New Lignans from *Kadsura coccinea* and Their Nitric Oxide Inhibitory Activities[1)]

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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 RAW 264.7. Further fractionation of the chloroform extract led to the isolation of three new lignans, including two dibenzocyclooctadiene lignans and one arylnaphthalene lignan, together with other three known dibenzocyclooctadiene lignans. This is the first report of NO production inhibitory activity of *Kadsura coccinea* and first report about the isolation of arylnaphthalene lignan from *K. coccinea*.**

Key words *Kadsura coccinea*; kadsuralignan; arylnaphthalene lignan; nitric oxide (NO)

Kadsura coccinea (LEM.) A. C. SMITH is widely distributed throughout southwestern of China. Plant extracts have been used in Chinese folk medicine for treatment of cancer and dermatosis and as an anodyne to relieve aches in China.[2)] Previous investigations of *K. coccinea* have yielded some lignans and triterpenoids.[3—12)] However, little work on the isolation and characterization of anti-inflammatory 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.

The roots of *K. coccinea* were extracted with 80% acetone. The extract was then partitioned with hexane, chloroform, ethyl acetate, *n*-butanol and water, successively. The chloroform fractions showed strong NO production inhibitory activity. Further bioassay-directed fractionation of this fraction led to the isolation of two new dibenzocyclooctadiene lignans, kadsuralignan A (**1**), kadsuralignan B (**2**), and one new arylnaphthalene lignan kadsuralignan C (**3**), with three known dibenzocyclooctadiene lignans gomisin R (**4**), schizanrin F (**5**), and schizanrin H (**6**).

To our knowledge, it's the first report about arylnaphthalene lignan was isolated from *K. coccinea*.

Here, we describe the isolation, structure elucidation and biological evaluation of these compounds.

The configuration of biphenyl groups in all isolated dibenzocyclooctadiene lignans was determined basing on their characteristic circular dichroism (CD) spectra. The CD spectra of **1**, **2**, **4**—**6** showed a positive Cotton effect around 215—225 nm and a negative Cotton effect around 230—255 nm, suggesting that these dibenzocyclooctadiene lignans possessed an *S*-biphenyl configuration as gomisin B.[13,14)]

Kadsuralignan A (**1**) was obtained as colorless needles and assigned to possess a molecular formula of $C_{22}H_{26}O_7$ by high resolution electron impact ionization MS (HR-EI-MS) ($[M]^+$, *m*/*z* 402.1679) with ten unsaturation. The UV spectrum of **1** possessed characteristic UV spectrum (λ_{max} 220, 255, 289sh nm) of dibenzocyclooctadiene lignans.[15)] It possessed a biphenyl moiety due to two aromatic protons at δ 6.67 and 6.34 (each 1H, s, H-4 and H-11) and twelve carbon atoms at δ 150.6, 138.3, 148.5, 113.5, 133.7 and 121.1 (C-1, 2, 3, 4, 5, 16, respectively); δ 138.2, 102.5, 148.7, 135.7, 141.1, 119.2 (C-10, 11, 12, 13, 14, 15, respectively). Moreover, one methylenedioxy moiety at δ 5.97 (2H, s, CH_2-19) and three methoxyl groups at δ 3.63, 3.79 and 3.95 (each 3H, s) occurred, and predictably located them at the biphenyl rings. In the cyclooctadiene ring, two secondary methyl groups at δ 0.93 (3H, d, J=7.5 Hz) and 1.16 (3H, d, J=6.9 Hz) were assigned to CH_3-17 and CH_3-18, respectively. Moreover, the signal of oxygenated methine at δ 83.9, 4.61 (1H, d, J=11.7 Hz, C-9 and H-9).

The detailed analysis of **1** using ^1H–^1H 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 suggested the substitution of cyclooctadiene ring.

Detailed analysis of HMBC spectrum showed the correlations between the protons of methylenedioxy moiety CH_2-19 at δ 5.97 (2H, s) 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-3 with the carbon at C-2 and 4. These facts affirmed the planar structure of **1** showed in Fig. 1.

The relative configuration at C-7, C-8 and C-9 were determined by the difference NOE. The irradiation of H-11 shows NOE with H-9β; the irradiations of H-4 shows NOE with H-6α, indicating the hydroxyl group in C-9 were located at the 9α. Moreover the NOE between H-4 and OH-3 affirmed the position of aryl hydroxyl at C-3. Like the other cyclooctadiene,[13)] **1** has a twist-boat conformation due to the correlated peaks between H-4 and CH_3-17; H-11 and H-8 (Fig. 2).

Thus, the structure of **1** was assigned as shown in Fig. 2.

