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NEW LIGNANS FROM *KADSURA COCCINEA* AND THEIR NITRIC OXIDE INIHIBITOTY ACTIVITIES[1]

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Abstract - *In vitro* anti-allergic screening of medicinal herbal extracts revealed that the chloroform extract of the rhizoma of *Kadsura coccinea* inhibited nitric oxide (NO) production in a lipopolysaccharide (LPS) and recombinant mouse interferon-γ (IFN-γ) activated murine macrophage like cell line, RAW264.7. Further fractionation of the chloroform extract led to the isolation of two new lignans kadsuralignans D (**1**) and E (**2**) with other two known dibenzocyclooctadiene lignans kadsulignan N (**3**) and neokadsuranin (**4**).

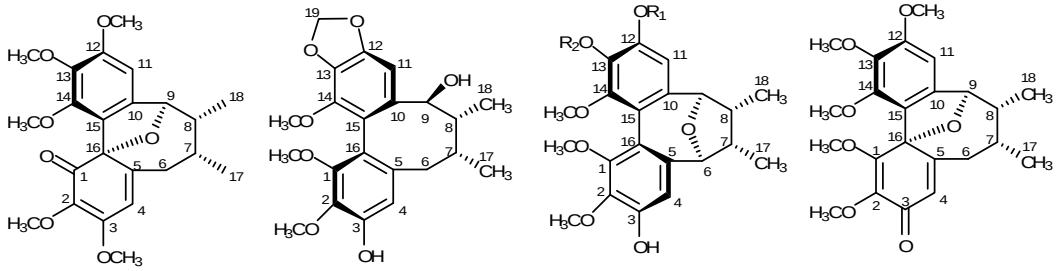

Figure 1.

INTRODUCTION

A considerable number of studies have been performed on plants of the family *Schisandraceae*, which contains only two genera, *Schisandra* and *Kadsura. Kadsura coccinea* is a plant indigenous to Guangxi

Province, People's Republic of China.[2] Plant extracts have been used in Chinese folk medicine for treatment of cancer and dermatosis and as an anodyne to relieve aches.[2] Previous investigations of *k. coccinea* have yielded some lignans and triterpenoids.[3-12] However, no report on the isolation and characterization of anti-allergic constituents from this plant has been made. As a part of our continuing phytochemical investigation of anti-allergic agents from Natural Sources,[1] the chemical composition of the rhizoma of *k. coccinea* has been examined.

Despite its common occurrence compounds in the isolation of *Schisandraceae* plants, kadsuralignan D, possessing a spirodienone structure and with 9,16-oxygen bridged lignan is encountered more rarely within the lignans. To our knowledge, former report of this kind of lignan dates back to 1988, where kadsulignan A (**5**) and B,[10] two lignans from the seeds of the same species were reported. Some later reports of new C_{19} homolignans with dienone structure but without oxygen bridge from another genus *Schisandra chinensis* [13] and same genus plant *K. heteroclita* have been reported. [14]

RESULTS AND DISCUSSION

Kadsuralignan D (**1**) was obtained as yellow gum, and assigned to possess a molecular formula of $C_{23}H_{28}O_7$ by HR-EIMS ($[M]^+$, *m/z* 416.1838) with ten unsaturation. The spectra of which display different features in comparison with those of dibenzocyclooctadiene lignans commonly found in this family of plants. The IR spectrum of **1** revealed the presence of an α,β,γ,δ-dienone groups [1652 cm^{-1} (C=CC=CC=O)].[13, 14] In the ^{13}C-and ^{1}H-NMR spectra, the signals at δ184.6, 157.6, 137.6, 122.3, 159.5 and 87.4 (C-1, 2, 3, 4, 5 and 16 , respectively), together with long-range correlation (HMBC) spectrum, which showed coupling between an olefinic proton (H-4, δ5.89, d, J = 1.7 Hz) with three double-bond carbons at C-5, 3, and 2, revealed the presence of a cyclohexanedienone moiety in **1**. The other aromatic signals at δ138.6, 99.0, 155.5, 141.5, 148.1 and 122.6 were assigned to C-10, 11, 12, 13, 14 and 15, respectively, on the basis of long-range correlations (Figure 2).

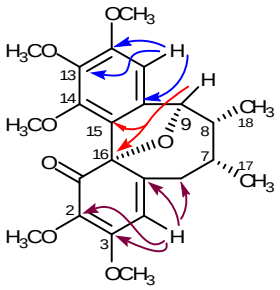

Figure 2. Key HMBC Correlations of **1**

The detailed analysis of **1** using ^{1}H-^{1}H COSY and HMQC disclosed partial structure unit with correlated protons: CH_2-$CH(CH_3)$-$CH(CH_3)$-CH(OH); and a cross peak (H-H long-rang coupling) between H-4 and H-6 in the ^{1}H-^{1}H COSY spectrum would explain that the signal of H-4 is a doublet.

