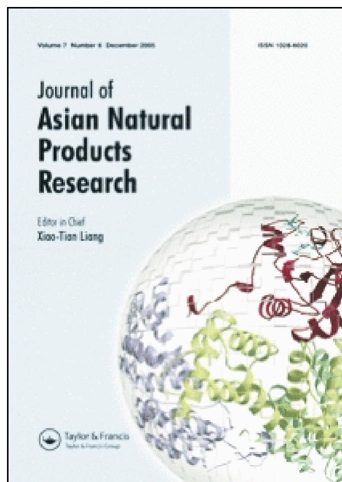
This article was downloaded by:
On: *22 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

Constituents from the seeds of *Brucea javanica* with inhibitory activity of *Tobacco mosaic virus*

Qi-Jian Chen[a]; Ming-An Ouyang[ab]; Qing-Wei Tan[b]; Zheng-Kun Zhang[a]; Zu-Jian Wu[a]; Qi-Ying Lin[a]
[a] Institute of Plant Virology, Fujian Agriculture and Forestry University, Fuzhou, Fujian, China [b] Key Laboratory of Bio-pesticide and Chemistry-Biology, Ministry of Education, Fujian Agriculture and Forestry University, Fuzhou, Fujian, China

To cite this Article Chen, Qi-Jian , Ouyang, Ming-An , Tan, Qing-Wei , Zhang, Zheng-Kun , Wu, Zu-Jian and Lin, Qi-Ying(2009) 'Constituents from the seeds of *Brucea javanica* with inhibitory activity of *Tobacco mosaic virus*', Journal of Asian Natural Products Research, 11: 6, 539 — 547

To link to this Article: DOI: 10.1080/10286020902932708

URL: http://dx.doi.org/10.1080/10286020902932708

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

Journal of Asian Natural Products Research
Vol. 11, No. 6, June 2009, 539–547

Taylor & Francis
Taylor & Francis Group

Constituents from the seeds of *Brucea javanica* with inhibitory activity of *Tobacco mosaic virus*

Qi-Jian Chen[a], Ming-An Ouyang[ab]*, Qing-Wei Tan[b], Zheng-Kun Zhang[a], Zu-Jian Wu[a] and Qi-Ying Lin[a]

[a]*Institute of Plant Virology, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China;* [b]*Key Laboratory of Bio-pesticide and Chemistry-Biology, Ministry of Education, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China*

(*Received 16 January 2009; final version received 30 March 2009*)

Three new constituents were obtained along with 10 known compounds from the seeds of *Brucea javanica*. The structures of these compounds were determined based on spectral and chemical evidence. These new compounds included a monoterpenoid glycoside and two sesquiterpenes. Bioactivity screening of these constituents showed that compounds **1**, **3**, **8**, **9**, and **13** with obvious activities in inhibiting multiplication of the *Tobacco mosaic virus*.

Keywords: Simaroubaceae; *Brucea javanica*; monoterpenoid glycoside; sesquiterpene; lignan; *Tobacco mosaic virus*

Downloaded At: 18:40 22 January 2011

1. Introduction

Brucea javanica is a shrub of Simaroubaceae. It distributes from southeast Asia to northern Australia, and grows in southern regions of China widely [1]. This plant has been deeply studied and found as a rich source of quassinoids. Its seed has still been used for the treatment of dysentery, malaria, and cancer in Chinese traditional medicine. Okano *et al.* [1–3] had previously reviewed the bioactive quassinoids from Simaroubaceae plants during the period of 1972–2000, which showed cytotoxic antitumor, antitumor-promoter, anti-HIV, antituberculosis, insect antifeedant, and other biological activities, and *B. javanica* is still now an interesting resource for new quassinoids and bioactivity studies [4–6].

It is a part of our search for antiphytoviral natural products; a chloroform fraction from the seeds of *B. javanica* showed significant inhibitory activity against the infection and the replication of *Tobacco mosaic virus* (TMV) *in vitro*. Previously, our institute reported the antiphytoviral activity of bruceine-D, a quassinoid isolated from the seeds of *B. javanica* [7]. It exhibited significant inhibitory activity against the infection and the replication of TMV, with IC_{50} values of 13.98 and 7.13 $\mu g\ ml^{-1}$, respectively. Bioassay-guided fractionation of the chloroform extract of the seeds of *B. javanica* using chromatography in silica gel and preparative HPLC led to the isolation of three new compounds. We report, herein, the structural elucidation and antiphytoviral activities of these new compounds and 10 known compounds.