Kadsuralignan B (**2**) was obtained as colorless needles and assigned to possess a molecular formula of $C_{27}H_{32}O_{11}$ by HR-EI-MS ($[M]^+$, *m*/*z* 532.1938) with twelve unsaturation, and the IR spectrum with bands at 3400 (OH), 1720 (ester), 1610 and 1590 (aromatic) cm^{-1}. Like **1**, **2** also possessed a biphenyl moiety due to two aromatic protons at δ 6.74, 6.45 (H-4 and H-11, respectively) and twelve carbon atoms at δ 151.1, 140.6, 151.8, 110.3, 130.0, and 121.8 (C-1, 2, 3, 4, 5,

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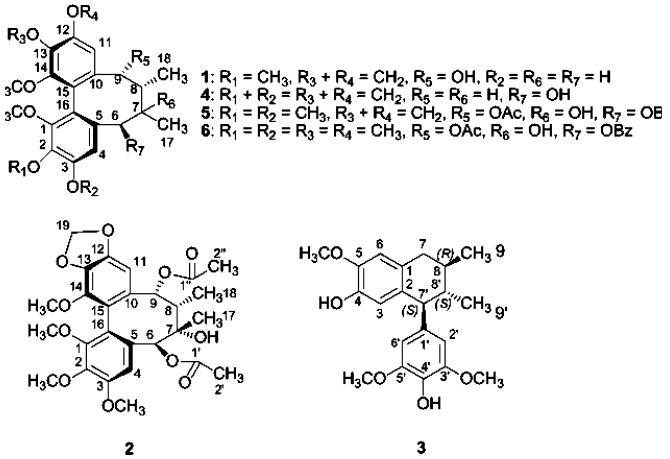

Fig. 1. Structure of Compounds **1**—**6**

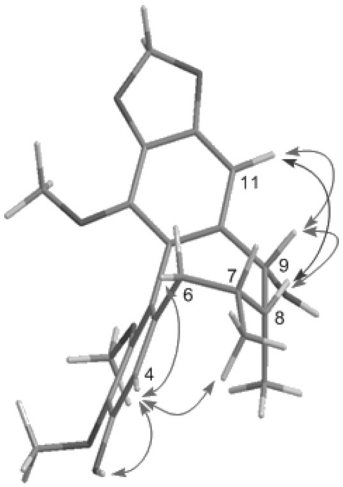

Fig. 2. Key NOEs of **1**

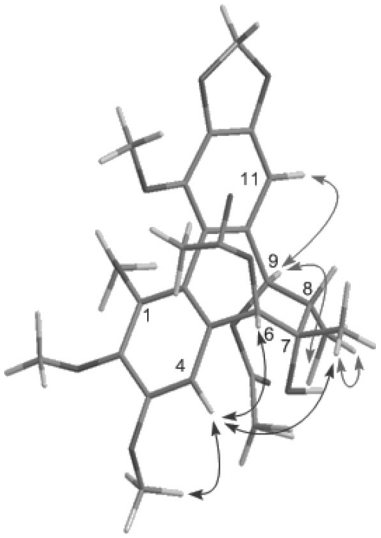

Fig. 3. Key NOEs of **2**

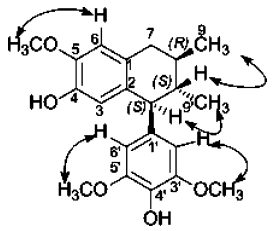

Fig. 4. Key NOEs of **3**

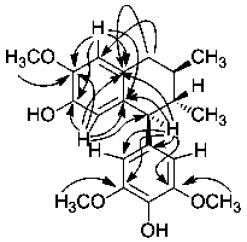

Fig. 5. Partial HMBC Correlations of **3**

16, respectively); δ 132.9, 102.0, 148.6, 135.4, 141.1 and 120.4 (C-10, 11, 12, 13, 14, 15, respectively). And **2** also have a methylenedioxy moiety at δ 5.94 and 5.96 (each 1H, d, J=1.4 Hz) and four methoxyl groups at δ 3.62, 3.85, 3.91 and 3.88 (each 3H, s) occurred, and predictably they were located at the biphenyl rings. In the cyclooctadiene ring, one secondary methyl group at δ 1.23, (3H d, J=9.0 Hz) and one singlet methyl group at δ 1.30 (3H, s), were assigned to CH_3-18 and CH_3-17, respectively, together with an oxygenated carbon at δ 73.9 was assignable to C-7. Moreover, the signals of two acetyl groups were found in the NMR spectrum. Detailed inspection of the HMBC spectrum, the couplings between H-6 and C-1′ (δ 168.8), and between H-9 and C-1″ (δ 169.3) were found and further suggested that two ester groups were present at C-6 and C-9, respectively. Thus, the structure of **2** was similar to the known schizanrin **F** (**5**)[15] except for a benzoate at C-6 in **5** replaced by an acetyl groups. Moreover, the mass spectrum of **2** showed two intense peaks at *m*/*z* 472 [M^+-CH_3COOH] and *m*/*z* 412 [$M^+-CH_3COOH-CH_3COOH$], both of them reflected the elimination of acetic acid. The NOESY spectrum of **2** showed correlations between H-4 and H-6α, and between H-11 and H-9β, indicating that the acetyl groups were located at the 6β and 9α position, respectively. Moreover, like the other cyclooctadiene lignans,[16,17] **2** has a twist-boat-chair conformation due to the correlated peaks between CH_3-17 and CH_3-18, H-9β and CH_3-18, and CH_3-17 and H-6α (Fig. 3).

