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Cyclic diarylheptanoids from *Myrica nana* inhibiting nitric oxide release

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ABSTRACT

Investigation of the roots of *Myrica nana* afforded five new cyclic diarylheptanoids, myricananins A–E (**1–5**), two new artifacts of myricananins A and B (**6–7**), and four known compounds, 12-hydroxymyricanone (**8**), alnusonol (**9**), myricatomentogenin (**10**), and actinidione (**11**). The structures of these new compounds were established by detailed spectroscopic methods. The stereochemistry of compounds **1** and **2** were determined by single-crystal X-ray diffraction. In exception of compounds **2**, **6** and **10**, all the other compounds were examined for their inhibitory effects on nitric oxide production in lipopolysaccharides-activated macrophages. Compounds **1**, **3**, **7**, **8** and **9** inhibited the release of nitric oxide with IC_{50} values of 45.32, 63.51, 52.81, 30.19 and 46.18 μM, respectively. Furthermore, compound **1** was found to inhibit the expression of inducible nitric oxide synthase.

1. Introduction

The family Myricaceae contains two genera and about 50 species in total. Besides *Comptonia peregrines* as the only plant in the genus *Comptonia* is native to North America, most of the *Myrica* plants are widely distributed in tropical, subtropical and temperate zones of the earth.[1] *Myrica nana* Cheval, shrub evergreen, is a species endemic to China, and mainly grown in the Provinces of Guizhou and Yunnan.[1] The roots and barks of this plant are commonly used for the treatment of diarrhea and burns in traditional Chinese medicine.[2] Previously we reported the isolation and characterization of five diarylheptanoids from the roots of this plant,[3] however, more minor diarylheptanoidal derivatives were not available. Diaryheptanoids are a class of natural products typical of 1,7-diphenylheptane, found in the families of Myricaceae, Betulaceae, Zingiberaceae, Aceraceae, Dioscoreae,[4] Juglandaceae,[5] Rhoipteleaceae,[6] and Burseraceae.[7,8] They are known to possess a broad biological activities.[4] In our recent efforts to seek bioactive constituents from traditional Chinese medicine against inflammation, we reinvestigated this plant by a chemical screening strategy, and five new cyclic diarylheptanoids were isolated in this study. Herein, we report the isolation, structural elucidation of these compounds and their inhibitory effects against nitric oxide production.

1.1. Structures of myricananins A–E (1–5)

The roots of *M. nana* were extracted with 80% EtOH, and the extract was sequentially partitioned with EtOAc and *n*-BuOH. The EtOAc soluble extract was chromatographed on silica gel, Sephadex LH-20 and Rp-18 to yield myricananins A–E (**1–5**), acetonides of myricananins A and B (**6** and **7**), together with 12-hydroxymyricanone,[9] alnusonol,[10] myricatomentogenin,[11] and actinidione.[12]

Myricananin A (**1**) was obtained as colorless needles. Its molecular formula $C_{20}H_{24}O_5$ was established on the basis of HRESIMS. The ^1H and ^{13}C NMR spectra of **1** (Tables 1 and 2, respectively) were similar to those of 12-hydroxymyricanon[9] and alnusonol,[10] implying **1** to be a cyclic diarylheptanoid analogue. The ^1H NMR spectrum of **1** displayed three aromatic signals at δ 6.82, 7.02, and 7.06 assignable to a typical ABX system, in addition, two singlets at δ 6.78 (H-5) and 6.72 (H-19) in the olefinic region were also observed. ^1H–^1H COSY experiment disclosed the presence of H-7 to H-13. The chemical shifts at δ 69.1 and 70.4 indicated that C-11 and C-12 were, respectively, oxygenated. The substitution pattern of two phenyl groups and the linkage of aliphatic chain with benzene rings were assembled by HMBC interactions. The HMBC spectrum of **1** (Fig. 1) showed the following key correlations: H-5 with

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Table 1
^{1}H NMR data for compounds **1**–**5**[a]

