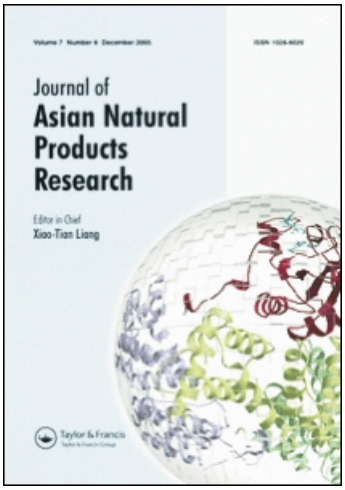

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Antifungal stilbenoids from *Stemona japonica*

Ya-Zhong Zhang[a]; Guo-Bing Xu[a]; Tong Zhang[b]
[a] Anhui Institute for Drug Control, Hefei, China [b] Department of TCM Pharmaceutics, Shanghai University of Traditional Chinese Medicine, Shanghai, China

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Antifungal stilbenoids from *Stemona japonica*

Ya-Zhong Zhang[a], Guo-Bing Xu[a] and Tong Zhang[b]*

[a]*Anhui Institute for Drug Control, Hefei, China;* [b]*Department of TCM Pharmaceutics, Shanghai University of Traditional Chinese Medicine, Shanghai, China*

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Three new dihydrostilbenes, stilbostemins P–R (**1**–**3**), and a new dihydrophenanthrene, stemanthrene G (**4**), were isolated from the roots of *Stemona japonica* together with three known bibenzyls, 3,5-dihydroxy-2′-methoxy bibenzyl (**5**), 3,3′-dihydroxy-2,5′-dimethoxy bibenzyl (**6**), and 3,5,2′-trihydroxy-4-methyl bibenzyl (**7**). Their structures were elucidated by spectroscopic analyses. Compounds **5** and **6** exhibited strong antifungal activities against *Candida albicans*.

Keywords: *Stemona japonica*; stilbenoids; stilbostemins P–R; stemanthrene G; antifungal

1. Introduction

The roots of *Stemona japonica* (Bl.) Miq have long been prescribed in traditional Chinese medicine for various medicinal biological properties [1]. Especially, the extracts from the flesh tuberous roots are still used in the treatment of respiratory disorders, including pulmonary tuberculosis and bronchitis, and externally used to kill insect pests [2,3]. In previous chemical investigations on *S. japonica*, many alkaloids [4,5] and stilbenoids [6] have been reported. As a continuation of our search for new bioactive principles from the title plant, three novel dihydrostilbenes, named stilbostemins P–R (**1**–**3**); a new dihydrophenanthrene, stemanthrene G (**4**); and three known bibenzyls, 3,5-dihydroxy-2′-methoxy bibenzyl (**5**), 3,3′-dihydroxy-2,5′-dimethoxy bibenzyl (**6**), and 3,5,2′-trihydroxy-4-methyl bibenzyl (**7**) were isolated. All the compounds were subjected to antimicrobial tests against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Candida albicans*. Compounds **5** and **6** exhibited strong antifungal activities against *C. albicans*.

2. Results and discussion

Stilbostemin P (**1**) was obtained as colorless oil. The molecular formula of **1** was deduced as $C_{17}H_{20}O_4$ from a molecular ion peak at *m/z* 288.1362 in the HR-EI-MS spectrum, which was consistent with the EI-MS and ^{13}C NMR spectral data. UV absorption maxima at 279 and 219 nm revealed the presence of benzyl moieties.[7] Its ^1H NMR spectrum showed one 1,2,4-trisubstituted benzene [δ_H 7.02 (1H, d, $J = 8.1$ Hz), 6.48 (1H, d, $J = 2.5$ Hz), and 6.43 (1H, dd, $J = 8.1$, 2.5 Hz)], two *meta*-coupling protons in the other benzene [δ_H 6.29 (1H, d, $J = 2.3$ Hz) and 6.22 (1H, d, $J = 2.3$ Hz)], two methoxy groups (δ_H 3.81 and 3.80, each 3H, s), a methyl group (δ_H 2.14, 3H, s), and two methylenes [δ_H 2.75 (4H, br s)], which indicated that **1** should be a