*Corresponding author. Email: maouyang@yahoo.com

ISSN 1028-6020 print/ISSN 1477-2213 online
© 2009 Taylor & Francis
DOI: 10.1080/10286020902932708
http://www.informaworld.com

Table 1. ^{1}H and ^{13}C NMR spectral data of compound **1**[a].

No.	δ_H	δ_C		NOESY	HMBC	H–H COSY
1		78.4	C			
2		108.9	C			
3	1.96 (1H, dt, 6.8, 5.3), 1.80 (1H, dt, 5.3, 3.4)	34.4	CH_2	H4	C5, C1	H4
4	2.13 (1H, dt, 6.8, 5.3), 1.73, (1H, dt, 5.3, 3.4)	36.4	CH_2	H8	C2, C8	H3
5		85.7	C			
6	1.23 (3H, s)	24.8	CH_3	H7	C2, C1	
7	1.34 (3H, s)	22.8	CH_3	H6	C2, C1	
8	1.29 (3H, s)	24.6	CH_3		C9, C5, C4	
9	6.14 (1H, dd, 15.8, 10.0)	146.7	CH	$H10_C$	C4, C8	H10 (H_T, H_C)
10	5.12[b] (1H_T, dd, 15.8, 1.7), 4.90[c] (1H_C, dd, 10.0, 1.7)	111.6	CH_2	H9	C5	H9
1′	4.57 (1H, d, 7.7)	95.0	CH	H3′	C2	H2′
2′	3.57 (1H, t, 9.3)	73.2	CH			H1′, H3′
3′	3.52 (1H, dd, 9.3, 3.1)	74.7	CH	H1′, H5′		H2′, H4′
4′	3.40 (1H, dd, 4.6, 3.1)	72.2	CH			H3′, H5′
5′	3.35 (1H, m)	79.3	CH			H4′, H6′
6′	3.80 (1H, dd, 11.2, 3.4), 3.64 (1H, brd, 11.2)	62.8	CH_2			H5′

[a] Measured in acetone-d_6 at 400 MHz for ^{1}H and 100 MHz for ^{13}C.
[b] *trans*-Proton.
[c] *cis*-Proton.

Downloaded At: 18:40 22 January 2011

2. Results and discussion

Chloroform extract of *B. javanica* was chromatographed on silica gel and eluted with 40% EtOAc–hexane to afford five fractions. The components were separated by preparative HPLC on silica gel developing with EtOAc–hexane (40:60). Thirteen compounds were obtained including the three new compounds, named as brucojavans **1**, **2**, and **3**. The 10 known compounds were identified to be pinoresinol (**4**) [8], cleomiscosin B (**5**) [9], 7,8-epoxylignans (**6**) [10], dihydrodehydrodiconiferyl alcohol (**7**) [11], 7′-hydroxylariciresinol (**8**) [12], secoisolariciresinol (**9**) [13], 4-methoxy-guaiacylglycerol (**10**) [14,15], 7-carbonyl-guaiacylglycerol (**11**) [15], 4-hydroxy-3-methoxy-benzoic acid (**12**), and 3,4-dihydroxy-benzoic acid (**13**) by comparison of their NMR spectral data with those reported data in the literature.

Brucojavan **1** was obtained as a white amorphous powder, whose molecular formula was determined to be $C_{16}H_{28}O_8$ by observation of a quasi-molecular ion peak at m/z 347.1705 $[M-H]^-$ in the negative-ion HR-FAB mass spectrum. The IR spectrum indicated the presence of hydroxyl group (3310 cm^{-1}) and double bond (1652 cm^{-1}). Acid hydrolysis of **1** with 2 M trifluoroacetic acid afforded galactose which was identified by HPLC. The ^{1}H NMR spectrum of **1** showed three methyl signals at δ_H 1.34 (3H, s), 1.29 (3H, s), and 1.23 (3H, s), a terminal double bond signals at δ_H 6.14 (1H, dd, $J = 15.8, 10.0$ Hz), 5.12 (1H, dd, $J = 15.8, 1.7$ Hz), and 4.90 (1H, dd, $J = 10.0, 1.7$ Hz), and an anomeric proton signal at δ_H 4.57 (1H, d, $J = 7.7$ Hz). The ^{13}C NMR spectrum of **1** gave a terminal double bond signal at δ_C 146.7 and 111.6, a ketal signal at δ_C 108.9, two quaternary carbon signals bearing oxygen at δ_C 85.7 and 78.4, two methene signals at δ_C 36.4 and 34.4, three methyl signals at δ_C 24.8, 24.6, and 22.8, and a hexose signals (Table 1). Evaluation of spin–spin coupling and chemical shifts of the hexose