Kadsuralignan C (**3**) was obtained as colorless needles and assigned to possess a molecular formula of $C_{21}H_{26}O_5$ by HR-EI-MS ([M]$^+$, *m*/*z* 358.1781) with nine degrees of unsaturation. Detailed analysis of the NMR and DEPT spectra showed that **3** contained three methoxyl groups, one methylene, seven methines and eight quaternary carbons. The IR spectrum showed a wide absorption band at 3400 cm^{-1} for hydroxyl group.

The ^{1}H-NMR of **3** accounted for the presence of four aromatic hydrogens by three singlets, two of them at δ 6.55 and 6.39 (H-6, H-3, respectively), and both of them have cross peaks with δ 127.5, 132.8, 143.4 and 144.9 (C-1, 2, 4, 5) in the HMBC spectrum (Fig. 5), suggested that they are in the same phenyl ring; while the third singlet at δ 6.24 (2H, s), integrating for two hydrogens, representing the methine protons of another phenyl ring, was evident. It was also proved by the cross peaks with the carbon at δ 130.7, 105.9, 105.9, 146.6, 146.6 and 138.0 (C-1′, 2′, 6′, 3′, 5′, 4′, respectively)

Table 1. ^{1}H- and ^{13}C-NMR Data for Compounds **1**—**3** in $CDCl_3$[a)]

No.	**1**		**2**		No.	**3**	
	δ_C	δ_H (mult; *J*, Hz)	δ_C	δ_H (mult; *J*, Hz)		δ_C	δ_H (mult; *J*, Hz)
1	150.6		151.1		1	127.5	
2	138.3		140.6		2	132.8	
3	148.5		151.8		3	115.9	6.39 s
4	113.5	6.67 s	110.3	6.74 s	4	143.4	
5	133.7		130.0		5	144.9	
6	38.3	2.59 m	84.4	5.65 s	6	110.5	6.55 s
7	34.7	2.07 m	73.7		7	35.4	2.44 (dd, 16.3, 5.3)
							2.90 (dd, 16.3, 5.3)
8	43.0	1.91 m	42.6	2.08 (dd, 11, 5)	8	40.5	1.95 m
9	83.9	4.61 (d, 11.7)	83.4	5.66 s	9	15.5	0.91 (d, 7)
10	138.2		132.9		1′	130.7	
11	102.5	6.34 s	102.0	6.45 s	2′	105.9	6.24 s
12	148.7		148.6		3′	146.6	
13	135.7		135.4		4′	138.0	
14	141.1		141.1		5′	146.6	
15	119.2		120.4		6′	105.9	6.24 s
16	121.1		121.8		7′	50.7	3.54 m
17	15.1	0.93 (d, 7.5)	28.5	1.30 s	8′	29.6	2.05 m
18	19.7	1.16 (d, 6.9)	16.8	1.23 (d, 9)	9′	16.0	0.87 (d, 7)
19	101.1	5.97 s	101.0	5.94 (d, 1.4)			
				5.96 (d, 1.4)			
1′			168.8				
2′			20.5	1.58 s			
1″			169.3				
2″			20.1	1.62 s			
1-OCH_3	60.1	3.63 s	60.3	3.62 s			
2-OCH_3	61.0	3.95 s	59.3	3.85 s	5-OCH_3	55.7	3.85 s
3-OCH_3			56.0	3.91 s	3′-OCH_3	56.3	3.79 s
14-OCH_3	59.7	3.79 s	60.5	3.88 s	5′-OCH_3	56.3	3.79 s
3-OH		5.76 s					

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

in the HMBC spectrum.

The $^{3}J_{CH}$ correlations obtained from the HMBC experiment provided sound evidence for the structure of **3** as having the basic arylnaphthalene framework, with one methoxyl groups at C-5, and the other methoxyl groups at 3′ and 5′ of ring C.

The detailed analysis of **3** using ^{1}H–^{1}H COSY and HMQC disclosed partial structure unit with correlated protons: CH_2–$CH(CH_3)$–$CH(CH_3)$–CH. Thus, the unit can be assigned to the partial structure of ring B.

