

## Lignans from the Roots of *Wikstroemia indica* and Their DPPH Radical Scavenging and Nitric Oxide Inhibitory Activities<sup>1)</sup>

Li-Yan WANG, Noriko UNEHARA, and Susumu KITANAKA\*

Department of Pharmacognosy, College of Pharmacy, Nihon University; 7-7-1 Narasinodai, Funabashi, Chiba 274-8555, Japan. Received May 23, 2005; accepted July 19, 2005; published online July 27, 2005

**In our investigation of *in vitro* anti-allergic screening of medicinal herbal extracts, the ethyl acetate extract of the root of *Wikstroemia indica* was observed to inhibit nitric oxide (NO) production in a lipopolysaccharide (LPS) and recombinant mouse interferon- $\gamma$  (IFN- $\gamma$ ) activated murine macrophage-like cell line, RAW 264.7. Fractionation of the active extract led to the isolation of two new lignan dimer bis-5,5-nortrachelogenin (**1**) and bis-5,5'-nortrachelogenin (**2**), and three known lignans. Compound **1** and liriioresinol B (**5**) inhibited NO production with IC<sub>50</sub> values at 48.6 and 53.5  $\mu$ M, respectively. Compound **5** also showed free radical scavenging effect on 1,1-diphenyl-2-picrylhydrazil (DPPH) at IC<sub>50</sub> values at 19.5  $\mu$ M.**

**Key words** *Wikstroemia indica*; Thymelaeaceae; bis-5,5-nortrachelogenin; bis-5,5'-nortrachelogenin; dilignan; nitric oxide

*Wikstroemia indica* (Thymelaeaceae) is distributed in the southeast of China. It has long been used as a traditional crude drugs for the treatment of pneumonia, rheumatism, and bronchitis<sup>2)</sup> in China. In the previous paper, we reported the isolation of one new guaiane type sesquiterpene, indicanone, and two known biflavonoids, sikokianin B and sikokianin C from 80% methanol extract of the roots of *Wikstroemia indica*.<sup>3)</sup> Indicanone inhibited nitric oxide (NO) production with IC<sub>50</sub> values at 9.3  $\mu$ M and also inhibit the iNOS gene expression. Further fractionation of the 80% methanol extract has led to the isolation of two new dilignans, and three known lignans. In this paper, we report the structure characterization and the NO and 1,1-diphenyl-2-picrylhydrazil (DPPH) evaluation of these compounds.

The IR spectrum of **1** showed absorptions at 3440 (OH) and 1770 ( $\gamma$ -lactone). The <sup>1</sup>H spectrum of **1** exhibited the presence of two methoxyls ( $\delta_{\text{H}}$  3.86 s 3H,  $\delta_{\text{H}}$  3.69 s 3H), a set of AB system signals of a methylene [ $\delta_{\text{H}}$  2.87 d (13.0), 3.10 d (13.0)], a 1,2,4-trisubstituted benzene ring [ $\delta_{\text{H}}$  6.70 d (1.8),  $\delta_{\text{H}}$  6.55 dd (8.0, 1.8),  $\delta_{\text{H}}$  6.66 d (8.0)], and a 1,2,3,5-tetrasubstituted benzene ring [ $\delta_{\text{H}}$  6.67 d (3.1),  $\delta_{\text{H}}$  6.69 d (3.1)]. The <sup>1</sup>H spectrum of **1** showed a very similar pattern to that of **3** (Table 1),<sup>4)</sup> a known compound named (+)-nortrachelogenin which was isolated from this plant formerly, except for the difference in the aromatic region. That is, the signals of 1,2,4-trisubstituted benzene ring due to the aromatic ring B of **3** [ $\delta_{\text{H}}$  6.66 d (2.1),  $\delta_{\text{H}}$  6.69 d (8.0),  $\delta_{\text{H}}$  6.58 dd (8.0, 2.1)] was changed to be two *meta*-coupling signals. The distortionless enhancement by polarization transfer (DEPT) spectrum of **1** were compared to those of **3** and found they showed a completely identical pattern except that C-5 in the aromatic ring of **1** changed to be a quaternary carbon, whereas C-5 in the same aromatic ring of **3** appeared as a tertiary carbon bearing one aromatic proton. Electron impact ionization (EI)-MS showed [M]<sup>+</sup> at *m/z* 746, which corresponding to MW of a dimer of **3** [2 $\times$ 374 (MW of **3**)–2H]. The high resolution (HR)-EI-MS of **1** indicated a molecular ion at *m/z* 746.2551, which corresponded to the molecular formula C<sub>40</sub>H<sub>46</sub>O<sub>12</sub>. From all these results, **1** was suggested to be a dimer of **3** and the two nortrachelogenin (**3**) units were linked in a magnetically symmetric mode at C-5. The C-5 symmetric dilignan of **1** was also supported by the down field

