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ISOAURONES FROM THE STEM OF *CASSIA SIAMEA* AND THEIR ANTI-TOBACCO MOSAIC VIRUS (ANTI-TMV) ACTIVITY

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Abstract – Two new isoaurones, siamaurones A and B (1-2), together with three known isoaurones (**3-5**) were isolated from the stems of *Cassia siamea*. The structures of **1-5** were elucidated by spectroscopic methods including 1D- and 2D-NMR techniques. Compounds **1-5** were evaluated for their anti-tobacco mosaic virus (anti-TMV) activity. As a result, compound **2** showed high anti-TMV activity with inhibition rate of 31.8%, which is higher than that of Ningnanmycin (26.5%). Compounds **1**, **3**, and **5** showed modest anti-TMV activity with inhibition rate of 15.2%, 16.4%, and 12.8%, respectively.

Cassia siamea Lam. (Leguminosae) is a common tree in the southern part of China. It has been used widely in traditional medicine, particularly for treatment of periodic fever, alaria, aperient, antiarthritic and swellings.[1,2] In previous work, a number of bioactive compounds, such as anthraquinone,[3,4] alkaloids,[5,6] triperpenoids,[7,8] steroids,[8,9] chromones,[10-12] and their homologous, were isolated from this plant. Motivated by a search for new bioactive metabolites from this plant, our group has investigated the chemical constituents of the stems of *C. siamea*, which led to the isolation and characterization of two

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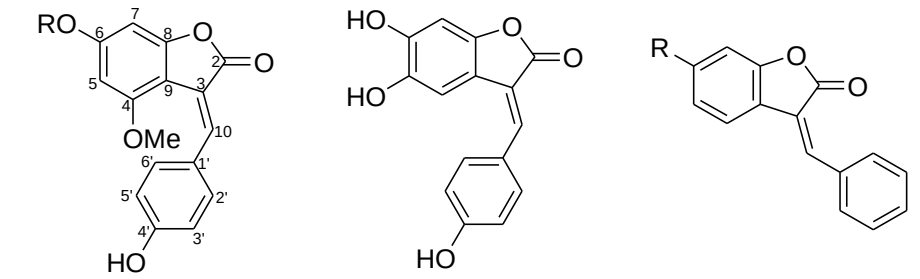
new isoaurones (**1**-**2**), along with three known isoaurones (**3**-**5**). The structures of the isolated compounds were established by means of spectroscopic methods including extensive 1D and 2D NMR techniques. This paper deals with the isolation, structural characterization, and anti-tobacco mosaic virus (anti-TMV) activity of these compounds.



1 R = -Glc; **3** R = -H **2** **4** R = H; **5** R = -OMe

Figure 1. The structures of Isoaurones from the stems of *C. siamea*

The stems of *C. siamea* were extracted with 95% aqueous methanol. The extract was subjected repeatedly to column chromatography on silica gel, Sephadex LH-20, RP-18, and semi-preparative RP-HPLC separation to afford two new isoaurones, siamaurones A and B (**1**-**2**), together with three known isoaurones (**3**-**5**). The structures of the compounds **1**-**5** were as shown in Figure 1, and the ^{1}H and ^{13}C NMR data of compounds **1** and **2** were listed in Table 1. Compared with literature data, the known compounds were identified as: 4′,6-dihydroxy-4-methoxyisoaurone (**3**),[13] isoaurone A (**4**),[14] and 6-methoxyisoaurone (**6**).[14]

Compound **1** was obtained as a yellow solid. It gives a parent ion by HR-ESIMS at *m/z* 445.1139 $[M-H]^-$ (calcd for 445.1135) corresponding to a molecular formula of $C_{22}H_{22}O_{10}$, requiring twelve degrees of unsaturation. The ^{1}H NMR spectrum of **1** showed the presence of an AA′BB′ aromatic system at δ_H 8.12 (2H, d, *J*=8.9 Hz, H-2′,6′) and 6.90 (2H, d, *J*=8.8 Hz, H-3′,5′), two *meta*-coupled aromatic protons at δ_H 6.16 (1H, d, *J*=1.8 Hz, H-7) and 6.26 (1H, d, *J*=1.8 Hz, H-5), an isolated olefinic proton at δ_H 7.80 (1H, s, H-10), a methoxy group proton (δ_H 3.88 s), and a glucosyl moiety [δ_H 5.30 (1H, d, *J*=7.3, H-1″); δ_H 3.22 ~ 3.70 (6H, m, H-2″, H-3″, H-4″, H-5″, H-6″)]. The ^{13}C NMR spectrum of **1** revealed the