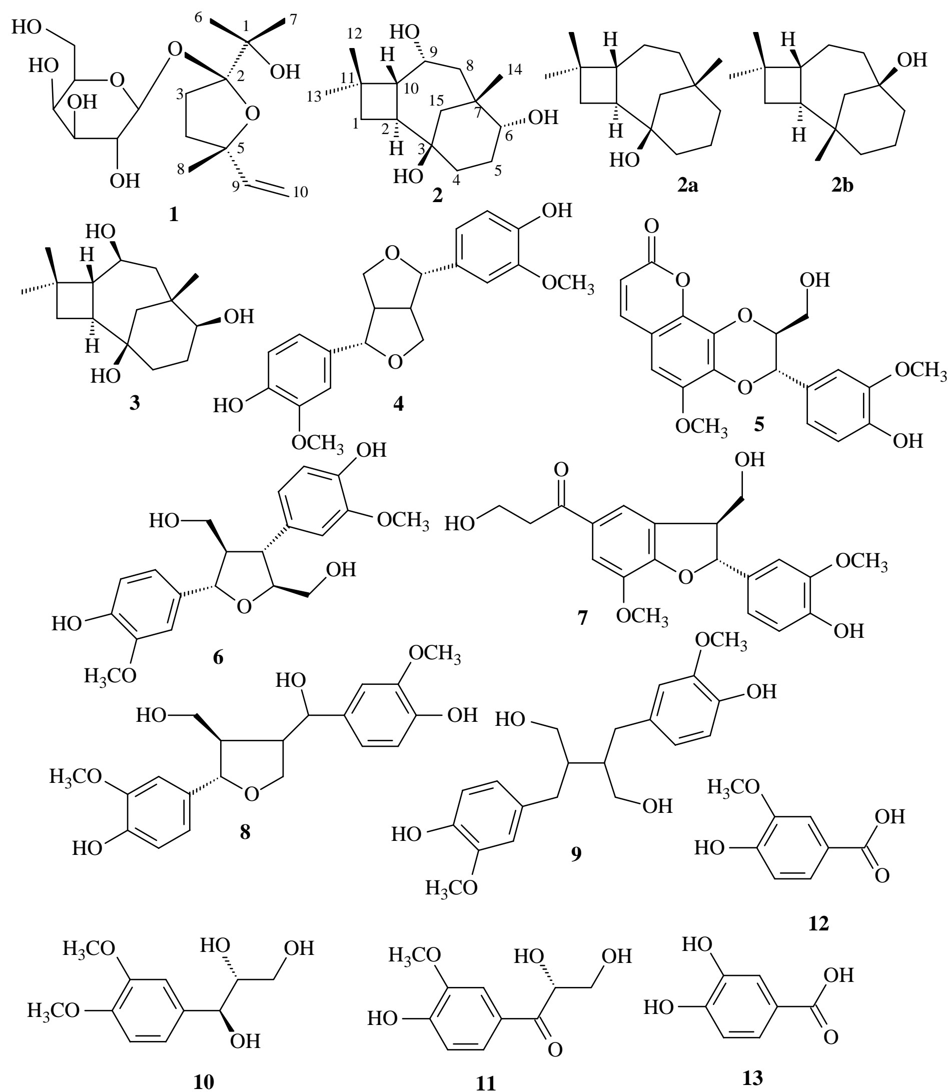


Figure 1. Structures of compounds **1**–**13**.

Downloaded At: 18:40 22 January 2011

allowed the identification of one β-galactopyranosyl unit [16]. The ^{1}H and ^{13}C NMR spectra of **1** suggested the presence of 16 carbon signals including a galactose unit signals, so compound **1** was a monoterpenoid glycoside. In the HMBC spectrum, the key correlations were observed between the anomeric proton signal at δ_H 4.57 and the ketal signal at δ_C 108.9 (C-2), between the two olefinic proton signals at δ_H 5.12 (H-10), 4.90 (H-10) and the oxygen-bearing quaternary carbon at δ_C 85.7 (C-5), between two methyl signals at δ_H 1.34 (H-7), 1.23 (H-6) and the ketal signal at δ_C 108.9 (C-2) (Figure 1). Because the unsaturated degree of compound **1** was three, C-5 and C-2 had to form a furan ring, this ring was confirmed by the signal splittings (coupling constant) of H-4 and H-3 (Table 1) [17]. The relative stereochemistry of **1** was displayed by its NOESY spectrum, the