shifts of C-5 and C-6 [ $\Delta\delta_{\text{C}} = +11.0, +2.4, (\Delta\delta_{\text{C}} = \delta_{\text{1}} - \delta_{\text{3}})$ ], and up field shifts of C-4 [ $\Delta\delta_{\text{C}} = -2.5$ ]. The relative stereochemistry of butyrolactone ring was assigned on the basis of nuclear magnetic resonance (NOESY) experiment.  $\delta_{\text{H}}$  3.10 (H-7') showed a NOE correlation with  $\delta_{\text{H}}$  2.46 (H-8) indicated a 8 $\alpha$ -H, 8' $\beta$ -OH. Based on the above evidence, **1** was identified as a new dilignan, bis-5,5-nortrachelogenin.

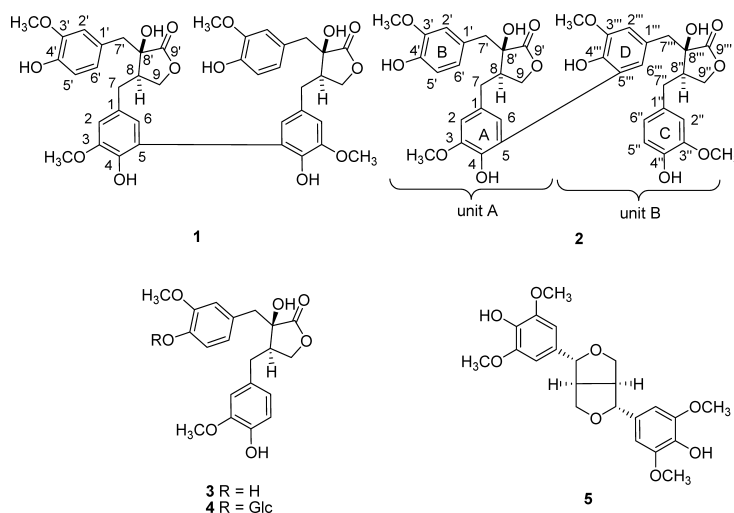
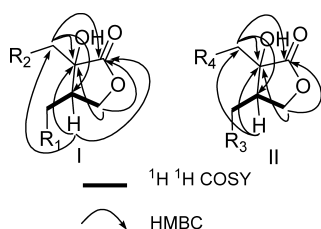
The molecular composition of **2** was found to be C<sub>40</sub>H<sub>42</sub>O<sub>14</sub>, the same as **1**, by the negative high resolution FAB-MS (HR-FAB-MS) (*m/z* 745.2489). **2** displayed IR absorptions at 3360 (OH) and 1750 ( $\gamma$ -lactone). <sup>1</sup>H- and <sup>13</sup>C-NMR spectra of **2** showed two pairs signal of **3** (Table 1). This suggested that **2** was a dimeric lignan of **3** and the two units linked in an asymmetric mode. The <sup>1</sup>H-NMR spectrum of **2** exhibited the presence of four methoxyls ( $\delta_{\text{H}}$  3.62 s 3H,  $\delta_{\text{H}}$  3.68 s 3H,  $\delta_{\text{H}}$  3.83 s 3H,  $\delta_{\text{H}}$  3.86 s 3H), two sets of AB system signals of two methylenes [ $\delta_{\text{H}}$  2.86 d (13.7), 3.10 d (13.7);  $\delta_{\text{H}}$  2.90 d (13.4), 3.17 d (13.4)], two 1,2,4-trisubstituted benzene rings [ $\delta_{\text{H}}$  6.68 d (1.8),  $\delta_{\text{H}}$  6.55 dd (7.9, 1.8),  $\delta_{\text{H}}$  6.65 d (7.9),  $\delta_{\text{H}}$  6.62 d (1.8),  $\delta_{\text{H}}$  6.64 d (7.9),  $\delta_{\text{H}}$  6.56 dd (7.9, 1.8)], and two 1,2,3,5-tetrasubstituted benzene rings [ $\delta_{\text{H}}$  6.66 d (1.8),  $\delta_{\text{H}}$  6.69 d (1.8),  $\delta_{\text{H}}$  6.70 d (1.8),  $\delta_{\text{H}}$  6.72 d (1.8)]. The <sup>13</sup>C-NMR and DEPT spectra of **2** also showed the presence of four aromatic rings (A, B, C, D), two carbonyls, four methoxyls, two oxygen-bearing methylenes, two oxygen-bearing quaternary carbons, four methylenes, and two methines. By the analysis of the <sup>1</sup>H–<sup>1</sup>H correlation spectroscopy (<sup>1</sup>H–<sup>1</sup>H COSY) and heteronuclear multiple quantum coherence (HMQC) spectrum, the correlation of H-8 ( $\delta_{\text{H}}$  2.58 m) with H-9 ( $\delta_{\text{H}}$  4.05 m) and H-7 ( $\delta_{\text{H}}$  2.55 m, 2.82 m), and H-8'' with H-9'' and H-7'' gave the partial structure C-7–C-9 and C-7''–C-9''. In the analysis of heteronuclear multiple bond connectivity (HMBC) spectrum, the long-range correlations of the carbons (C-7–C-9 and C-7'–C-9') with proton signals (H-7–H-9 and H-7') connected the partial structure C-7–C-9 with C-7'–C-9' and established the partial structure I. The chemical shift of C-9 ( $\delta_{\text{C}}$  72.0) and C-9' ( $\delta_{\text{C}}$  180.8), and the HMBC correlation between H-9 and C-9' confirmed the lactone unit at C-9 and C-9'. The same partial structure II (C-7''–C-9'' and C-7'''–C-9'') was ascertained in the same manner (Fig. 2). Furthermore, the HMBC spectrum showed correlations between H-8 and C-1, H-7 and C-1, C-2,