No.	**1**[b]	**2**[b]	**3**[c]	**4**[d]	**5**[c]
5	6.78 (s)		6.67 (s)		
7	2.90 (m), 2.49 (m)	2.74 (m), 2.51 (m)	2.78 (m)	2.58 (m)	2.70 (m), 2.64 (m)
8	1.97 (m), 1.85 (m)	1.90 (m)	1.92 (m)	1.82 (m)	2.14 (m), 1.61 (m)
9	1.69 (m), 1.45 (m)	1.64 (m), 1.46 (m)	1.86 (m)	1.59 (m)	1.99 (m), 1.75 (m)
10	2.26 (m), 1.37 (m)	2.27 (m), 1.36 (m)	2.78 (overlap)	2.84 (m)	2.79 (m)
11	4.05 (m)	4.02 (m)			
12	4.24 (m)	4.27 (m)	2.78 (overlap)	5.86 (d, 13.0)	4.79 (m)
13	2.96 (m)	2.97 (m), 2.85 (m)	3.03 (m)	6.52 (d, 13.0)	3.08 (m), 2.88 (m)
15	7.02 (dd, 8.1, 1.7)	7.11 (dd, 7.9, 1.8)	7.06 (dd, 8.2, 2.0)	6.75 (d, 2.2)	7.02 (d, 2.0)
16	6.82 (d, 8.1)	6.80 (d, 7.9)	6.89 (d, 8.2)		
18	7.06 (d, 1.7)	7.03 (d, 1.8)	6.75 (d, 2.0)	6.31 (d, 2.2)	6.27 (d, 2.0)
19	6.72 (s)	6.78 (s)	6.55 (s)	6.44 (s)	6.57 (s)
OMe-3		3.88 (s)		3.76 (s)	3.99 (s)
OMe-4	3.86 (s)	3.89 (s)	3.91 (s)	3.79 (s)	3.83 (s)
OMe-12					3.49 (s)

[a] ^{1}H NMR data of **1** at 500 MHz, of **2**–**4**, and **5** at 400 MHz.
[b] Measured in acetone-d_6.
[c] Measured in $CDCl_3$.
[d] Measured in DMSO-d_6.

Table 2
^{13}C NMR data for compounds **1**–**5**[a]

No.	**1**[b]	**2**[b]	**3**[c]	**4**[c]	**5**[c]
1	126.3	123.3	124.1	121.8	122.3
2	126.6	123.7	125.4	125.8	125.7
3	140.9	147.6	138.9	145.7	145.8
4	148.8	140.3	146.4	138.7	138.6
5	112.1	149.9	110.2	148.5	147.9
6	131.8	130.3	131.9	123.0	123.4
7	30.9	26.2	32.0	27.0	27.2
8	27.3	26.5	25.4	20.4	24.5
9	23.2	23.3	21.7	23.7	21.6
10	35.6	35.4	45.8	43.4	47.4
11	69.1	69.0	213.3	209.1	211.3
12	70.4	70.4	42.3	128.6	79.1
13	36.8	36.9	28.6	132.4	48.8
14	130.5	126.1	132.2	127.3	134.4
15	130.3	130.3	129.0	114.8	111.2
16	117.2	117.1	117.1	141.0	145.8
17	152.8	152.6	151.8	145.7	139.4
18	134.7	134.4	132.2	124.9	121.1
19	126.5	130.0	125.5	129.4	128.6
OMe-3		61.5		61.7	61.4
OMe-4	56.6	61.4	56.2	61.4	61.7
OMe-12					57.3

[a] Assignments based on HMQC and HMBC correlations; ^{13}C NMR data of **1** at 125 MHz, of **2**–**4**, and **5** at 100 MHz.
[b] In acetone-d_6.
[c] In $CDCl_3$.

Table 3
^{1}H and ^{13}C NMR data for compounds **6** and **7**[a]

No.	**6**[b]		**7**[c]	
	^{1}H	^{13}C	^{1}H	^{13}C
1		125.9		122.8
2		127.7		126.1
3		141.2		146.0
4		148.9		138.7
5	6.84 (s)	112.0		147.8
6		132.6		123.5
7	2.89 (m), 2.64 (m)	33.2	2.87 (m), 2.66 (m)	27.8
8	2.04 (m), 1.34 (m)	27.4	2.21 (s), 1.47 (m)	24.5
9	2.04 (m), 1.44 (m)	26.2	2.20 (m), 1.63 (m)	26.7
10	2.04 (m), 1.85 (m)	39.3	2.08 (m), 1.97 (m)	38.5
11	4.17 (m)	85.5	4.18 (m)	85.0
12	3.85 (m)	83.0	3.99 (m)	81.9
13	2.98 (m)	41.3	3.11 (m), 3.01 (m)	40.7
14		129.6		128.9
15	7.11 (dd, 8.2, 2.2)	131.1	7.12 (dd, 8.2, 2.2)	130.6
16	6.82 (d, 8.2)	117.3	6.93 (d, 8.2)	116.9
17		153.8		152.4
18	6.94 (d, 2.2)	135.3	6.95 (d, 2.2)	133.9
19	6.60 (s)	126.5	6.75 (s)	129.3
OMe	3.87 (s)	56.6	4.16 (s)	61.5
OMe			3.89 (s)	61.4
20		108.3		107.9
Me	1.34 (s)	27.4	1.47 (s)	27.0
Me	1.44 (s)	27.0	1.47 (s)	26.6