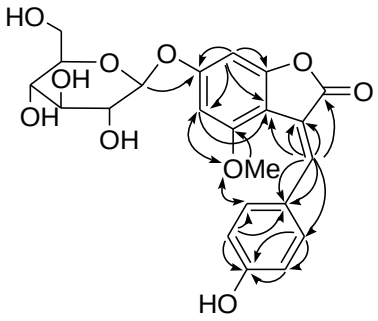


Figure 2. Key HMBC (↷) and NOESY (↶↷) correlations of **1**

presence of an isoaurone nucleus[13] (δ_C 170.2 s, 119.0 s, 158.8 s, 96.2 d, 159.4 s, 93.0 d, 155.0 s, 105.1 s, 142.1 d, 128.1 s, 135.4 d (2C), 116.1 d (2C), 161.7 s), a glucosyl moiety (δ_C 103.5 d, 75.7 d, 78.5 d, 71.3 d, 78.0 d, 62.6 t), and a methoxy group (δ_C 56.1 q). The HMBC correlations of **1** (Figure 2) showing the deshielded H-10 (δ_H 7.80) coupled to C-2 (δ_C 170.2) suggested that the structure of **1** could be an isoaurone too.[15,16] The AA′BB′ spin system in the ^{1}H NMR spectrum indicated one hydroxy group attached to C-4′ (δ_C 161.7). In the class of C6-C3-C6 compounds possessing hydroxy substituents at C-5 and C-7, the chemical shift of C-6 (or C-5 in the case of **1**) appears at lower field than C-8 (or C-7 in the case of **1**);[14] therefore, the signals at δ_C 96.2 and 93.0 were assigned to C-5 and C-7, respectively. The HSQC spectrum indicated that the *meta*-coupled aromatic protons at δ_H 6.27 and 6.18 associated with C-5 and C-7, respectively. The HMBC spectrum revealed the coupling from the methoxy group (δ_H 3.94) to the carbon atom at δ_C 158.8, which further showed the ^{1}H-^{1}H COSY correlation with H-5 (δ_H 6.27) but not with H-7 (δ_H 6.18). This indicated that the methoxy group was located at C-4. The long-range correlations in the HMBC spectrum between H-1″ (δ_H 5.30 d) and C-6 (δ_C 159.4 s) indicated the glucosyl was linked to C-6, and the coupling constant value of H-1″ (J=7.3 Hz) indicated that the glucosyl moiety was connected to the aglycone by a β-linkage.[17,18] The configuration of *E*- and *Z*-isoaurones is determined on the basis of the chemical shift of H-10, which is anisotropically and diamagnetically affected by the C-2 carbonyl group. It is known that the chemical shift of H-10 in *Z*-isoaurone resonates at higher field (7.4~7.5 ppm) than in the *E*-isomer (7.8~8.0 ppm).[16,19] The differences in ^{13}C shifts of the olefinic carbon between *E* (169 ppm) and *Z* (166 ppm) isomers were reportedly too small to be useful.[16] Therefore, the H-10 chemical shift at δ_H 7.80 suggested that **1** is *E*-isoaurone. This conclusion was supported by the cross-peak between the methoxy group (δ_C 3.88) and H-2′,6′ (δ_H 8.12) in a NOESY experiment. Thus, compound **1** was evident, and given the name as siamaurone A.

