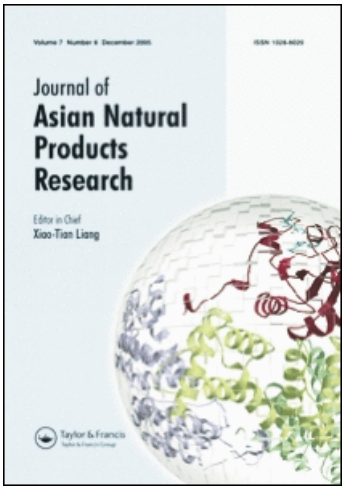

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Stilbenoids from *Stemona japonica*

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Three new dihydrostilbenes, stilbostemins **J**–**L** (**1**–**3**), and a new dihydrophenanthrene, stemanthrene **F** (**4**), were isolated from the roots of *Stemona japonica* together with two known bibenzyls, 3,5-dihydroxy-4-methylbibenzyl (**5**) and 3,5-dihydroxy-2′-methoxy-4-methylbibenzyl (**6**). Their structures were elucidated by spectroscopic analyses. Compounds **3**–**6** exhibited strong antibacterial activities against *Staphylococcus aureus* and *Staphylococcus epidermidis*.

Keywords: *Stemona japonica*; Stilbenoids; Stilbostemins **J**–**L**; Stemanthrene **F**

1. Introduction

The roots of *Stemona japonica* (Bl.) Miq, known as "Baibu", have long been prescribed in traditional Chinese medicine as insecticides and antitussive agents [1–3]. Up to now, extracts from roots of this plant are still being used to treat respiratory disorders, including pulmonary tuberculosis and bronchitis, and externally used to kill insect pests [4,5]. In previous chemical studies on *S. japonica*, many alkaloids [3,7–10] have been reported. Some stemofoline-type alkaloids were found to exhibit strong insecticidal activity [4,6]. In our further investigation on the non-alkaloid extracts of the title plant, four new stilbenoids, along with two known bibenzyls, were isolated and identified (figure 1). All the stilbenoids were subjected to antimicrobial tests against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and *Candida albicans*. Compounds **3**–**6** exhibited strong antibacterial activities against *S. aureus* and *S. epidermidis*. Previously, such stilbenoids have been only reported from the ethanol extract of *S. tuberosa* [11] and chloroform extract of *S. collinsae* [12,13].

2. Results and discussion

Stilbostemin **J** (**1**) was obtained as colourless oil. The molecular formula of **1** was deduced as $C_{16}H_{18}O_4$ from a molecular ion peak at *m*/*z* 274.1204 in the HREI-MS spectrum, which was

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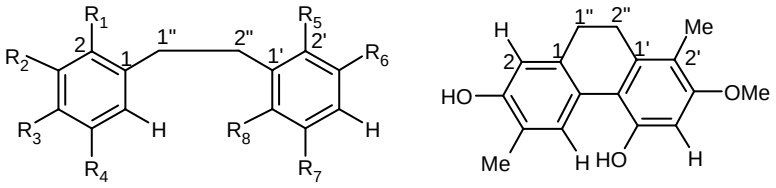
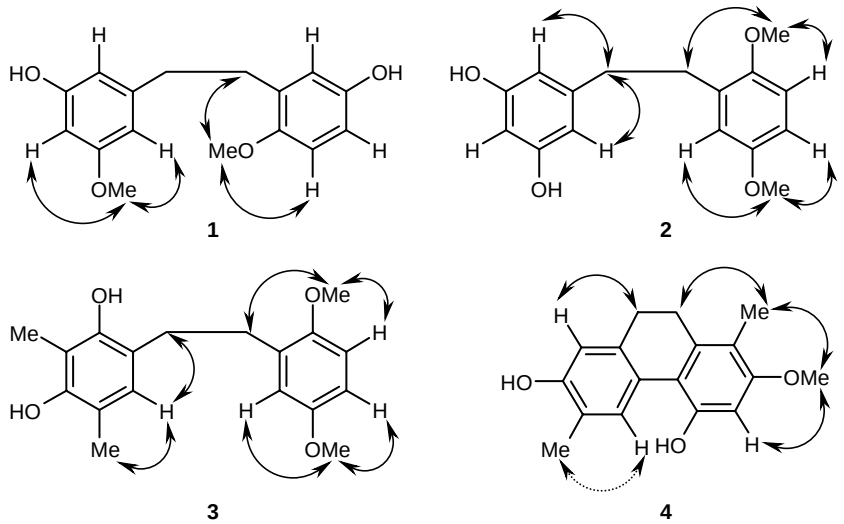