\* To whom correspondence should be addressed. e-mail: kitanaka@pha.nihon-u.ac.jp

Table 1.  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR Data for Compounds **1**, **2**, and **3** [ $\text{CD}_3\text{OD}$ , TMS,  $\delta$  (ppm) ( $J=\text{Hz}$ )]<sup>a)</sup>

	<b>1</b> <sup>b)</sup>		Unit A-		Unit B-		<b>3</b> <sup>c)</sup>	
	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$
1	131.4		131.3		1''	131.9	131.9	
2	112.4	6.69 d (3.1)	112.4	6.69 d (1.8)	2''	113.4	113.6	6.66 d (2.1)
3	149.7		149.8		3''	149.0	149.1	
4	143.7		143.9		4''	146.1	146.2	
5	127.3		127.44 <sup>d)</sup>		5''	116.2	116.3	6.693 d (8.0)
6	124.8	6.67 d (3.1)	124.7	6.66 d (1.8)	6''	122.4	122.4	6.58 dd (8.0, 2.1)
7	32.4	2.53 m	32.5	2.55 m	7''	32.3	32.2	2.46 dd (14.0, 9.6)
		2.82 dd (12.5, 7.9)		2.82 m				2.78 dd (14.0, 4.8)
8	44.7	2.46 m	44.4	2.58 m	8''	44.6	44.6	2.44 m
9	72.0	4.04 m	72.0	4.05 m	9''	72.1	71.9	3.97 d (7.2)
1'	128.3		128.3		1'''	127.45 <sup>d)</sup>	128.3	
2'	115.0	6.70 d (1.8)	115.0	6.68 d (1.8)	2'''	113.8	115.0	6.687 d (2.1)
3'	148.8		148.8		3'''	149.6	148.9	
4'	146.6		146.6		4'''	144.8	146.7	
5'	116.1	6.66 d (8.0)	116.1	6.65 d (7.9)	5'''	127.43 <sup>d)</sup>	116.1	6.71 d (8.0)
6'	124.2	6.55 dd (8.0, 1.8)	124.2	6.55 dd (7.9, 1.8)	6'''	126.4	124.1	6.57 dd (8.0, 2.1)
7'	41.9	2.87 d (13.0)	41.8	2.86 d (13.7)	7'''	41.9	41.9	2.84 d (13.7)
		3.10 d (13.0)		3.10 d (13.7)				3.11 d (13.7)
8'	77.5		77.5		8'''	77.6	77.5	
9'	180.8		180.8		9'''	180.7	180.7	
3-OCH <sub>3</sub>	56.7	3.86 s	56.3	3.62 s	3'''-OCH <sub>3</sub>	56.6	56.47 <sup>d)</sup>	3.81 s
3'-OCH <sub>3</sub>	56.4	3.69 s	56.4	3.68 s	3'''-OCH <sub>3</sub>	56.7	56.41 <sup>d)</sup>	3.78 s