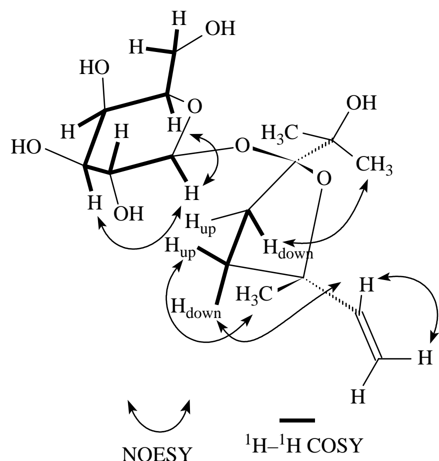


Figure 2. Key NOESY and ^{1}H–^{1}H COSY correlations of compound **1**.

correlations of which were between H_{up}-4 and H-8, between H_{down}-4 and H-9, between H_{down}-3 and H-6, H-7 (Figure 2). On the basis of above evidences, **1** was determined to be 5-ethenyltetrahydro-2-hydroxyl-1,1,5-trimethyl-2-furanmethanol-2-*O*-β-D-galactopyranoside, named brucojavan **1**.

Compound **2**, $[\alpha]_D^{21} + 74.0$, gave molecular formula, $C_{15}H_{26}O_3$, which is based on HR-EI-MS peak at *m/z* 254.1881 $[M]^+$. The IR spectrum showed the presence of hydroxy group (3302 cm^{-1}). The ^{1}H NMR spectrum displayed three methyl proton signals at δ 1.00 (3H, s), 1.05 (3H, s), and 1.07 (3H, s); two signals at δ 3.42 (1H, brd, $J = 8.3$ Hz), 3.91 (1H, m), the protons are on the carbon atom bonded to oxygen. The ^{13}C NMR spectrum exhibited 15 carbon signals including three methyl, five methene, four methine, and three quaternary carbons (Table 2). The ^{1}H and ^{13}C NMR spectral data of **2** suggested its structure rather similar to that of β-caryolanol (**2a**) or isocaryolanol (**2b**) [18]. Its HMBC experiment (Figure 3) confirmed the correlations between H-12, H-13, and C-1; between H-2 and C-9, C-11; between H-9 and C-2, C-11; between H-14 and C-6, C-8; between H-4 and C-2, C-6; between H-10 and C-3, C-8; and between H-5 and

Table 2. ^{1}H and ^{13}C NMR spectral data of compounds **2** and **3**[a].

	2			**3**		
No.	δ_C		δ_H	δ_C		δ_H
1	35.1	CH_2	1.43 (1H, dd, 9.1, 8.7), 1.63 (1H, overlap)	35.3	CH_2	1.45 (1H, dd, 13.2, 7.7), 1.42 (1H, dd, 13.2, 7.7)
2	41.3	CH	2.10 (1H, m)	47.9	CH	1.90 (1H, m)
3	70.1	C		70.1	C	
4	35.5	CH_2	1.42 (1H, m), 1.65 (1H, overlap)	27.7	CH_2	1.67 (1H, m), 1.71 (1H, m)
5	29.0	CH_2	1.68 (1H, m), 1.84 (1H, m)	29.8	CH_2	1.64 (1H, m), 2.17 (1H, m)
6	71.8	CH	3.42 (1H, brd, 8.3)	74.7	CH	3.27 (1H, brd, 7.5)
7	39.4	C		38.1	C	
8	47.1	CH_2	1.27 (1H, dd, 8.0, 4.0), 1.64 (1H, overlap)	50.8	CH_2	1.19 (1H, dd, 10.4, 3.4), 1.73 (1H, dd, 10.4, 8.5)
9	69.3	CH	3.91 (1H, m)	71.2	CH	3.68 (1H, m)
10	53.1	CH	1.90 (1H, dd, 8.2, 5.3)	53.5	CH	1.83 (1H, dd, 8.7, 2.0)
11	34.9	C		36.5	C	
12	21.8	CH_3	1.07 (3H, s)	20.2	CH_3	1.15 (3H, s)
13	31.5	CH_3	1.05 (3H, s)	31.9	CH_3	1.08 (3H, s)
14	29.3	CH_3	1.00 (3H, s)	32.3	CH_3	1.00 (3H, s)
15	44.4	CH_2	1.53 (1H, d, 12.9), 1.71 (1H, d, 12.9)	47.5	CH_2	1.25 (1H, d, 14.2), 1.84 (1H, d, 14.2)

[a] Measured in acetone-d_6 at 400 MHz for ^{1}H and 100 MHz for ^{13}C.