[a] ^{1}H NMR data of **6** at 400 MHz, ^{13}C NMR data of **6** at 125 MHz. ^{1}H NMR data of **7** at 400 MHz, ^{13}C NMR data of **7** at 100 MHz.
[b] Measured in acetone-d_6.
[c] Measured in $CDCl_3$.

C-3, C-4, C-7 and C-19, H-19 with C-1, C-2, C-3, C-5 and C-7, OMe with C-4, H-7 with C-5, C-6 and C-19, H-13 with C-14, C-15 and C-18, H-18 with C-1 and C-2. This evidence suggested the structure of **1** as shown. Due to the flexibility of side chain, the stereochemistry of two chiral centers in **1** was eventually determined by X-ray crystallographic analysis (Fig. 2). The X-ray structure of **1** apparently displayed the twisting of two phenyl moieties with torsion angle 141.3° of C-1-C-17-C-2-C-19 and the orientations of two hydroxyl groups attached at C-11 and C-12. Consequently, the structure of **1** was assigned as 17-methoxytricyclo[12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaen-3,8β,9β,17-tetraol, with trivial name myricananin A. It was noted that compound **6** was a new artifact of **1** produced during isolation procedure by HPLC evidence.

Myricananin B (**2**), colorless crystals, had the molecular formula $C_{21}H_{26}O_6$ and it was derived from the HRESIMS. The ^{13}C NMR spectrum of **2** (Table 2) was similar to that of **1** and suggested **2** to be a cyclic diarylheptanoid. On inspection of HMBC interactions (Fig. 1), it was found that the main difference between **1** and **2** occurred at one phenyl group. HMBC correlations of H-7 with C-5, C-6 and C-19, H-19 with C-1, C-2 and C-3, and two OMe groups at δ 3.88 and 3.89 with C-3 and C-4, respectively, indicated a 5-hydroxy-3,4-dimethoxyl substitution pattern in **2**. It was noted that the coupling of H-11 and H-12 was not observed in the ^{1}H-^{1}H COSY spectrum, this may be due to the dihedral angle of H-C-11 and H-C-12 which approached 90°. After many efforts in different solvents, a single crystal of **2** was obtained from anhydrous ethanol, the relative position of two phenyl groups and the stereochemistry of two hydroxyls at C-11 and C-12 were thus determined by X-ray diffraction as shown (Fig. 2). Hence, the structure of **2** was assigned as 16,17-dimethoxytricyclo[12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaen-3,8 β,9 β,15-tetraol, with trivial name myricananin B.

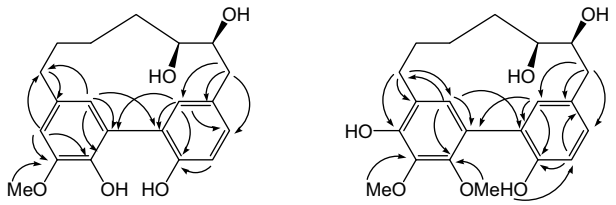


Figure 1. Selected HMBC correlations of compounds **1** and **2**.

Note that compound **7**, a new acetonide of **2**, was also isolated in this study.