1 R_1,R_3,R_5,R_7=H, R_2,R_6=OH,R_4,R_8=OMe
2 R_1,R_3,R_6,R_8=H, R_2,R_4=OH, R_5, R_7=OMe
3 R_1,R_3=OH, R_2,R_4=Me, R_5,R_7=OMe, R_6,R_8=H
5 R_1,R_5,R_6,R_7,R_8=H, R_2,R_4=OH, R_3=Me
6 R_1,R_6,R_7,R_8=H, R_2,R_4=OH, R_3=Me, R_5=OMe

4

Figure 1. Structures of **1**–**6**.

in agreement with the EI-MS and ^{13}C NMR data. ^{1}H NMR, ^{13}C NMR and HREI-MS showed one 1,2,4-trisubstituted benzene [δ_H 6.72 (1H, d, $J = 8.4$ Hz), 6.64 (1H, dd, $J = 8.4$, 2.9 Hz), and 6.58 (1H, d, $J = 2.9$ Hz)], one 1,3,5-trisubstituted benzene [δ_H 6.35 (1H, t, $J = 2.2$ Hz), 6.27 (1H, t, $J = 2.2$ Hz), and 6.25 (1H, t, $J = 2.2$ Hz)], two methoxy groups (δ_H 3.78 and 3.75, each 3H, s), two methylenes [δ_H 2.76 (2H, m), 2.83 (2H, m)], and two hydroxyl groups, which indicated **1** could be a tetra substituted bibenzyl. ROESY experiments were carried out to determine the substituted positions of methoxys and hydroxyls (figure 2). NOE correlation between one methoxy group (3.75, 3H, s) and H-4, H-6 revealed that the methoxy was attached to C-5. NOE correlation between the other methoxy group (3.78, 3H, s) and H-5′ as well as H-2″ showed it was in position 6′. Thus the remaining positions 3 and 3′ should be substituted by two hydroxys. HMBC experiments provided further evidences for the substituted positions (figure 3). The correlations were observed between H-2, H-4 and C-3 and between H-4, H-6 and C-5, which suggesting a 3-hydroxy-5-methoxy substitution in ring A. The HMBC cross peaks were also found between H-2′, H-4′ and C-3′ and between H-5′, H-2″ and C-6′, confirming a 3′-hydroxy-6′-methoxy substitution in ring B. Moreover, the



1 **2** **3** **4**

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

presence of the ion fragments at *m/z* 137 ($C_8H_9O_2$) in EIMS spectrum also proved this substituted pattern. Thus, **1** was identified as 1-(3-hydroxy-5-methoxyphenyl)-2-(5-hydroxy-2-methoxyphenyl)-ethane (figure 1).

Compound **2** was also isolated as oil and possessed the same molecular formula of $C_{16}H_{18}O_4$ as **1** which was deduced from its HREI-MS and ^{13}C NMR. Its UV, IR and ^1H NMR spectra were almost superposed with those of **1**. All of those suggested that **2** was an isomer of **1**. The ^1H NMR spectrum showed the proton signals of one 1,3,5-trisubstituted benzene ring [δ_H 6.25 (1H × 2, t, $J = 1.8$ Hz), 6.18 (1H, t, $J = 1.9$ Hz)] and one 1,2,4-trisubstituted benzene [δ_H 6.76 (1H, d, $J = 8.4$ Hz), 6.70 (1H, dd, $J = 8.4$, 2.8 Hz), 6.66 (1H, d, $J = 2.8$ Hz)], indicating that **2** has the same substituted pattern with **1**. The differences between two compounds could be deduced from ROESY experiments (figure 2). The cross peaks between the methoxy at δ_H 3.74 and H-2″ and H-3′ showed it was attached to C-2′ and the ones between the other methoxy at δ_H 3.72 and H-4′ and H-6′ confirmed it was in position 5′. So, two remaining hydroxyls could be attached to C-3 and C-5 of ring A, respectively. HMBC experiments provided more evidences to elucidate the structure of **2** (figure 3). These findings led to a conclusion that **2** had the structure as 1-(3,5-dihydroxyphenyl)-2-(2,5-dimethoxyphenyl)-ethane.