*Corresponding author. Email: zhangtdmj@sohu.com

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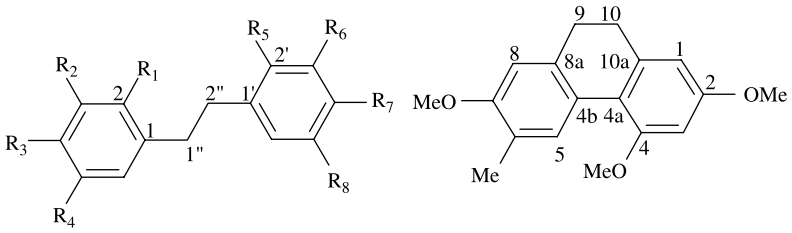
pentasubstituted bibenzyl. The substitution pattern of **1** was established by the NOESY experiments (Figure 2). The NOE correlation chain H-2″ ↔ 2′-OMe ↔ H-3 ↔ 4′-OMe confirmed the 2′,4′-dimethoxy substitution in ring B. Additionally, the cross-peaks between the methyl group at δ_H 2.14, the proton at δ_H 6.29, and H-1″ confirmed that the proton was in position 6 and the methyl group was attached to C-2. Taking into account the presence of the special ion fragments at *m/z* 137 ($C_8H_9O_2$) in EI-MS spectrum, the remaining positions 3 and 5 should be substituted by two hydroxyl groups in ring A. The substitution patterns of rings A and B were further confirmed by the HMBC correlations (Figure 3) between H-4, H-6, H-1″, and C-2; between 2-Me, H-4, and C-3; and between H-4, H-6, and C-5, as well as between H-2″, H-3′, 2′-methoxy, and C-2′, and between H-3′, 4′-methoxy, H-5′, H-6′, and C-4′. Thus, **1** was identified as 3,5-dihydroxy-2′,4′-dimethoxy-2-methyl bibenzyl (Figure 1).

Compound **2** was also isolated as an oil, possessing the same molecular formula $C_{17}H_{20}O_4$ as **1**, which was deduced from its HR-EI-MS and ^{13}C NMR spectra. Its UV, IR, and ^1H NMR spectra were almost superposed with those of **1**. All of these suggested that **2** was an isomer of **1**. Two similar *meta*-coupling protons [δ_H 6.27 (1H, d, $J = 2.2$ Hz) and 6.22 (1H, d, $J = 2.2$ Hz)] to those of **1** suggested the 3,5-dihydroxy-2-methyl substitution in ring A, which was further confirmed by the NOESY correlations between the methyl



1 R_1=Me, R_2,R_4=OH, R_3,R_6,R_8=H, R_5,R_7=OMe
2 R_1=Me, R_2,R_4=OH, R_3,R_6,R_7=H, R_5,R_8=OMe
3 R_1,R_5,R_6,R_8=H, R_2,R_4,R_7=OMe, R_3=Me

4

Figure 1. Structures of **1**–**4**.

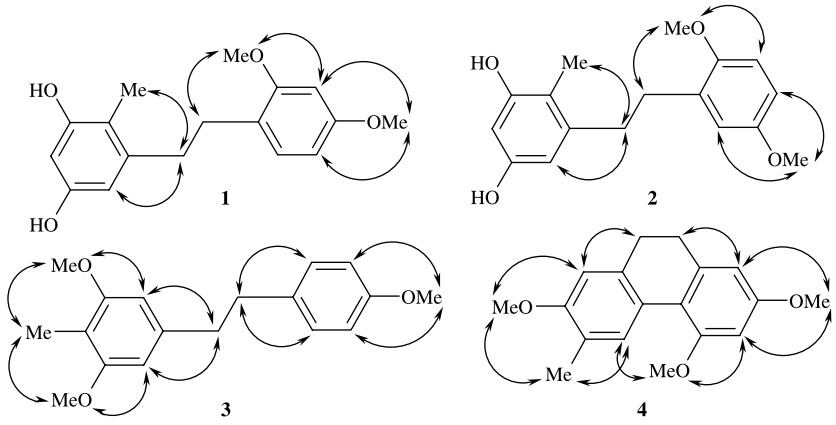


Figure 2. Key NOESY correlations for **1**–**4**.