a) Assignments confirmed by decoupling,  $^1\text{H}$ - $^1\text{H}$  COSY, HMQC, HMBC, and NOESY spectra. b) Spectra obtained at 500 MHz and 125 MHz. c) Spectra obtained at 600 MHz and 150 MHz. d) Interchangeable signal.

Fig. 1. Structures of Compounds **1**—**5** from the Roots of *Wikstroemia indica*Fig. 2.  $^1\text{H}$ - $^1\text{H}$  COSY and Key HMBC Correlations of Partial Structure I and II

and C-6, H-2 and C-1, C-3, C-4, and C-7, H-6 and C-1, C-4, C-5, and C-7. From these results, the assignments at benzene ring A were confirmed and the benzene ring A was con-

nected with C-7 at C-1. The HMBC correlations of H-7' to C-1', C-2', and C-6', H-2' to C-1', C-3', and C-7', and H-6' to C-1', C-4', and C-5' confirmed the assignments at benzene ring B and the benzene ring B was connected with C-7' at C-1'. From these results, the half-structure (Unit A) of **2** was confirmed. The other half-structure of **2** (Unit B) was also confirmed by the same carefully analysis of HMBC correlations (Fig. 3). Since C-5 of unit A and C-5''' of unit B are quaternary carbon, **2** was suggested to be a dilignan linked at C-5 and C-5'''. The C-5,5''' dimer of **2** was also supported by the down field shifts of C-5 and C-6 [ $\Delta\delta_{\text{C}} = +11.1, +2.3, (\Delta\delta_{\text{C}} = \delta 2 - \delta 3)$ ], and up field shifts of C-4 [ $\Delta\delta_{\text{C}} = -2.3$ ] in the unit A, and the down field shifts of C-5''' and C-6''' [ $\Delta\delta_{\text{C}} = +11.3, +2.3, (\Delta\delta_{\text{C}} = \delta 2 - \delta 3)$ ], and up field shifts of

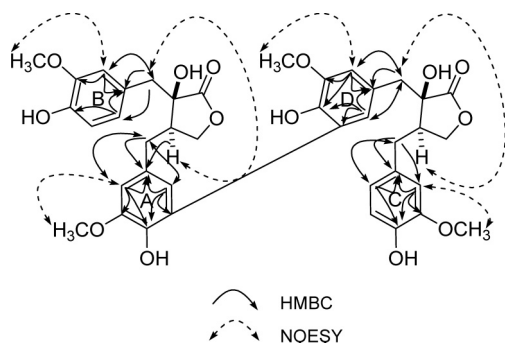


Fig. 3. Key HMBC and NOE Correlations of **2**

Table 2. Inhibitory Effects on NO Production and Free Radical Scavenging Effects on DPPH of Compounds **1**–**5**

Compounds	NO	DPPH
	IC <sub>50</sub> (μM)	
<b>1</b>	48.6	133.2
<b>2</b>	>200	>200
<b>3</b>	>200	90.1
<b>4</b>	>200	84.2
<b>5</b>	53.5	19.5

C-4''' [ $\Delta\delta_C = -1.9$ ] in the unit B comparing to **3**. **2** showed the significant NOE correlation between methoxyl  $\delta_H$  3.62 s (3-OCH<sub>3</sub>) and H-2,  $\delta_H$  3.68 s (3'-OCH<sub>3</sub>) and H-2',  $\delta_H$  3.86 s (3''-OCH<sub>3</sub>) and H-2'',  $\delta_H$  3.83 s (3'''-OCH<sub>3</sub>) and H-2'''. These facts indicated the positions of the methoxyls.  $\delta_H$  3.10 (H-7') showed a NOE correlation with  $\delta_H$  2.58 (H-8) and  $\delta_H$  3.17 (H-7'') showed a NOE correlation with  $\delta_H$  2.48 (H-8'') indicated a 8 $\alpha$ -H, 8' $\beta$ -OH and 8'' $\alpha$ -H, 8'' $\beta$ -OH.