Due to its chemical shift of δ 143.4, C-4 was an oxygenated quaternary carbon, thus pointing at the position of hydroxyl (OH-4) substitution; meanwhile, H-2′ and 6′ have cross peaks with another hydroxyl quaternary carbon in ring C, which only can be assigned as C-4′.

The ^{1}H, ^{13}C, ^{1}H–^{1}H COSY, HMQC and HMBC spectra supported the planar structure of **3**. The relative configurations at C-8, C-8′ and C-7 were determined by the difference NOE. The irradiation of H-7′ showed NOE with CH_3-9′; the irradiation of H-8′ showed NOE with CH_3-9. These results indicated that the phenyl and the two methyl groups are in *trans–trans* relationship to one another.[18)] In addition, a negative CD cotton effect at 288 nm let us conclude that **3** is a (7′*S*,8′*S*,8*R*)-aryl-tetralin derivative.[19)] Thus, the structure of **3** was assigned as shown in Fig. 4.

Compounds **4**,[20)] **5**,[15)] and **6**,[15)] were known compounds, whose structure were elucidated by comparison of their spectral data with those of the literature.

Table 2. Inhibitory Effects on NO Production of Compounds **1**—**6**

Compound	IC_{50} (μM)
1	137.0
2	61.0
3	21.2
4	50.7
5	>200
6	97

Compounds **1**—**6** 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 **3** (IC_{50}=21.2 μM) showed somewhat stronger inhibition than quercetin (IC_{50}=24.8 μM). Quercetin is reported to have an inhibitory effect on the production of NO by LPS stimulated macrophage cell RAW 264.7.[21,22)]

Recently, Rao and coworkers have reported that some arylnaphthalene type lignans significantly inhibited LPS-induced NO production.[23)] Which is well in agreement with our findings that **3** showed inhibition effect on NO production.

Experimental

General Procedures The UV spectra were obtained in MeOH on a Shimadzu UV-160 spectrophotometer, and the IR spectra were recorded on a JASCO IR A-2 spectrophotometer. Optical rotations were measured in MeOH on a JASCO DIP-360 polar meter. The NMR spectra were recorded on a JEOL-ECA600 MHz spectrometer, with TMS as an internal standard.

The MS data were obtained on a JEOL GC mate spectrometer. Column chromatographies were conducted with a silica gel column (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 instrument equipped with a JASCO UV-2075 detector. A Senshu Pak PEGASIL silica 60-5 (10×250 mm i.d.) column 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), respectively. 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 (13 ϕ×65 cm, eluted with $CHCl_3$ and MeOH 95 : 5→70 : 30). The column chromatographic fractions (500 ml each) were combined according to TLC monitoring into eleven portions. Fraction 10 was subjected to silica gel column chromatography (3 ϕ×21 cm, 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 four was isolated and further purified by HPLC (Fluofix 120N, 10 ϕ×250 mm, $CH_3CN : H_2O$, 70 : 30) to give **1** (5 mg), **2** (14 mg) and **5** (6 mg). Portion five, was subjected to silica gel column chromatography (3 ϕ×21 cm, eluted with $CHCl_3$: MeOH 95 : 5→70 : 30). The column chromatographic fractions (100 ml each) were combined according to TLC monitoring into six portions. Portion four was isolated and further purified by HPLC (Fluofix 120N, 10 ϕ×250 mm, $CH_3CN : H_2O$, 80 : 20) to give **3** (9 mg), **4** (12 mg) and **6** (7 mg).

Compound **1**: Colorless needles from hexane; mp 157—158 °C; 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 (80), 208 (65), 188 (55); 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.

Compound **2**: Colorless needles from hexane; mp 140—142 °C; UV (MeOH) λ_{max} nm (log ε): 230 (3.88), 255 (sh), 280 (sh); EI-MS *m/z* (rel. int. %) : 532 $[M]^+$ (35), 472 (100), 412 (40), 358 (65), 328 (55); HR-EI-MS *m/z*: 532.1938 ($[M]^+$, Calcd 532.1944 for $C_{27}H_{32}O_{11}$); ^{1}H- and ^{13}C-NMR: see Table 1.

Compound **3**: Colorless needles from hexane; mp 209—210 °C; UV (MeOH) λ_{max} nm (log ε): 215 (4.00), 240 (sh), 288 (3.50); IR (KBr) cm^{-1}: 3400 (OH), 1605, 1511, 1459, 1372, 1259; EI-MS *m/z* (rel. int. %): 358 $[M]^+$ (100), 271 (80), 204 (40), 151 (30), 135 (28); HR-EI-MS *m/z*: 358.1781 ($[M]^+$, Calcd 358.1780 for $C_{21}H_{26}O_5$); ^{1}H- and ^{13}C-NMR: see Table 1.

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-well 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 coli* 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 microliters 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-naphthyletyl-enediamide 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.

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