Compound **2** was obtained as pale yellow solid, and showed quasi molecular ion at *m/z* 269.0443 $[M-H]^-$ in the HRESIMS (calcd *m/z* 269.0450), corresponding to the molecular formula of $C_{15}H_{10}O_5$. The ^{1}H and ^{13}C NMR signals revealed that compound **2** also have isoaurone skeleton with an AA′BB′ spin system. By the comparison NMR data of compounds **1** and **2**, the obvious chemical shift differences resulted from the substituent group variations in the aromatic ring. In the class of C6-C3-C6 compounds possessing the substituents at C-5 and C-6, the chemical shift of C-4 appears at lower field than C-7;[14,19] therefore, the carbons signals at δ_C 109.4 and 104.2 were assigned to C-4 and C-7, and the HSQC spectrum indicated that two singlets δ_H 6.61 and 6.36 was associated with C-4 and C-7, respectively. Since no other substituents signals were obverved in its ^{1}H and ^{13}C spectrometry, all substituents in **2** should be hydrogen. On the basis of the above evidence, the structure of **2** was established as shown, and given the name as siamaurone B.

Table 1. ^{1}H and ^{13}C NMR Data of compounds **1** and **2** (CD_3OD, 400 MHz)

No.	Compound 1		Compound 2	
	δ_C (mult.)	δ_H (mult, *J*, Hz)	δ_C (mult.)	δ_H (mult, *J*, Hz)
2	170.2 s		169.5 s	
3	119.0 s		120.0 s	
4	158.8 s		109.4 d	6.61 s
5	96.2 d	6.26 d, *J*=1.8	145.2 s	
6	159.4 s		147.8 s	
7	93.0 d	6.16 d, *J*=1.8	104.2 d	6.36 s
8	155.0 s		154.8 s	
9	105.1 s		118.7 s	
10	142.1 d	7.80 s	142.5 d	7.81 s
1′	128.1 s		126.8 s	
2′, 6′	135.4 d	8.12 d, *J*=8.9	133.0 d	8.07 d, *J*=8.7
3′, 5′	116.1 d	6.90 d, *J*=8.9	116.3 d	6.89 d, *J*=8.7
4′	161.7 s		162.4 s	
OMe-4	56.1 q	3.88 s		
1″	103.5 d	5.30, d, *J*=7.3		
2″	75.7 d	3.45 m		
3″	78.5 d	3.43 m		
4″	71.3 d	3.30 m		
5″	78.0 d	3.22 m		
6″	62.6 t	3.52 m 3.70 m		

The compounds **1**-**5** were tested for their anti-TMV activity. The inhibitory activities of compounds **1**-**5** against TMV replication were tested using the half-leaf method.[20,21] Ningnanmycin, a commercial product for plant disease in China, was used as a positive control.[22] The antiviral inhibition rates of compounds **1**-**5** at the concentration of 20 μM were listed in Table 2. The results showed that compound **2** showed high anti-TMV activity with inhibition rate of 31.8%, which is higher than that of Ningnanmycin (26.5%). Compounds **1**, **3**, and **5** showed modest anti-TMV activity with inhibition rate of 15.2%, 16.4%, and 12.8%, respectively. Compound **4** also showed weak anti-TMV activity with inhibition rate of 8.25%.

Table 2. The antiviral inhibition rates of compounds **1**-**5**

Compounds		Compounds	
1	15.2 ± 1.8	**4**	8.25 ± 2.0
2	31.8 ± 3.1	**5**	12.8 ± 2.3
3	16.4 ± 2.4	ningnanmycin	26.5 ± 2.2

All results are expressed as mean ± SD; n = 3 for all groups.

EXPERIMENTAL

General. UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. IR spectra were obtained in KBr disc on a Bio-Rad Wininfmred spectrophotometer. ESI-MS were measured on a VG Auto Spec-3000 MS spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on Bruker DRX-400 instrument with TMS as internal standard. 2D NMR spectra were recorded on Bruker DRX-500 instrument with TMS as internal standard. Column chromatography was performed on silica gel (200-300 mesh), or on silica gel H (10～40 μm, Qingdao Marine Chemical Inc., China). Preparative HPLC was used an Agilent 1100 HPLC equipped with ZORBAX-C_{18} (21.2 mm × 250 mm, 7.0 μm) column and DAD detector.