(+)-Nortrachelogenin (**3**),<sup>4</sup> nortracheloside (**4**),<sup>5</sup> and liriorelin B (**5**)<sup>6</sup> were known compounds, whose structures were elucidated by comparisons with the literature.

Compounds **1** and **5** showed most potent NO production inhibitory activity with IC<sub>50</sub> value of 48.6 and 53.5 μM, which is not stronger than that of N<sup>G</sup>-monomethyl-L-arginine (L-NMMA) used as a positive control having an IC<sub>50</sub> value of 25.5 μM.

Compounds **1**–**5** were also tested for their DPPH radical scavenging activity, and all compounds, except **2** showed significant scavenging activity. Liriorelin B (**5**) possessed the strongest DPPH radical scavenging activity with IC<sub>50</sub> value of 19.5 μM. L-Ascorbic acid, a well known antioxidant used as a positive control, had an IC<sub>50</sub> value of 20.3 μM.

## Experimental

**General Procedures** The UV spectrum was obtained in MeOH on a Shimadzu UV-160 spectrophotometer, and the IR spectrum was recorded on a JUSCO FT/300E spectrophotometer. Optical rotations were measured in MeOH on a JASCO DIP-360 polarimeter. The NMR spectra were recorded on a JNM-ECX-500 and JNM-ECA 600 spectrometer, with TMS as an internal standard. MS was obtained on a JMS GC mate spectrometer. Column chromatography was carried out with silica gel (Wako gel C-300, Wako Pure Chemical Industry Ltd.). Thin-layer chromatography (TLC) was performed on Merck TLC plates (0.25 mm thickness), with compounds visualized by spraying with 5% (v/v) H<sub>2</sub>SO<sub>4</sub> in ethanol solution and then heating on a hot plate. HPLC was performed on a JASCO PU-2089 apparatus equipped with a JASCO UV-2075. YMC pack pro C-18 (10×250 mm) and Fluofix (10×250 mm) were used for preparative purposes.

**Plant Materials** The dried root of *W. indica* was collected in Guangdong Province, People's Republic of China, in October 1998 and was identified by Professor Weichun Wu (Department of Medical Plants, Shenyang Pharmaceutical University, People's Republic of China). Voucher specimens (NK-01017) have been deposited at the Department of Pharmacognosy, College of Pharmacy, Nihon University.

**Extraction and Isolation** The dried roots of *W. indica* (2.3 kg) were extracted twice with 80% methanol. Evaporation of the solvent under reduced pressure from the combined extract gave the 80% MeOH extract 286 g (NO inhibitory effect 100 μg/ml, 38.2%). The extract was dissolved and suspended in water (2 l) and partitioned with hexane (3×2 l), ethyl acetate (3×2 l), and *n*-butanol (3×2 l). The amounts extracted were 6.5 g (76.1%), 62.0 g (86.9%), and 25.0 g (−6.4%), respectively, and the residual aqueous extract yielded 88.3 g (3.8%).

The ethyl acetate fraction was subjected to sephadex LH-20 column chromatography (13×20 cm, eluted with MeOH:H<sub>2</sub>O 30:70→90:10). The column chromatographic fractions (200 ml each) were combined according to TLC monitoring into four portions. Fraction 2, was subjected to silica gel column chromatography (3×21 cm, eluted with chloroform and methanol in increasing polarity). The column chromatographic fractions (100 ml each) were combined according to TLC monitoring into eleven portions. Portion three was isolated and further purified by HPLC (YMC pack pro C-18, 10×250 mm, MeOH:H<sub>2</sub>O, 45:55) to give **3** and **5** (55.5, 10.1 mg). Portion five was isolated and further purified by HPLC (Fluofix, 10×250 mm, MeOH:H<sub>2</sub>O, 35:65) to give **1** and **2** (5.7, 4.5 mg). Portion 7, was further purified by HPLC (YMC pack pro C-18, 10×250 mm, MeOH:H<sub>2</sub>O, 35:65) to give **4** (4.9 mg).