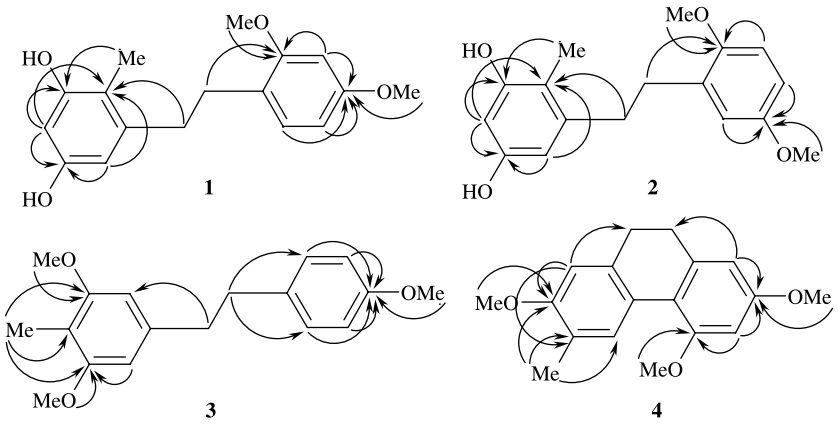


Figure 3. Key HMBC correlations for **1**–**4** (H–C).

group at δ_H 2.08, the proton at δ_H 6.27, and H-1″, and the presence of the special ion fragment at *m/z* 137 ($C_8H_9O_2$) in the EI-MS spectrum. Moreover, the cross-peaks between the methoxy at δ_H 3.84 and H-2″ and H-3′ showed that it was attached to C-2′, and the ones between the other methoxy at δ_H 3.78 and H-4′ and H-6′ confirmed that it was in position 5′. The HMBC experiments provided more evidence to elucidate the structure of **2** (Figure 3). These findings have led to a conclusion that **2** has the structure 3,5-dihydroxy-2′,5′-dimethoxy-2-methyl bibenzyl.

The molecular formula of **3** was determined as $C_{18}H_{22}O_3$ by the HR-EI-MS and ^{13}C NMR spectra. The ^1H NMR spectrum (Table 1) disclosed signals for four aromatic protons in one 1,4-disubstituted benzene ring, two equivalent singlet protons in the other benzene ring, three methoxy groups and a methyl group, as well as two methylenes, which indicated **3** was a tetrasubstituted bibenzyl. Meanwhile, the ^{13}C NMR spectrum exhibited four signals in the aromatic region (δ_C 140.9, 107.7, 158.2, and 111.8), which was consistent with the symmetrical 3,5-dimethoxy-4-methyl or 2,6-dimethoxy-4-methyl substitution pattern in ring A. This was confirmed by the presence of two fragment ions at *m/z* 165 ($C_{10}H_{13}O_2$) and 121 (C_8H_9O) in the EI-MS spectrum. The NOE correlations between one methoxy signal at δ_H 3.79 and H-3′ and H-5′, and between two methoxyl signals at δ_H 3.85 and H-2, 4-methyl, and H-6 revealed that three methoxy groups were in positions 4′, 3 and 5, respectively. HMBC data (Figure 3) also supported the substitution pattern. Compound **3** was concluded to be as 3,5,4′-trimethoxy-4-methyl bibenzyl.

The molecular formula of **4** was established as $C_{18}H_{20}O_3$ by its HR-EI-MS and ^{13}C NMR spectra, which suggested the presence of nine degrees of unsaturation. Compared with the above compounds, **4** showed more intense UV absorption maxima at 306, 281, and 213 nm, which were typical for dihydrophenanthrenes [7,8]. The positions of the functional groups in rings A and B were confirmed by the NOESY spectrum (Figure 2). The NOE contacting chain H-9 ↔ H-8 ↔ 7-methoxy ↔ 6-methyl ↔ H-5 ↔ 4-methoxy ↔ H-3 ↔ 2-methoxy established the 2,5,7-trimethoxy-6-methyl substitution of **4**. Furthermore, the HMBC experiments confirmed this substitution pattern and also allowed the assignment of the ^{13}C resonances (Figure 3). Therefore, the structure of **4** was determined to be 2,5,7-trimethoxy-6-methyl-9,10-dihydrophenanthrene.

By comparison with the previously published data [7], the three known compounds were identified as 3,5-dihydroxy-2′-methoxy bibenzyl (**5**), 3,3′-dihydroxy-2,5′-dimethoxy bibenzyl (**6**), and 3,5,2′-trihydroxy-4-methyl bibenzyl (**7**).

All compounds were tested against *S. aureus* (ATCC 25923), *S. epidermidis* (ATCC 12228), *E. coli* (ATCC 15628), and *C. albicans* (ATCC 16000) *in vitro* by a microdilution assay [9]. Two most potential natural antibacterial agents, bakuchiol [10] and magnolol [11], were used as positive controls in the tests. Among these compounds, **5** and **6** showed strong antifungal activities against *C. albicans* at the level of MICs 12.5–25 μg/ml (Table 3). In comparison with the positive controls, compounds **1**–**6** exhibited less antibacterial activities against *S. aureus*, *S. epidermidis*, and *E. coli*.