The molecular formula of **3** was determined as $C_{18}H_{22}O_4$ by HREI-MS, ^{13}C NMR and DEPT NMR data. Compared with **2**, two low-field protons were observed disappearing while two methyl groups emerging at δ_H 2.21 and 2.27 in **3**. Moreover, the proton signals attributed to one 1,2,4-trisubstituted benzene [δ_H 6.78 (1H, d, $J = 8.4$ Hz), 6.71 (1H, dd, $J = 8.4$, 2.9 Hz), 6.67 (1H, d, $J = 2.9$ Hz)] were found to be identical with that of **2**. The ^{13}C NMR spectrum also resembled those of **2** except for the presence of two methyl groups in **3** instead of two protons in **2**, which implied that **3** had the same substituted ring B with **2**, but in ring A two protons in C-3 and C-5 were replaced by two methyl groups. The above conclusion was confirmed by the ROESY and HMBC spectra (figures 2 and 3). The ROESY contacting chain 5-CH_3 ↔ H-6 ↔ H-1″ and the HMBC cross peaks between 5-CH_3 and C-4 and C-6 and between 3-CH_3 and C-2 and C-4 allowed the unambiguous identification of a 2,4-dihydroxy-3,5-dimethyl substituted ring A. The substitution pattern of ring A and ring B was

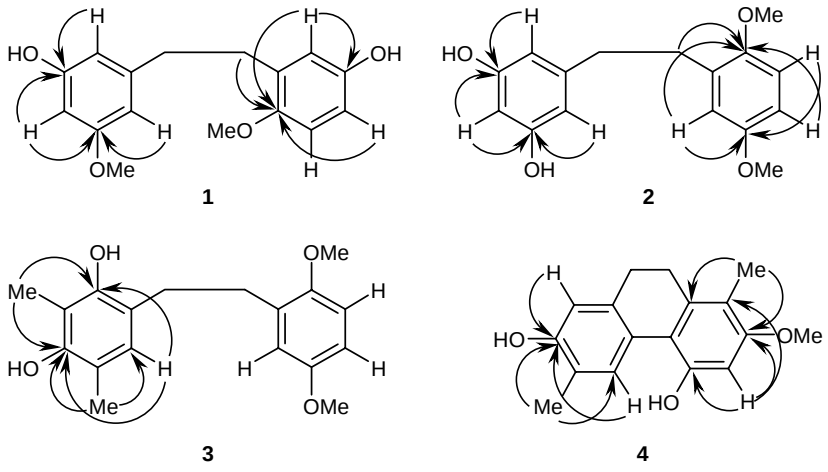


Figure 3. Selective HMBC correlations for **1**–**4** (H to C).

also confirmed by the presence of the only one ion fragment at *m/z* 151 ($C_9H_{11}O_2$) in EI-MS spectrum. Thus the structure of **3** was established as 1-(2,4-dihydroxy-3,5-dimethylphenyl)-2-(2,5-dimethoxyphenyl)-ethane.

The molecular formula of **4** was established as $C_{17}H_{18}O_3$ by its HREI-MS and ^{13}C NMR spectra, which suggesting the presence of nine unsaturations. Compared with the above stilbenoids, **4** was observed with more intense UV absorption bands at 300 and 213 nm, which were typical for dihydrophenanthrenes [12,13]. The ^{13}C NMR and DEPT spectra (table 2) displayed 17 carbon atoms including two benzene rings (δ 99–160, 3 × CH and 9 × C), one methoxy group (δ 55.6), two methyl groups (δ 11.3 and 15.9) and two methylene groups (δ 29.3 and 26.4). Supposed by ROESY experiments (figure 2), the structure of **4** was identified as a dihydrophenanthrene with a methoxy group in position 3′, two hydroxyl groups in 3 and 5′, and two methyl groups in 4 and 2′, respectively. Furthermore, 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-dihydroxy-7-methoxy-3,8-dimethyl-9,10-dihydrophenanthrene.

The isolated 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. Two most potential natural antibacterial agents, bakuchiol [14] and magnolol [15], were used as positive controls in the tests. Among these compounds, **3–6** showed strong antibacterial activities against two hospital pathogenic gram-positive bacteria *S. aureus* and *S. epidermidis* at the level of MICs 12.5–50 μg/ml (table 3). In comparison with the positive controls, compounds **1–6** exhibited less antibacterial and antifungal activities against *E. coli* and *C. albicans*.