Downloaded At: 18:40 22 January 2011

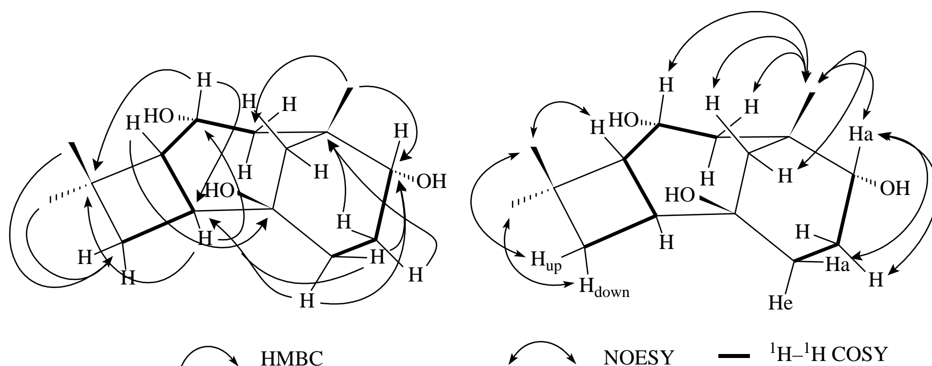


Figure 3. Key HMBC, NOESY, and $^{1}H-^{1}H$ COSY correlations of compound **2**.

Downloaded At: 18:40 22 January 2011

C-7. These correlations indicated the existence of 6,9-dihydroxy-β-caryolanol skeleton. Moreover, the NOESY experiment gave the correlations (Figure 3) between H-14 and H-6, H-8, H-9, H-15; between H-12 and H-10, H-1_{up}; between H-6 and H-4_a, H-14. These correlations disclosed the axial H-6, H-9_{up}, and H-10_{up}. So, the two hydroxyl groups were all α form. Thus, the structure of compound **2** was elucidated as 6α,9α-dihydroxy-β-caryolanol, and named brucojavan **2**.

Brucojavan **3** gave the same molecular formula, $C_{15}H_{26}O_3$ and similar NMR spectral data with those of **2**. Carefully comparing the data of compound **3** with those of compound **2** indicated the hydroxyl configurations of C-6 and C-9 position in **3** differed from **2**, which was confirmed by the NOESY correlations between H-9 and H-8, H-13, and between H-6 and H-5, H-8, H-14. The correlations also displayed that two hydroxy groups were all β form. Thus, **3** was determined as 6β,9β-dihydroxy-β-caryolanol.

Inhibitory replication and infection activities of compounds **1–13** against TMV were tested *in vitro* using local lesion assay and leaf-disk method, and the data were listed in Table 3. The compounds were tested at a final concentration of 50 μg ml^{-1}, five compounds (**1**, **3**, **8**, **9**, and **13**) showed a moderate inhibitory activity against TMV replication (Figure 4), while the inhibitory activities of other compounds against TMV infection was much lower. Noticeably, compound **9** displayed an inhibitory rate of 73.5 and 52.7% against TMV replication and infection, respectively. The inhibitory activity of compound **9** was lower than bruceine-D at the equal concentration, but the chemical structure of **9** was simpler than that of bruceine-D. In this case, it could be that these compounds were assistant agents in the antiphytoviral activity of the main active constituent, bruceine-D, in *B. javanica* to TMV.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured with a Jasco DIP-180 digital polarimeter spectrophotometer. IR spectra were recorded with a Perkin-Elmer 1750 FT-IR spectrometer as KBr disks. The ^{1}H, ^{13}C, DEPT, $^{1}H-^{1}H$ COSY, NOESY, HMQC, and HMBC NMR spectra were performed using a Bruker AM-400 spectrometer. Mass spectra were recorded on a Jeol JMS-HX 110 instrument. Chromatographic stationary phases were RP-18 (40–60 μm; Merck, Darmstadt, Germany), silica gel (160–200 mesh; Qingdao Oceanic Chemical Co., Qingdao, China), Sephadex LH-20 (25–100 μm; Pharmacia Fine Chemical Co., Ltd, Tokyo, Japan), MCI-gel CHP20P (75–150 μm;

Table 3. The inhibitory activities of TMV in compounds **1**–**13** (%)[a].

Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Rate of inhibitory replication	56.0	42.8	59.0	40.1	10.4	15.5	14.7	53.4	73.5	31.5	46.8	22.9	60.3
Rate of inhibitory infection	23.7	3.5	–	–	5.2	7.6	–	13.6	52.7	19.1	8.1	5.8	–

[a]Compound concentration in 50 μg ml^{-1}.

Downloaded At: 18:40 22 January 2011

Mitsubishi Chemical Industries Ltd, Tokyo, Japan). HPLC was P-230-UV-230 (Dalian Elite Analytical Instruments Co., Ltd, Dalian, China) and HPLC column (S-5 μm, 250 × 10 mm; YMC-Pack ODS-A, Kyoto, Japan). The following solvent systems were used: (a) $CHCl_3$–MeOH (50:3), $CHCl_3$–MeOH–H_2O (80:20:3), and MeOH–H_2O (0–100%) for the glycosides and (b) $CHCl_3$–MeOH–H_2O (7:3:1) lower-layer 9 + 1 ml HOAc for sugars. Compounds on TLC were detected by spraying with 5% H_2SO_4 followed heating. Sugars were detected by spraying with aniline–phthalate reagent.

3.2 *Plant material*

The seeds of *B. javanica* were collected from Fuzhou City, Fujian Province, China in 2003 and were identified by Prof. K.Z. He, Department of Botany, Fujian Agriculture and Forestry University, Fujian, China. A voucher specimen (No. 19720031) is deposited in the Herbarium of the Key Laboratory of Biopesticide and Chemistry and Biology of the Ministry of Education, Fujian, China.

3.3 *Extraction and isolation*

The seeds of *B. javanica* (2 kg) were extracted (2× 4 l) with MeOH at room temperature (7 days ×2). The extract was concentrated to dryness under reduced pressure and the residue (700 g) was suspended in water (2.5 l) and partitioned with ethyl acetate (3× 3 l). The water layer was subsequently extracted with *n*-butanol (3× 3 l). The *n*-butanol extract was evaporated *in vacuo* to give a residue of 130 g. The residue was subjected to dry column chromatography on silica gel (1.0 kg) and eluted with $CHCl_3$–MeOH (10:1), producing 13 fractions. Each fraction was then separated on Sephadex LH-20 and RP-18 columns, eluted with methanol–water (10–90%), and finally purified by a silica gel column with $CHCl_3$–EtOAc–MeOH

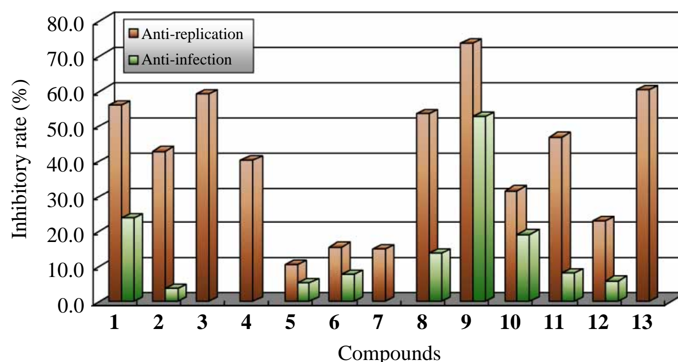


Figure 4. Inhibitory activity of **1**–**13** from the seeds of *B. javanica* against TMV at 50 μg ml^{-1}.

(10:10:1) and $CHCl_3$–EtOH (10:0.5–10:2) to yield **1** (34 mg), **2** (14 mg), **3** (11 mg), **4** (26 mg), **5** (84 mg), **6** (22 mg), **7** (15 mg), **8** (35 mg), **9** (45 mg), **10** (25 mg), **11** (26 mg), **12** (84 mg), and **13** (22 mg).

3.3.1 Compound (**1**)

A colorless amorphous powder; $[\alpha]_D^{21} - 29$ ($c = 0.24$, MeOH); IR ν_{max} (film; cm^{-1}): 3310, 2965, 1640, 1340, 1210, 965; ^{1}H and ^{13}C NMR spectral data, see Table 1; FAB-MS *m/z*: 347 [M−H]$^-$, 185 [M−H−162]$^-$; HR-FAB-MS *m/z*: 347.1705 [M−H]$^-$ (calcd for $C_{16}H_{27}O_8$, 347.1706).