3. Experimental

3.1 General experimental procedures

UV spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer, and IR spectra on a Nicolet Magna 750 FTIR (KBr) spectrophotometer. All MS data were obtained with MAT-95 mass spectrometer. NMR spectra were recorded on a Bruker AM-400 instrument with TMS as internal standard; the chemical shift values are reported in units (δ) and coupling constants (J) are given in Hertz. Silica gel (100–200, 200–300 mesh) and silica gel GF_{254} for precoated plates (produced by Qingdao Haiyang Chemical Group Co. Qingdao, China) were used for column chromatography (CC) and preparative TLC, respectively.

3.2 Plant material

S. japonica (Stemonaceae) was collected, in September 2003, at Anji County (Zhejiang Province, China) and identified by Dr. Ya-zhong Zhang of Shanghai University of Traditional Chinese Medicine where a voucher specimen (No. 2003/SJ005/TCM) is deposited.

3.3 Extraction and isolation

The air-dried and powdered roots of *S. japonica* (4.5 kg) were percolated with 95% EtOH (10 l × 3 times) at room temperature. The extract (340 g) was suspended in H_2O (2 l) and partitioned successively with petroleum ether (Pe), ether, EtOAc, and *n*-BuOH. The ether-soluble fraction (30 g) was subjected to CC over silica gel eluted with Pe–EtOAc gradients (5:1 to 1:2) to obtain fractions 1–7. Fraction 1 (1.6 g) was chromatographed over silica gel with hexane–EtOAc–formic acid (100:15:1, 2 l) to

Table 1. ^{1}H NMR spectral data for compounds **1**–**4** in $CDCl_3$ (400 MHz).

No.	1	2	3	No.	4
2			6.23 s	1	6.41 d (2.1)
4	6.22 d (2.3)	6.22 d (2.2)		3	6.32 d (2.1)
5				5	7.80 s
6	6.29 d (2.3)	6.27 d (2.2)	6.23 s	8	6.69 s
2′			7.09 d (8.5)	9	2.65 m
3′	6.48 d (2.5)	7.04 d (8.4)	6.83 d (8.5)	10	2.69 m
4′		6.43 dd (8.4, 2.3)		2-OMe	3.77 s
5′	6.43 dd (8.1, 2.5)		6.83 d (8.5)	4-OMe	3.77 s
6′	7.02 d (8.1)	6.53 d (2.3)	7.09 d (8.5)	7-OMe	3.72 s
1″	2.75 br s	2.71 br s	2.73 m	6-Me	2.27 s
2″	2.75 br s	2.71 br s	2.82 m		
2-Me	2.14 s	2.08 s			
4-Me			2.11 s		
3-OMe			3.85 s		
5-OMe			3.85 s		
2′-OMe	3.81 s	3.84 s			
4′-OMe	3.80 s		3.79 s		
5′-OMe		3.78 s			

Table 2. ^{13}C NMR spectral data for compounds **1**–**4** in $CDCl_3$ (100 MHz).

No.	**1**	**2**	**3**	No.	**4**
1	143.2	143.5	140.9	1	106.4
2	113.6	114.7	107.7	2	160.5
3	156.5	155.3	158.2	3	100.1
4	100.7	100.8	111.8	4	158.2
5	156.2	154.5	158.2	4a	114.1
6	108.1	108.8	107.7	4b	125.4
1′	123.3	131.9	133.7	5	128.3
2′	158.8	152.3	128.5	6	121.4
3′	98.9	111.7	113.6	7	156.9
4′	160.2	111.5	157.4	8	114.2
5′	104.6	153.7	113.6	8a	137.6
6′	130.5	116.7	128.5	9	29.1
1″	35.3	34.3	37.5	10	31.2
2″	32.7	31.7	36.6	10a	141.3
2-Me	10.4	10.4		2-OMe	55.6
4-Me			7.8	4-OMe	56.4
3-OMe			55.7	7-OMe	56.1
5-OMe			55.7	6-Me	15.5
2′-OMe	55.5	56.2			
4′-OMe	55.2		55.2		
5′-OMe		55.8			