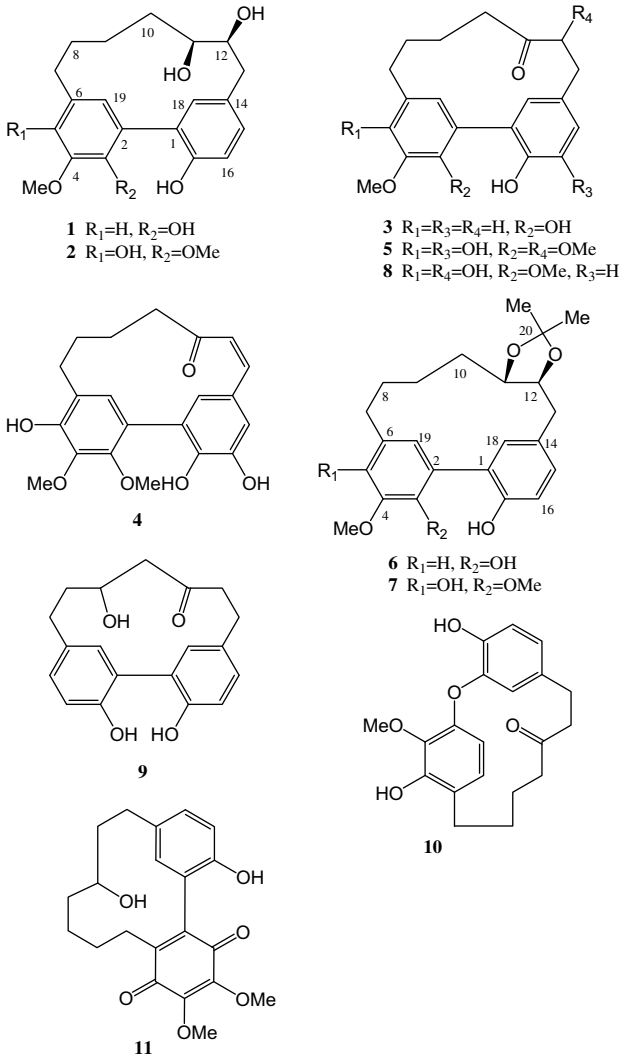
Myricananin C (**3**) was obtained as colorless crystals, with the molecular formula of $C_{20}H_{22}O_4$ deduced from its HRESIMS. The similarity of ^1H and ^{13}C NMR spectra of **3** (Tables 1 and 2, respectively) with **1** suggested that they are analogues. The key difference was at aliphatic chain, ^1H-^1H COSY of **3** showed two spin systems from H-7 to H-10, and from H-12 to H-13. HMBC correlations of H-9, H-10 and H-12 all with C-11 at δ 213.3 suggested an 11-oxo functionality. Hence, the structure of **3** was identified as 3,17-dihydroxy-16-methoxytricyclo[12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaen-9-one, with trivial name myricananin C.

Myricananin D (**4**) was obtained as amorphous white powders. The HRESIMS of **4** suggested that the molecular formula is $C_{21}H_{22}O_6$ with 11 degrees of unsaturation. The resemblance of ^{13}C NMR data of **4** (Table 2) with those of 12-hydroxymyricanon[9] indicated that they bear the same skeleton. However, some unexpected troubles occurred in the ^1H NMR measurements. Whatever in $CDCl_3$ (291 and 323 K) and DMSO-d_6 (291 K), the aliphatic ^1H spectral signals appeared quite broad and unresolved. Acceptable line widths were finally realized upon recording in DMSO-d_6 at 323 K. The reasons for these phenomena remained still unknown. The ^1H–^1H COSY spectrum of **4** (in DMSO-d_6) displayed two fragments of H-7 to H-10 and H-12 to H-13. The substitution pattern of two benzene rings was established by the following HMBC correlations: H-19 with C-1, C-2, C-3, C-5, C-6 and C-7, H-18 with C-1, C-2, C-13, C-15 and C-17, OH at δ 8.20 in $CDCl_3$ (δ 8.24 in DMSO-d_6) with C-1, C-16 and C-17, OH at δ 6.02 in $CDCl_3$ with C-4, C-5 and C-6,. Further HMBC interactions of H-12 (δ 5.86) with C-11 (δ 209.1), C-13 (δ 132.4) and C-14 confirmed the presence of a conjugated ketone in the side chain. The configuration of the double bond was assigned as *Z* form according to the large coupling constant of 13.0 Hz. As a result of this, the structure of **4** was determined to be 3,4,15-trihydroxy-16,17-dimethoxytricyclo[12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,7,14,16-heptaen-9-one,with trivial name myricananin D.

Myricananin E (**5**) was isolated as amorphous white powders, its molecular formula was determined as $C_{22}H_{26}O_7$ by HRESIMS. The ^1H and ^{13}C NMR data (Tables 1 and 2, respectively) of **5** were closely related to those of **4**, implying that they share the same backbone. Interpretation of 2 D NMR spectra of **5** suggested that compounds **4** and **5** had the same substitution pattern in the benzene rings, the main difference was that C-12-C-13 double bond of **4** was replaced by a OMe group (δ 57.3) attached at C-12 in **5**. The HMBC correlations of OMe with C-12, H-12 with C-11 and C-14 provided further evidence for substitution pattern on the side chain. The preferential conformation of **5** in chloroform was determined by the observed ROESY interactions, which were H-19 with H-8 and H-9, H-18 with H-12 and H-13. However, the absolute configuration at C-12 remained still unknown. Therefore, the structure of **5** was assigned as 3,4,15-trihydroxy-8,16,17-trimethoxytricyclo[12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaen-9-one, with trivial name myricananin E.