3. Experimental

3.1 General experimental procedures

All melting points were determined on a Fisher–Johns melting point apparatus and are uncorrected. The UV spectra were detected on a Hewlett–Packard 8452A diode array spectrophotometer. IR spectra were recorded 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 unit (δ) and coupling constant (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 for preparative TLC, respectively.

3.2 Plant material

The fresh roots of *Stemona japonica* were collected in Anji County, Zhejiang Province, China, in September 2002, and identified by Professor Jin-gui Shen of Shanghai Institute of Materia Medica, Chinese Academy of Sciences. A voucher specimen (20020013B) is deposited in the Herbarium of the institute.

3.3 Extraction and isolation

The air-dried and powdered roots of *S. japonica* (8.5 kg) were percolated with 95% EtOH (10 L × 3) at room temperature. The ethanol extract was filtered and concentrated under reduced pressure. Then the concentrated extract was suspended in MeOH/H_2O (20/80, v/v, 1 L) and partitioned successively with petroleum ether (60–90°C), chloroform, EtOAc and *n*-BuOH (each 1 L × 3). The $CHCl_3$-soluble fraction (80 g) was subjected to the CC over silica gel eluted with petroleum ether–acetone gradients (4:1 to 1:2) to obtain fractions 1–5. Fraction 1 (7 g) was chromatographed over silica gel with hexane/EtOAc/formic acid (100:25:1, 2 L) to yield fractions 1.1–10, among which the fraction 1.1 and fraction 1.3 were further purified on a Sephadex LH-20 gel ($CHCl_3$/MeOH, 25:75, v/v, 1 L) to afford crude **1** (15 mg) and **3** (12 mg), respectively. **1** (9 mg) and 3 (7 mg) were obtained as pure compounds by preparative TLC (hexane/EtOAc/formic acid, 100:20:1). The Fraction 3 (12 g) was also subjected to the CC over silica gel. The elution was carried out with hexane/EtOAc/formic acid (100:40:1) to yield the fractions 3.1–12. Fraction 3.2 (250 mg) was further purified on a Sephadex LH-20 ($CHCl_3$/MeOH, 25:75, v/v, 0.5 L) to afford crude **2** (25 mg) and **4** (45 mg), respectively. Then 15 mg of **2** was obtained by repeated TLC (hexane/EtOAc/formic acid, 100:40:1) and 33 mg of **4** was gained by recrystallisation in Et_2O. Fraction 4 (8.5 g) was separated into nine subfractions (fractions 4.1–9) through silica gel column with $CHCl_3$/MeOH (10:1, 2 L) as eluent. Further purification of the fraction 4.3 (2.5 g) and fraction 4.4 (500 mg) followed by recrystallisation in acetone led to **5** (1.5 g) and **6** (320 mg).

3.4 Antimicrobial bioassay

Bioassay on antimicrobial activities against *S. aureus*, *S. epidermidis*, *E. coli* and *C. albicans in vitro* were carried out according to the protocols described in the literature [16].

3.5 Identification

3.5.1 Stilbostemin J (1). Colourless oil; UV (MeOH) λ_{max} (log ε) (nm): 278 (3.35), 214 (4.15); IR (KBr) ν_{max} (cm^{-1}): 3288, 2929, 2838, 1619, 1594, 1502, 1456, 1322, 1220, 1195, 1159, 1062, 1029, 958, 811, 744; EI-MS *m/z*: 274 $[M]^+$(47), 243 (12), 242 (12), 151 (13), 137 (100), 107 (24), 91 (3), 77 (8); HREI-MS *m/z*: 274.1204 (calcd for $C_{16}H_{18}O_4$, 274.1205); ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data are shown in tables 1 and 2, respectively.

3.5.2 Stilbostemin K (2). Colourless oil; UV (MeOH) λ_{max} (log ε) (nm): 281 (3.49), 216 (4.25); IR (KBr) ν_{max} (cm^{-1}): 3334, 2945, 2923, 1604, 1494, 1456, 1280, 1220, 1155, 1031, 838, 756; EI-MS *m/z*: 274 $[M]^+$(42), 242 (5), 151 (100), 123 (3), 121 (38), 91 (12), 78 (9); HREI-MS *m/z*: 274.1203 (calcd for $C_{16}H_{18}O_4$, 274.1205); ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data are shown in tables 1 and 2, respectively.