Plant material. The stems of *C. siamea* were collected on Dehong Prefecture, Yunnan Province, People's Republic of China, in September 2011. The identification of the plant material was verified by Dr. Yuan. N, of Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (YNNU 11-9-32) has been deposited in our Laboratory.

Extraction and Isolation. The air-dried and powdered stems of *C. siamea* (5.0 kg) were extracted four times with 90% aq. EtOH (4 × 50 L) at room temperature and filtered. The crude extract (321 g) was applied to silica gel (200 – 300 mesh) column chromatography, eluting with a $CHCl_3$-acetone gradient system (20:1, 9:1, 8:2, 7:3, 6:4, 5:5), to give six fractions A–F. The further separation of fraction C (8:2, 8.26 g) by silica gel column chromatography, eluted with $CHCl_3$-MeOH (9:1, 8:2, 7:3, 6:4, 1:1), yielded mixtures C1–C5. Fraction C1 (9:1, 1.57 g) was subjected to preparative HPLC (40% MeOH, flow rate 12 mL/min) to give **3** (34.5 mg), **4** (16.7 mg), and **5** (19.2 mg). The further separation of fraction D (7:3, 22.6 g) by silica gel column chromatography, and preparative HPLC (32% MeOH, flow rate 12 mL/min) to give **1** (28.6) and **2** (17.8 mg).

Siamaurone A (1). Obtained as pale yellow gum; UV (MeOH) max (log ε) 205 (4.22), 262 (3.87), 390 (3.64) nm; IR (KBr) ν_{max} 3420, 2910, 2854, 1688, 1624, 1548, 1430, 1356, 1135, 922, 846 cm^{-1}; ^{1}H NMR and ^{13}C NMR data (CD_3OD, 400 and 100 MHz), see Table 1; negative ESIMS *m/z* 445 $[M-H]^-$; negative HRESIMS *m/z* 445.1139 $[M-H]^-$ (calcd for $C_{22}H_{21}O_{10}$, 445.1135).

Siamaurone B (2). Obtained as yellow solid; UV (MeOH) max (log ε) 210 (4.31), 260 (3.90), 385 (3.68) nm; IR (KBr) ν_{max} 3434, 2915, 2862, 1668, 1624, 1462, 1375, 1126, 943, 835 cm^{-1}; ^{1}H NMR and ^{13}C NMR data (CD_3OD, 400 and 100 MHz), see Table 1; negative ESIMS *m/z* 269 $[M-H]^-$; negative HRESIMS *m/z* 269.0443 $[M-H]^-$ (calcd for $C_{15}H_9O_5$, 269.0450).

Anti-TMV Assays. TMV (U1 strain) was obtained from the Key Laboratory of Tobacco Chemistry of Yunnan Province, Yunnan Academy of Tobacco Science, P. R. China. The virus was multiplied in Nicotiana tabacum cv.K326 and purified as described in literature.[23] The concentration of TMV was

determined as 20 mg/mL with an ultraviolet spectrophotometer [virus concentration = (A260 × dilution ratio) / $E_{1\,cm}^{0.1\%,\,260nm}$]. The purified virus was kept at -20 ºC and was diluted to 32 μg/mL with 0.01 M PBS before use.

Nicotiana glutinosa plants were cultivated in an insect-free greenhouse. *N. glutinosa* was used as a local lesion host. The experiments could be conducted when the plants grow to the 5-6-leaf stage. The tested compounds were dissolved in DMSO and diluted with distilled H_2O to the required concentrations. The solution of equal concentration of DMSO was used as negative control. The commercial antiviral agent ningnanmycin was used as a positive control.

For Half-Leaf Method,[20] the virus was inhibited by mixing with the solution of compound. After 30 min, the mixture was inoculated on the left side of the leaves of *N. glutinosa*, whereas the right side of the leaves was inoculated with the mixture of DMSO solution and the virus as control. The local lesion numbers were recorded 3-4 days after inoculation. Three repetitions were conducted for each compound. The inhibition rates were calculated according to the formula:

$$\text{inhibition rate (\%)} = [\,(C-T) / C\,] \times 100\%$$

where C is the average number of local lesions of the control and T is the average number of local lesions of the treatment.

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