The correlations of HMBC also showed the correlation between H-6 with C-4, 5, and 16 of cyclohexanedienone and between H-9 and C-10, 11, 15 and 16 allowed us establish the connectivity using the same oxygen connects both of the tertiary carbon C-9 and quaternary carbon C-16 forming a 9,16-oxygen bridge. Moreover, the HMBC showed the correlations between H-4 with C-2, 3, 5, 6 and 16 (in Figure 2), these facts confirms **1** has an α,β,γ,δ- dienone moiety but not α,β,α',β' moiety, and more

important, the NOE spectrum showed the correlation between H-4 and OCH_3-3 (Figure 3), these facts above clearly indicate **1** has the planar structure showed in Figure1.

The relative configuration at C-7, C-8 and C-9 were determined by the NOEs of **1**. On irradiation of CH_3-18, H-9 and CH_3-17 showed NOE, respectively, indicating a *trans* relationship between H-9/CH_3-18 and a *cis* relationship between CH_3-17/CH_3-18. Furthermore, the difference NOE experiment also showed the correlations between H-11/H-9, H-7/H-8, H_α-6/CH_3-17 and H_α-6 /H-4. These results indicate a boat conformation of the cyclooctene ring and a chair conformation of the cycloheptoxide ring.

The absolute configurations of **1** are assigned as 7*R*, 8*R*, 9*R*, and 16*S* based on the similarity of the circular dichroism (CD) spectrum of **1** (Figure 4) to that of **5**[10]. The CD spectrum of **1** showed a negative cotton effect at 340-360 nm and positive cotton effects at 230-240 and 300-320 nm.

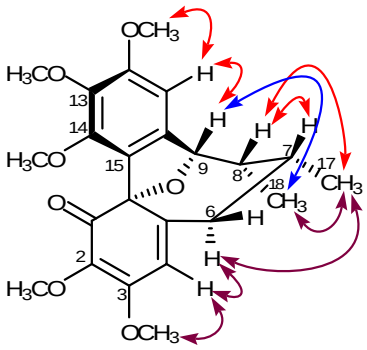

Figure 3. Key NOEs of **1**

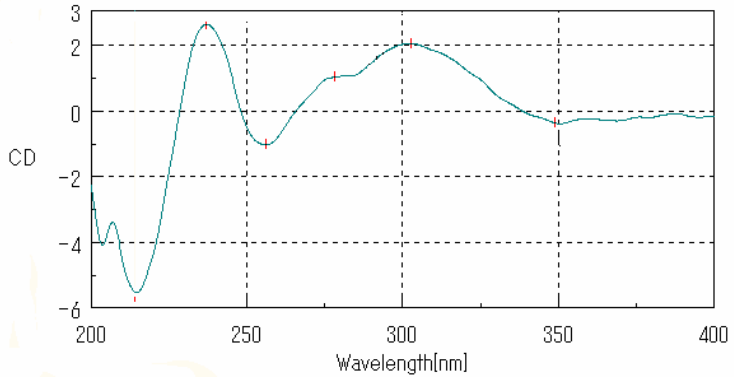

Figure 4. The CD spectrum of **1**

Kadsuralignan E (**2**) was obtained as colorless needles and assigned to possess a molecular formula of $C_{22}H_{26}O_7$ by HR-EIMS ($[M]^+$, *m/z* 402.1679) with ten unsaturation. The UV spectrum of **2** possessed characteristic UV spectrum (λ_{max} 225, 255sh and 290sh nm) of dibenzocyclooctadiene lignans.[15] It possessed a biphenyl moiety due to two aromatic protons at δ6.59 and 6.90 (each 1H, s, H-4 and H-11) and twelve carbon atoms at δ150.0, 137.4, 149.2, 110.1, 140.6 and 119.8 (C-1, 2, 3, 4, 5 and 16, respectively); δ140.1, 102.1, 148.6, 135.3, 140.1, 120.0 (C-10, 11, 12, 13, 14 and 15, respectively). Moreover, one methylenedioxy moiety at δ5.97 and 5.98 (each 1H, d, J = 1.4 Hz, CH_2-19) and three methoxyl groups at δ3.56, 3.78 and 3.90 (each 3H, s) were occurred, and predictably located them at the biphenyl rings. Moreover, ^{1}H-NMR spectra showed the presence of two secondary methyls groups, one of them shielded by the aromatic ring showed at δ0.68 (3H, d, J = 7.0), and the other at δ0.98 (3H, d, J = 7.0 Hz). They were assigned to CH_3-18 and CH_3-17, respectively. Furthermore, the signal of oxygenated methine was found at δ73.6, 4.53 (1H, d J = 1.2 Hz, C-9 and H-9). The detailed analysis of **2** using ^{1}H-^{1}H COSY and HMQC disclosed partial structure unit with correlated protons: CH_2-CH(CH_3)-CH(CH_3)-CH(OH), this unit should belong to the cyclooctadiene ring and clearly