3.5.3 Stilbostemin L (3). Colourless oil; UV (MeOH) λ_{max} (log ε) (nm): 274 (3.34), 215 (3.98); IR (KBr) ν_{max} (cm^{-1}): 3417, 2934, 2923, 2854, 1617, 1592, 1514, 1470, 1378, 1348, 1250, 1110, 1079, 989, 845; EIMS *m/z*: 302 $[M]^+$(46), 256 (8), 151 (100), 121 (36), 97 (9),

Table 1. ^{1}H NMR data for compounds **1**–**4** in $CDCl_3$.

No	***1***	***2***	***3***	***4***
2	6.27 t (2.2)	6.25 t (1.8)		6.73 s
4	6.25 t (2.2)	6.18 t (1.9)		
5				7.71 s
6	6.35 t (2.2)	6.25 d (1.8)	6.31 s	
2′	6.58 d (2.9)			
3′		6.76 d (8.4)	6.78 d (8.4)	
4′	6.64 dd (8.4, 2.9)	6.70 dd (8.4, 2.8)	6.71 dd (8.4, 2.9)	6.41 s
5′	6.72 d (8.4)			
6′		6.66 d (2.8)	6.67 d (2.9)	
1″	2.76 m	2.71 m	2.66 m	
2″	2.83 m	2.82 m	2.77 m	
3-Me			2.27 s	
4-Me				2.27 s
5-Me/OMe	3.75 s		2.21 s	
2′-Me/OMe		3.74 s	3.76 s	2.12 s
3′-OMe				3.81 s
5′-OMe		3.72 s	3.74 s	
6′-OMe	3.78 s			

91 (12), 73 (8); HREI-MS *m/z*: 302.1516 (calcd for $C_{18}H_{22}O_4$, 302.1518); ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data are shown in tables 1 and 2, respectively.

3.5.4 Stemanthrene F (4). Colourless crystal (Et_2O); mp 133–135°C; UV (MeOH) λ_{max} (log ε) (nm): 300 (3.23), 279 (3.81), 213 (4.34); IR (KBr) ν_{max} (cm^{-1}): 3417, 3382, 2946, 2935, 1612, 1596, 1459, 1394, 1284, 1222, 1194, 1064, 1000, 925, 819; EI-MS *m/z*: 270 $[M]^+$(100), 255 (52), 240 (5), 209 (5), 165 (5), 135 (7); HREI-MS *m/z*: 270.1256 (calcd for $C_{17}H_{18}O_5$, 270.1256); ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data are shown in tables 1 and 2, respectively.

Table 2. ^{13}C NMR data for compounds **1**–**4** in $CDCl_3$.

No.	***1***	***2***	***3***	***4***
1	145.0 s	145.5 s	120.6 s	137.9 s
2	108.2 d	108.2 d	151.6 s	114.8 d
3	156.5 s	156.6 s	115.8 s	152.0 s
4	98.9 d	100.5 d	153.4 s	121.6 s
5	160.8 s	156.6 s	118.5 s	128.0 d
6	106.9 d	108.2 d	130.5 d	125.7 s
1′	131.6 s	131.5 s	132.6 s	139.3 s
2′	117.2 d	151.8 s	149.8 s	114.4 s
3′	149.1 s	111.7 d	109.6 d	157.0 s
4′	113.0 d	111.4 d	108.4 d	99.1 d
5′	111.6 d	153.3 s	151.9 s	150.9 s
6′	151.8 s	116.4 d	117.5 d	115.9 s
1″	32.0 t	35.9 t	31.6 t	29.3 t
2″	36.1 t	31.9 t	27.8 t	26.4 t
3-Me			7.9 q	
4-Me				15.9 q
5-Me/OMe	55.3 q		14.2 q	
2′-Me/OMe		56.2 q	55.9 q	11.3 q
3′-OMe				55.6 q
5′-OMe		55.8 q	56.4 q	
6′-OMe	56.0 q			

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

Compound	*S. aureus*	*S epidermidis*	*E. coli*	*C. albicans*
1	>50	>50	>50	>50
2	>50	>50	>50	>50
3	50	12.5–25	>50	>50
4	25	12.5–25	>50	>50
5	12.5	12.5–25	>50	>50
6	25	25–50	>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.

Acknowledgements

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