yield the mixture of **1** and **2**. Compounds **1** (11 mg) and **2** (7 mg) were obtained by preparative TLC (hexane–acetone–formic acid, 100:10:1). Fraction 2 (2.3 g) was chromatographed over silica gel with hexane–EtOAc–formic acid (100:20:1, 2.5 l) to yield crude **3** (24 mg) and **4** (13 mg). Compounds **3** (9 mg) and **4** (5 mg) were obtained as pure ones by preparative TLC (hexane–EtOAc–formic acid, 100:15:1). Fraction 3 (6.9 g) was also subjected to CC over silica gel. The elution was carried out with hexane–EtOAc–formic acid (100:30:1) to yield fractions 3.1–9. Fraction 3.5 (3.4 g) was purified by CC on silica gel with 30% EtOAc in hexane to afford 11 mg of **5** and 25 mg of **6**. Fraction 6 (500 mg) was separated with Sephadex LH-20 (MeOH) to afford crude **7** (125 mg). Then 89 mg of **7** was obtained by recrystallization in acetone.

3.4 Antimicrobial bioassay

Bioassay on antimicrobial activities against *S. aureus*, *S. epidermidis*, *E. coli*, and

Table 3. Antimicrobial activities of **1**–**6** in MIC[a] values (μg/ml).

Compounds	*S. aureus*	*S. epidermidis*	*E. coli*	*C. albicans*
1	>50	>50	>50	>50
2	>50	>50	>50	>50
3	50	>50	>50	>50
4	>50	50	>50	>50
5	50	>50	>50	12.5
6	>50	>50	>50	25
7	>50	50	>50	>50
Bakuchiol[b]	25	12.5	50	25
Magnolol[b]	25	12.5	50	50

[a] MIC was defined as the lowest concentration that inhibited visible growth.
[b] Bakuchiol and magnolol were used as positive control agents.

C. albicans in vitro were carried out according to the protocols reported in the literature [9].

3.4.1 Stilbostemin P (**1**)

Colorless oil; UV (MeOH) λ_{max} (log ε; nm): 279 (3.37), 219 (4.02); IR (KBr) ν_{max} (cm^{-1}): 3349, 2927, 2854, 1614, 1594, 1508, 1455, 1290, 1258, 1213, 1154, 1142, 1045, 836; ^1H and ^{13}C NMR spectral data: see Tables 1 and 2; EI-MS *m/z*: 288 $[M]^+$(5), 167 (14), 151 (100), 149 (10), 121 (27), 91 (6), 77 (4); HR-EI-MS *m/z*: 288.1362 $[M]^+$ (calcd for $C_{17}H_{20}O_4$, 288.1361).

3.4.2 Stilbostemin Q (**2**)

Colorless oil; UV (MeOH) λ_{max} (log ε; nm): 281 (3.49), 216 (4.25); IR (KBr) ν_{max} (cm^{-1}): 3411, 2956, 2923, 2837, 1611, 1501, 1451, 1270, 1225, 1135, 1053, 1025, 801; ^1H and ^{13}C NMR spectral data: see Tables 1 and 2; EI-MS *m/z*: 288 $[M]^+$(46), 256 (8), 167 (6), 151 (100), 149 (2), 137 (6), 121 (29), 91 (10); HR-EI-MS *m/z*: 288.1358 $[M]^+$ (calcd for $C_{17}H_{20}O_4$, 288.1361).

3.4.3 Stilbostemin R (**3**)

Colorless oil; UV (MeOH) λ_{max} (log ε; nm): 271 (3.34), 207 (3.97); IR (KBr) ν_{max} (cm^{-1}): 3401, 2934, 2854, 1602, 1595, 1511, 1426, 1308, 1238, 1178, 1079, 832; ^1H and ^{13}C NMR spectral data: see Tables 1 and 2; EI-MS *m/z*: 286 $[M]^+$(22), 165 (100), 150 (16), 135 (9), 121 (69), 105 (13), 91 (47), 77 (9); HR-EI-MS *m/z*: 286.1573 $[M]^+$ (calcd for $C_{18}H_{22}O_3$, 286.1569).

3.4.4 Stemanthrene G (**4**)

Colorless oil; UV (MeOH) λ_{max} (log ε; nm): 306 (3.17), 281 (3.86), 216 (4.12); IR (KBr) ν_{max} (cm^{-1}): 3412, 3378, 2951, 2938, 1612, 1599, 1455, 1387, 1288, 1219, 1197, 1063, 1003, 819; ^1H and ^{13}C NMR spectral data: see Tables 1 and 2; EI-MS *m/z*: 284 $[M]^+$(100), 269 (11), 253 (45), 222 (17), 176 (8); HR-EI-MS *m/z*: 284.1416 $[M]^+$ (calcd for $C_{18}H_{20}O_3$, 284.1412).

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