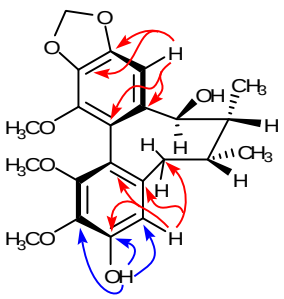

Figure 5. key HMBC correlations of **2**

suggested the substitution of cyclooctadiene ring.

Detailed analysis of HMBC spectrum (Figure 5) showed the correlations between the protons of methylenedioxy moiety CH_2-19 with carbons C-12 and C-13; H-4 with the carbon C-2, 3, 5, 6, and 16; H-11 with the carbon C-10, 12, 13, 15 and 9; the proton of hydroxyl in OH-1with the carbon at C-2 and 4. These facts affirmed the planar structure of **2** showed in Figure 1.

Table 1. ^{1}H- and ^{13}C-NMR data for compounds (**1** and **2**) (600 MHz, 125 MHz in $CDCl_3$) [a)]

	1		**2**	
	δ_c	δ_H (mult; *J*, Hz)	δ_c	δ_H (mult; *J*, Hz)
1	184.6		150.0	
2	157.6		137.4	
3	137.6		149.2	
4	122.3	5.89, (d, 1.7)	110.1	6.59, s
5	159.5		140.6	
6	37.5	H_α: 2.10, (d, 17.1) H_β: 2.74, m	34.4	H_α: 1.95, m H_β: 2.10, m
7	28.9	1.40, m	39.3	1.90, m
8	45.5	1.79, m	43.0	1.96, m
9	90.1	5.37, (d, 1.5)	73.6	4.53, (d, 1.2)
10	138.6		140.1	
11	99.0	6.46, s	102.1	6.90, s
12	155.5		148.6	
13	141.5		135.3	
14	148.1		140.1	
15	122.6		120.0	
16	87.4		119.8	
17	20.8	0.84, (d, 7.0)	22.0	0.98, (d, 7)
18	11.2	1.15, (d,7.0)	7.8	0.68, (d, 7)
19			101.0	5.97, (d, 1.4) 5.98, (d, 1.4)
1-OCH_3			60.3	3.56, s
2-OCH_3	60.5	3.95, s	61.0	3.90, s
3-OCH_3	60.7	3.74, s		
12-OCH_3	56.1	3.87 s		
13OCH_3	60.5	3.77, s		
14-OCH_3	60.7	3.73, s	59.7	3.78, s

[a)] Data were recorded on Jeol ECA-600MHz spectrometer (^{1}H, ^{13}C); chemical shifts (δ) are in ppm; assignments were confirmed by ^{1}H-^{1}H COSY, HMQC and HMBC

The relative configuration at C-7, C-8 and C-9 were determined by the difference NOE experiment. On irradiation of CH_3-18, H-11 and CH_3-17 showed NOE, respectively. On irradiation of H-6α, H-4 and H-7 showed NOE, respectively. These facts indicating compound (**2**) have a twist-boat confirmation.

Moreover the NOE between H-4 and OH-3 affirmed the position of OH-3 located at C-3 (Figure 6). The CD spectra of **2** showed a positive Cotton effect around 230-255 nm and a negative Cotton effect around 215-225 nm, suggesting **2** possessed an *R*-biphenyl configuration just like gomisin A.[15] Thus, the structure of **2** was assigned as shown in Figure 5

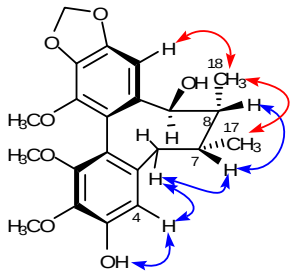

Figure 6. key NOEs of **2**

Compounds (**3**) [6] and (**4**) [11] were known compounds, whose structures were elucidated by comparisions with the literature.

Compounds (**1**-**4**) had been tested for their ability to inhibit nitric oxide (NO) production in a LPS and IFN-γ activated murine macrophage like cell line RAW 264.7 (Table 2).

