:1, v/v) as eluant to afford a depigmented fraction F_{3a} (2.0 g). Separation of F_{3a} followed the same procedure as F_2 and yielded an enriched fraction, a part (50 mg) of which was purified by semi-preparative HPLC to afford 15 mg of **2** ($t_R = 44.0$ min, recrystallized in MeOH). F_4 (2.7 g) was subjected to chromatography of Sephadex LH-20 ($CHCl_3$/MeOH, 1:1, v/v) to yield compounds **6** (100 mg), **10** (10 mg), and a bibenzyl mixture F_{4a} (120 mg). A part (60 mg) of F_{4a} was further fractionated by semi-preparative HPLC to afford **3** (10 mg, $t_R = 46.0$ min) and **4** (8 mg, $t_R = 53.7$ min). F_5 (2.0 g) was purified under the conditions used for F_4 and afforded **1** (10 mg, $t_R = 36.0$ min).

3.4.1. Asterelin A (*1*)

White powder, m.p. 203–204 °C; UV (MeOH) λ_{max} (log ε) 212 (4.83), 304 (3.56) nm; IR (KBr) ν_{max} 3422, 1611, 1503, 1436 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Table 1; ESIMS (negative) *m/z* 421 $[M-H]^-$; HREIMS *m/z* 422.1514 $[M]^+$ (calcd. for $C_{28}H_{22}O_4$, 422.1518).

3.4.2. Asterelin B (*2*)

Colorless needles (MeOH), m.p. 196–197 °C. UV (MeOH) λ_{max} (log ε) 212 (4.84), 304 (3.58) nm; IR (KBr) ν_{max} 3420, 1609, 1504, 1439 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Table 1; ESIMS (negative) *m/z* 435 $[M-H]^-$; HREIMS *m/z* 436.1655 $[M]^+$ (calcd. for $C_{29}H_{24}O_4$, 436.1675).

3.4.3. 11-O-demethyl marchantin I (*3*)

White powder, m.p. 200–202 °C; UV (MeOH) λ_{max} (log ε) 210 (4.78), 273 (3.67) nm; IR (KBr) ν_{max} 3494, 1609, 1576, 1506 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Table 2; ESIMS (negative) *m/z* 423 $[M-H]^-$; HREIMS *m/z* 424.1712 $[M]^+$ (calcd. for $C_{28}H_{24}O_4$, 424.1718).

3.4.4. Dihydroptychantol A (*4*)

White powder, m.p. 201–202 °C; UV (MeOH) λ_{max} (log ε) 210 (4.76), 273 (3.70); IR (KBr) ν_{max} 3494, 1609, 1583, 1505 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Table 2; ESIMS (negative) *m/z* 423 $[M-H]^-$. HREIMS *m/z* 424.1720 $[M]^+$ (calcd. for $C_{28}H_{24}O_4$, 424.1718).

3.5. X-ray crystallographic analysis of *2*

Single crystals suitable for X-ray analysis were obtained by recrystallization from methanol. A colorless platelet crystal with approximate dimensions of 0.39 mm × 0.07 mm × 0.01 mm was used for analysis. All measurements were made on a Bruker APEX2 CCD area-detector diffractometer employing graphite monochromated Mo Kα radiation ($\lambda = 0.71073$ Å) at 293 K and operating in the ϕ–ω scan mode. Crystal data: $C_{29}H_{24}O_4 \cdot H_2O$, M = 454.50, monoclinic, space group *P*2(1), $a = 15.2970(10)$ Å, $b = 6.162(8)$ Å, $c = 11.850(16)$ Å, $\beta = 105.643(4)°$, $V = 2254.4(2)$ $Å^3$, $Z = 4$, $D_{calcd} = 1.339$ Mg/m^3, $F(000) = 960$, and μ(Mo Kα) = 0.091 mm^{-1}. Cell refinement and data reduction: APEX2 Software Suite (Bruker, 2005). Program used to refine structure: SHELXL-97 (Sheldrick, 1997); refinement on F^2, full-matrix least-squares calculations. All non-hydrogen atoms were refined anisotropically, and all hydrogen atoms were placed in geometrically calculated positions and refined as riding atoms with the relative isotropic parameters. One lattice water molecule was contained in the structure. A total of 14,763 reflections (5125 unique, $R_{int} = 0.1613$) were collected from 1.60° to 27.71° in θ and index ranges: $12 \geqslant h \geqslant -19$, $7 \geqslant k \geqslant -5$, $33 \geqslant I \geqslant -34$. The final stage converged to $R_1 = 0.0811$ ($wR_2 = 0.1408$) for 5125 observed reflections [with $I > 2\sigma(I)$] and 310 variable parameters, $R_1 = 0.0680$ ($wR_2 = 0.1615$) for all unique reflections and GoF = 1.022. Details of crystallographic data (excluding structural factors) for the structure analysis have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC 631368. Copies of these data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: C44 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk].

3.6. Antifungal assays

3.6.1. Minimal inhibitory quantity (MIQ)

The bioautography procedure described by Rahalison et al. (1994) and Nostro et al. (2000) was adopted to determine the active fractions. The ether extract of *A. angusta* (50 mg/ml, in EtOH, 2 μl) was developed on a TLC plate with petroleum spirit/acetone (6:4, v/v). The plate was dried carefully to remove all solvents, and overlaid by agar seeded with an overnight culture of *C. albicans* (~10^7 CFU/ml). Then it was placed in a sterile Petri dish containing filter-paper humidified with water and incubated for 24 h at 30 °C. The bioassay plate was visualized by spraying with 2.5 mg/ml^{-1} methyl thiazolyl tetrazolium chloride (MTT) in a phosphate saline buffer solution (PBS), followed by incubation at ambient temperature for 30 min. Fluconazole (positive control) and the isolated bis(bibenzyl)s were diluted to different concentrations, and 2 μl of each solution was applied to a TLC plate to determine the MIQ values against *C. albicans* by the method described above. The quantity of the first spot, where no inhibition zone was visualized, was taken as MIQ.

3.6.2. Minimal inhibitory concentration (MIC)

MIC values were determined by the broth microdilution method (NCCLS, 2002). All tests were performed in RPMI-1640 broth supplemented with DMSO at a final concentration of 0.5% (v/v). *C. albicans* ATCC10231 was incubated for 48 h before MIC determination. Serial double dilutions of the bis(bibenzyl)s were prepared in a

96-well microtiter plate and ranged from 0.25 μg/ml to 512 μg/ml. The final inocula were adjusted to 2.5×10^4 CFU/ml. The inoculated plates were incubated aerobically at 35 °C for 24 h. Growth of the microorganism was indicated by the presence of turbidity and a pellet on the well bottom. The MIC value was recorded as the lowest concentrations at which no microorganism growth was observed. Fluconazole was used as a positive control drug.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2007.04.036.

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