3.3.2 Compound (**2**)

A colorless amorphous powder; $[\alpha]_D^{21} + 74.0$ ($c = 0.13$, acetone); IR ν_{max} (film; cm^{-1}): 3380, 2927, 1248, 1062, 903; ^{1}H and ^{13}C NMR spectral data, see Table 1; EI-MS *m/z*: 254 [M]; HR-EI-MS *m/z*: 254.1881 [M]$^+$ (calcd for $C_{15}H_{26}O_3$, 254.1882).

3.3.3 Compound (**3**)

A colorless amorphous powder; $[\alpha]_D^{21} + 12.0$ ($c = 0.21$, acetone); IR ν_{max} (film; cm^{-1}): 3367, 2947, 1221, 1042; ^{1}H and ^{13}C NMR spectral data, see Table 1; EI-MS *m/z*: 254 [M]; HR-EI-MS *m/z*: 254.1881 [M]$^+$ (calcd for $C_{15}H_{26}O_3$, 254.1882).

3.4 Acid hydrolysis

A solution of compound **1** (6 mg) was heated and refluxed at 100°C in 2 M aqueous CF_3COOH (3 ml) on a water bath for 3 h. The reaction mixture was then diluted with H_2O (15 ml) and extracted with CH_2Cl_2 (3× 5 ml). The aqueous layer was neutralized with Amberlite IR-45 and concentrated *in vacuo* to dryness. The form (D or L) of sugar was determined using HPLC (Shodex RSpak DC-613, 75% CH_3CN, 1 ml/min, 70°C) with refraction index detection and chiral detection (Shodex OR-1) to compare with authentic sugars (10 mmol each D-glucose and D-galactose). These sugars gave the following peaks: D-glucose 7.40 min and D-galactose 8.01 min. D-Galactose was detected in compound **1**.

3.5 Virus and host

The common strain of TMV was propagated and maintained in the systemic host *Nicotiana tabacum* L. cv. K326, purified as described in our previous study [7]. *N. tabacum* cv. K326 and *Nicotiana glutinosa* L. were cultivated in an insect-free glasshouse, using for systemic and local lesion host of TMV, respectively.

3.6 Screening material preparations

Different compounds were dissolved in a small quantity of DMSO (final

Downloaded At: 18:40 22 January 2011

concentration <0.5%), and diluted with water to provide solutions having a concentration of 100 μg ml^{-1}, and placed in a Ø 35 mm Petri dishes for the bioassay.

3.7 *Local lesion assay*

N. glutinosa of six-leaf stage was used for local lesion assay. The compound solution was mixed with an equal volume of TMV solution to make a mixture (50 μg ml^{-1} of test compound and 10 μg ml^{-1} of TMV, final concentration) to use for inoculation. Half leaves of *N. glutinosa* were mechanically inoculated with the test mixtures, the other half leaves were treated with a solution of 10 μg ml^{-1} of TMV and an equal volume of solvent as control. The mixtures were incubated for 30 min at room temperature before inoculation, and leaves were washed with distilled water 5 min after inoculation. All assays were performed in triplicate. Local lesion number was recorded 3 days later and inhibition rate of virus infection was calculated according to the following formula:

$$\text{Inhibition rate} = (1 - T/C) \times 100,$$

where T is the average lesion number of treated half leaves and C is the average lesion number of the control.

3.8 *Leaf-disk method*

Leaves of *N. tabacum* were mechanically inoculated with TMV at 10 μg ml^{-1}. Leaf disks of 10 mm diameter were punched and floated on the solutions of different compounds 7 h after inoculation and incubated at 25 ± 1°C for 48 h. Disks treated with solvent only were used as a positive control, whereas disks of healthy leaves were used as a negative control; 48 h later, leaf disks were ground in coating buffer (sodium carbonate buffer: $NaHCO_3$ 35 mM and Na_2CO_3 15 mM), and virus concentrations in them were measured by indirect ELISA. There were six repetitions in every treatment. A series of TMV solutions at known concentrations was incorporated into every microliter plate to provide an internal calibration curve. Linearity of absorbance with TMV concentrations was obtained within a range of about two orders of magnitude (0.04888–12.5 ng of TMV). Virus concentration was calculated by the TMV standard curve with OD_{405} value of indirect ELISA, and the inhibition of virus replication was calculated as follows:

$$\text{Inhibitory rate} = (1 - T_0/C_0) \times 100,$$

where T_0 is the virus concentration of compound-treated leaf disk and C_0 is the virus concentration of leaf disk of the positive control.