Compound (**1**) and **(2**) showed weak NO production inhibitory activity with an IC_{50} value of 39.3 and 40.5, respectively. Both of them showed weaker activity than that of quercetin (IC_{50} = 23.5 μM), which was reported to have an inhibitory effect on the production of NO by LPS stimulated macrophage cell RAW 264.7.[16, 17]

Table 2. Inhibitory effects on NO production of compounds (**1**-**4**)

Compound	NO IC_{50}(μM)
1	39.3
2	40.5
3	>200
4	50.7

EXPERIMENTAL

The UV spectrum was obtained in MeOH on a Shimadzu UV-160 spectrophotometer, and the IR spectrum was recorded on a JASCO IR A-2 spectrophotometer. Optical rotations were measured in MeOH on a JASCO DIP-360 polarimeter. The NMR spectra were recorded on a Varian JMercury-300BB spectrometer, with TMS as an internal standard. The mass spectra (MS) were obtained on a JEOL GCmate 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_2SO_4 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. A Senshu Pak PEGASIL Silica 60-5 (10×250 mm i.d.) column, Fluofix 120N (10×250 mm i.d.) and a Senshu Pak PEGASIL ODS 2 were used for preparative purposes.

Plant Materials The dried rhizoma of *K. coccinea* were collected in Guangxi Province, People's Republic of China, in April 2004 and was identified by Dr. Bao-Lin Guo, Peking Union Medical College,

Beijing, China. Voucher specimens were deposited in the Department of Pharmacognosy, College of Pharmacy, Nihon University

Extraction and Isolation The dried rhizoma of *K. coccinea* (1.75 kg) were extracted three times with 80% acetone. Evaporation of the solvent under reduced pressure from the combined extract gave the extract 82.5 g (NO inhibitory effect 100 μg/mL, 78.5%). The extract was dissolved and suspended in water (2 l) and partitioned with chloroform (3 × 2 L), ethyl acetate (3 × 2 L), and *n*-butanol (3 × 2 L). The amounts extracted were 44.7 g (47.8%), 4.0 g (4.3%), and 14.2 g (15.4%), respectively, and the residual aqueous extract yielded 30.6 g (33.2%). The chloroform fraction was subjected to silica gel column chromatography (13φ × 65 cm, eluted with $CHCl_3$ and MeOH in increasing polarity). The column chromatographic fractions (500 mL each) were combined according to TLC monitoring into eleven portions. **3** was amount of in the portion eight, the crude crystals in the portion eight were recrystallized gave **3** (105mg). Portion ten was subjected to silica gel column chromatography (3 × 21cm, eluted with $CHCl_3$ and MeOH in increasing polarity). The column chromatographic fractions (100 mL each) were combined according to TLC monitoring into seven portions. Portion six was isolated and further purified by HPLC (Fluofix 120N, 10φ× 250 mm, CH_3CN: H_20, 70: 30) to give **1** (5 mg), **2** (14 mg) and **4** (26 mg).

Inhibitory Activity on NO Production from Activated Macrophages-Like Cell Line, RAW 264.7 The cells were seeded at 1.2×10^6 cells/mL onto 96-wells flat bottom plate (Sumitomo Bakelite, #8096R, Tokyo) and then incubated at 37 °C for 2 h. Next, the test extract was added to the culture simultaneously with both *Escherichia coil* LPS. (100 ng/mL) and recombinant mouse IFN- γ (0.33 ng/mL). Then cells were incubated at 37 °C for approximately 16 h and subsequently chilled on ice. One hundred micro liters of the culture supernatant was placed in duplicate in the wells of 96-well flat bottomed plates. A standard solution of $NaNO_2$ was placed in alternate wells on the same plate. To quantify nitrite, 50 μL of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-1-naphthyletylenediamide dihydrochloride) was added to each well. After 10 min the reaction products were colorimetrically quantified at 550 nm using a Model 3550 Microplate Reader (BIO-RAD) 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.

Compound (**1**): yellow gum from $CHCl_3$; UV (MeOH) λ_{max} nm (log ε) : 210 (4.61), 242 (3.77) and 300 (3.51); IR (KBr) cm^{-1}: 1745, 1652, 1602, 1465; EI-MS *m/z* (rel. int. %) : 416 $[M]^+$ (100), 401 (18), 346 (80), 385 (18), 331 (40); HR-EI-MS *m/z*: 416.1838 ($[M]^+$, Calcd 416.1835 for $C_{23}H_{28}O_7$); ^1H- and ^{13}C-NMR: see Table 1.

Compound (**2**): Colorless needles from hexane; mp 157-158℃; UV (MeOH) λ_{max} nm (log ε): 220 (3.90), 255 (sh), 289 (sh); EI-MS *m/z* (rel. int. %) : 402 $[M]^+$ (100), 384 (55), 346 (22), 208 (22), 168 (15); HR-EI-MS *m/z*: 402.1679 ($[M]^+$, Calcd 402.1678 for $C_{22}H_{26}O_7$); ^{1}H- and ^{13}C-NMR: see Table 1.

ACKNOWLEDGEMENTS

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