1.2. Nitric oxide release inhibition

Considering the roots of this plant have been used for the treatment of burns, compounds **1**, **3–5**, **7–9**, and **11** were therefore examined for their inhibitory effects on nitric oxide production in lipopolysaccharides-activated mouse macrophages, with *N*-monomethyl-L-arginine (L-NMMA) as a positive control, and the results were summarized in Table 4. The results showed that compounds **1**, **3**, **7**, **8** and **9** inhibited the production of nitric oxide with IC_{50} values of 45.32, 63.51, 52.81, 30.19 and 46.18 μM, respectively.

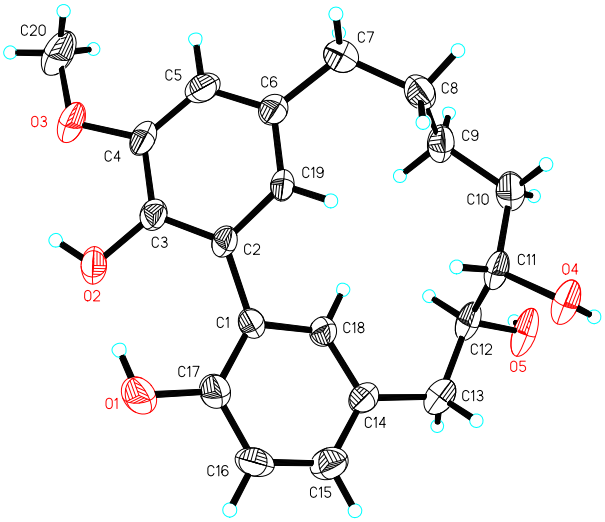


Furthermore, the action of **1** on inducible nitric oxide synthase (iNOS) induction was examined by Western blotting analysis (Fig. 3). It was found that **1** inhibited the iNOS expression in lipopolysaccharides-activated macrophages, which suggested that the mechanism of **1** on nitric oxide production inhibition was mainly related with its inhibitory effect against iNOS induction. These results lend support for the traditional use of this plant as remedies for burns.

2. Experimental

2.1. General methods

Melting points were obtained on an X-4 apparatus and were uncorrected. Optical rotations were measured on a Horiba SEPA-



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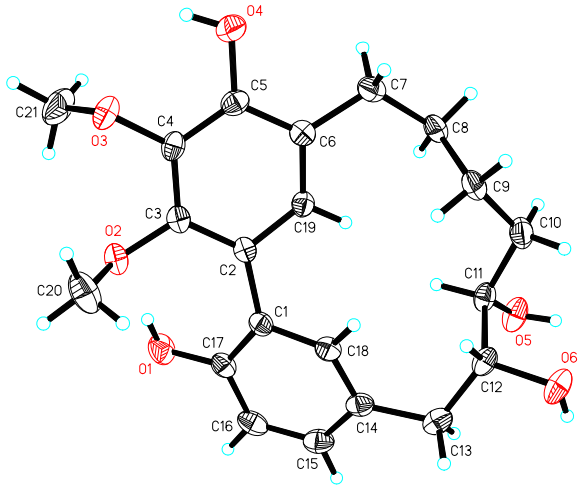

2

Figure 2. X-ray structures of **1** and **2** showing relative configuration.

300 high sensitive polarimeter. UV spectra were recorded on a Shimadzu double-beam 210A spectrometer. IR spectra were recorded on a Bio-Rad FTS-135 spectrometer with KBr pellets. NMR spectra were obtained on a Bruker AM-400 and DRX-500 spectrometer with TMS as an internal standard. HREISMS was measured by a VG Auto Spec 3000 spectrometer. X-ray data were collected on a CCD APEX diffractometer by a method of full-matrix least-squares on F^2.

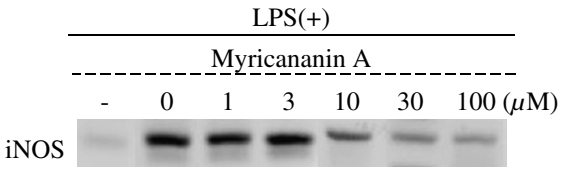

Figure 3. Inhibitory effect of **1** on iNOS induction in LPS-activated macrophages.