3.9 *Indirect ELISA procedure*

One hundred microliters of diluted antigen were added to each well of a micro-ELISA plate and incubated overnight at 4°C. After incubation, the antigen solution was discarded and the plates were washed three times in phosphate-buffered saline (PBS), pH 7.2, containing 0.001% Tween 20 (PBS-T). Rabbit anti-TMV serum (1:2000) was added to the antigen-coated wells. The plates were incubated for 1 h at and then washed three times in PBS-T. Goat anti-rabbit alkaline phosphatase (Sigma, Pool, Dorset, UK) conjugate (1:30,000) in PBS-T was then added and a further incubation for 1 h carried out at 37°C. The plates were again washed three times, 100 μl of *p*-nitrophenyl phosphate substrate (1000 mg l^{-1}) per well was added, and after 10 min, the reaction was stopped by adding 100 μl of 3 M sodium hydroxide. The intensity of color development was determined by measuring absorbance using a micro-ELISA reader equipped with a 405 nm filter.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (Grant

Downloaded At: 18:40 22 January 2011

No. 30770089) and Fujian Administration of Education (Grant Nos 2006NZ0002C4, 2008J0073, and 2006F5017). We thank the staff of the analytical group of Department of Chemistry, Taiwan University for measurements of NMR and FAB-MS spectra.

References

[1] M. Okana, N. Fukamiya, and K.H. Lee, *Studies in Natural Products Chemistry* (Elsevier, Amsterdam, 1990), Vol. 7, pp. 369–404.

[2] M. Okana, N. Fukamiya, and K.H. Lee, *Studies in Natural Products Chemistry* (Elsevier, Amsterdam, 2000), Vol. 23, pp. 285–333.

[3] N. Fukamiya, K.H. Lee, I. Muhammad, C. Murakami, M. Okano, I. Harvey, and J. Pelletier, *Cancer Lett.* **220**, 37 (2005).

[4] I.H. Kim, S. Takashima, Y. Hitotsuyanagi, T. Hasuda, and K. Takeya, *Heterocycles* **3**, 691 (2004).

[5] H. Subeki, K. Matsuura, K. Takahashi, M. Nabeta, Y. Yamasake, K. Maede, and Katakura, *J. Nat. Prod.* **10**, 1654 (2007).

[6] I.H. Kim, S. Takashima, Y. Hitotsuyanagi, T. Hasuda, and K. Takeya, *J. Nat. Prod.* **67**, 863 (2004).

[7] J.G. Shen, Z.K. Zhang, Z.J. Wu, M.A. Ouyang, L.H. Xie, and Q.Y. Lin, *Pest Manage. Sci.* **64**, 191 (2008).

[8] F. Abe and T. Yamauchi, *Phytochemistry* **28**, 1737 (1989).

[9] A.B. Ray, S.K. Chattopadhyay, S. Kumar, C. Konno, and Y. Kiso, *Tetrahedron* **41**, 209 (1985).

[10] A. Hernandez, C. Pascual, and S. Valverde, *Phytochemistry* **1**, 181 (1981).

[11] S.F. Fonseca, P.J. Campello, L.E.S. Barata, and E.A. Ruveda, *Phytochemistry* **17**, 499 (1978).

[12] L.A.C. Pieters, D.A. Berghe, and A.J. Vlierinck, *Phytochemistry* **29**, 348 (1990).

[13] M.A. Ouyang, Y.S. Wein, and Y.H. Kuo, *Helv. Chim. Acta* **6**, 1099 (2007).

[14] L.H. Briggs, R.C. Cambie, and J.L. Hoare, *Tetrahedron Lett.* **3-4**, 262 (1959).

[15] K. Ishimaru, G. Nonaka, and I. Nishioka, *Phytochemistry* **26**, 1147 (1987).

[16] D.Q. Yu and J.S. Yang, *Analysis of NMR Spectroscopy* (Chemical Industry Press, Beijing, 1999), Vol. 7, pp. 496, 902.

[17] M. Miyazawa, K. Tsurno, and H. Kameoka, *Nat. Prod. Lett.* **10**, 291 (1997).

[18] L. Fitjer, A. Malich, C. Paschke, S. Kluge, R. Gerke, B. Rissom, J. Weiser, and M. Noltemeyer, *J. Am. Chem. Soc.* **117**, 9180 (1995).

Downloaded At: 18:40 22 January 2011