Bis-5,5-nortrachelogenin (**1**): Light yellow oil; [ $\alpha_D^{23}$ ] +55.0° (*c*=0.10, MeOH); UV (MeOH)  $\lambda_{max}$  (log  $\epsilon$ ) nm: 284 (4.07), 205 (4.98); IR (KBr)  $\nu_{max}$  3440, 1770, 1600, 1520, 1460, 1430 cm<sup>−1</sup>; CD (MeOH)  $\lambda_{ext}$  232 nm ( $\Delta\epsilon$  +2.48), 205 nm ( $\Delta\epsilon$  +3.28); <sup>1</sup>H- and <sup>13</sup>C-NMR data, see Table 1; HR-EI-MS *m/z*: 746.2551 (Calcd for C<sub>40</sub>H<sub>42</sub>O<sub>14</sub>, 746.2574).

Bis-5,5'-nortrachelogenin (**2**): Light yellow oil; [ $\alpha_D^{23}$ ] +68.1° (*c*=0.42, MeOH); UV (MeOH)  $\lambda_{max}$  (log  $\epsilon$ ) nm: 280 (4.22); IR (liquid film)  $\nu_{max}$  3360, 1750, 1600, 1520, 1470 cm<sup>−1</sup>; CD (MeOH)  $\lambda_{ext}$  232 nm ( $\Delta\epsilon$  +2.41), 205 nm ( $\Delta\epsilon$  +2.31); <sup>1</sup>H- and <sup>13</sup>C-NMR data, see Table 1; negative HR-FAB-MS *m/z*: 745.2489 (Calcd for C<sub>40</sub>H<sub>41</sub>O<sub>14</sub>, 745.2495).

(+)-Nortrachelogenin (**3**): See ref. 4; CD (MeOH)  $\lambda_{ext}$  234 nm ( $\Delta\epsilon$  +2.62), 206 nm ( $\Delta\epsilon$  +3.28).

**Inhibitory Activity on NO Production from Activated Macrophages-Like Cell Line, RAW 264.7** The cells were seeded at 1.2×10<sup>6</sup> cells/ml onto 96-well flat bottom plate and then incubated at 37 °C for 2 h. Next, the test sample was added to the culture simultaneously with both *Escherichia coli* LPS (100 ng/ml) and recombinant mouse IFN- $\gamma$  (0.33 ng/ml). Then cells were incubated at 37 °C for approximately 16 h and subsequently chilled on ice. One hundred microliters of the culture supernatant was placed in duplicate in the wells of 96-well flat-bottomed plates. A standard solution of NaNO<sub>2</sub> was placed in alternate wells on the same plate. To quantify nitrite, 50 μl of Griess reagent (1% sulfanilamide in 5% H<sub>3</sub>PO<sub>4</sub> and 0.1% *N*-1-naphthylethylenediamide dihydrochloride) was added to each well. After 10 min the reaction products were colorimetrically quantified at 550 nm using a Model 3550 Microplate Reader and the background absorbance (630 nm) was subtracted. Cytotoxicity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method.

**DPPH Radical Scavenging Activity** 0.2 M acetic acid buffer (40 μl), 12% methanol (40 μl), and sample (0.4 μl) was mixed with 0.5 mM DPPH (40 μl). The resulting solution was thoroughly mixed and the absorbance was measured at 520 nm after 30 min. The scavenging activity was determined by comparing the absorbance with that of the control (100%) containing only DPPH and solvent.

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## References and Notes

- Part 13 in the series of anti-allergic agents from natural sources. Part 12: Ishii R., Horie M., Koyama K., Ishikawa Y., Kitanaka S., *Biol. Pharm. Bull.*, **28**, 786–790 (2005).
- "Zhong Hua Ben Cao," Shanghai Xin Hua Press, Shanghai, 1999, pp. 425–426.

- 3) Wang L. Y., Unehara N., Kitanaka S., *Chem. Pharm. Bull.*, **53**, 137—139 (2005).
- 4) Kato A., Hashimoto Y., Kidokoro M., *J. Nat. Prod.*, **42**, 159—162 (1979).
- 5) Li T. Z., Zhang W. D., Gu Z. B., Liu W. Y., Zhou J., Chen W. S., *Yao Xue Xue Bao*, **38**, 520—522 (2003).
- 6) Tong W. Y., Mi L., Liang H., Zhao Y. Y., *Beijing Daxue Xuebao, Yixueban*, **35**, 180—183 (2003).