2.2. Material

The roots of *M. nana* were collected from Songhua dam, a Kunming suburb of Yunnan Province, China, in May 2007. The species was identified by Prof. Y.M. Shui, Kunming Institute of Botany, and a voucher specimen (CHYX0391-2) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, China.

2.3. Extraction and isolation

The dried and powdered roots of *M. nana* (20 kg) were extracted with 80% EtOH under reflux for 3 × 2 h. Removal of the solvent in vacuo afforded a brown residue, which was suspended in H_2O followed by successive partition with EtOAc and *n*-BuOH. The EtOAc extract (1100 g) was subjected to column chromatography (CC) over silica gel (200–300 mesh) eluting with gradient $CHCl_3$/MeOH (1:0 to 0:1) to afford nine fractions (I–IX). To obtain cyclic diarylheptanoids exclusively, a chemical screening strategy was utilized during isolation procedure to differentiate cyclic diarylheptanoids from triterpenoids based on their UV absorption difference. Fraction III (112 g) was fractionated by CC on silica gel with gradient acetone in petroleum ether (10–100%) to yield six fractions (IIIA–IIIF). Fractions IIIB and IIIE were further chromatographed over Sephadex LH-20 with $CHCl_3$/MeOH (6:4) to yield compounds **3** (12 mg, 0.00006% yield) and **10** (4 mg, 0.00002% yield). Fraction IV (196 g) was separated over silica gel eluting with gradient EtOAc in petroleum ether (20–100%) resulted in seven fractions (IVA–IVG). Fractions IVC and IVF were further purified by CC on Sephadex LH-20 using MeOH as solvent to give compounds **8** (21 mg, 0.000105% yield), **9** (17 mg, 0.000085% yield) and **11** (158 mg, 0.00079% yield). Fraction V (131 g) was fractionated by CC over silica gel with gradient acetone in petroleum ether (10–100%) to yield eight fractions (VA–VH). Fraction VB and VF were subjected to gel filtration over Sephadex LH-20 eluting with MeOH to give compounds **1** (33 mg, 0.000165% yield), **6** (5 mg, 0.000025% yield) and **7** (13 mg, 0.000065% yield). Fraction VI (56 g) was separated by CC over silica gel followed by Rp-18 column with gradient aqueous MeOH (30–80%) to afford compounds **4** (38 mg, 0.00019% yield) and **5** (11 mg, 0.000055% yield). Fraction VII (146 g) was chromatographed over silica gel eluting with gradient EtOAc in petroleum ether

Table 4
Inhibitory effects of compounds **1**, **3**–**5**, **7**–**9**, and **11** on nitric oxide release in LPS-activated peritoneal macrophages

Compound	Inhibition (%)						
	0 (μM)	1 (μM)	3 (μM)	10 (μM)	30 (μM)	100 (μM)	IC_{50} (μM)
1	0.0 ± 10.87	0.84 ± 0.84	−0.13 ± 8.67	35.95 ± 11.74**	50.76 ± 17.4**	84.39 ± 13.34**	45.32
3	0.0 ± 2.49	17.31 ± 8.09	32.82 ± 11.42	39.12 ± 14.46	42.57 ± 7.96**	50.07 ± 14.84**	63.51
4	0.0 ± 4.48	6.12 ± 2.32	17.72 ± 9.12	23.8 ± 6.26**	19.08 ± 6.39**	31.83 ± 6.63**	
5	0.0 ± 2.69	−3.27 ± 5.45	9.22 ± 2.89	15.27 ± 2.94	29.76 ± 7.22**	49.25 ± 8.64**	
7	0.0 ± 12.05	17.3 ± 5.03	32.84 ± 16.2	39.16 ± 13.88**	49.67 ± 12.03**	55.82 ± 8.54**	52.81
8	0.0 ± 5.09	35.07 ± 1.36**	48.7 ± 4.44**	41.83 ± 8.77**	55.66 ± 21.36**	65.53 ± 16.04**	30.19
9	0.0 ± 9.02	−3.39 ± 8.15	34.82 ± 2.95**	45.83 ± 14.31**	41.15 ± 23.89**	54.54 ± 24.07**	46.18
11	0.0 ± 4.93	9.52 ± 5.17	21.08 ± 3.44**	22.92 ± 9.93**	31.43 ± 18.74**	38.72 ± 17.47**	
L-NMMA	0.0 ± 2.55	7.0 ± 4.92	11.87 ± 2.19	17.64 ± 1.35**	45.73 ± 10.04**	69.9 ± 3.61**	64.24

Each value represents the mean ± SEM (n = 6). Significantly different from control, $^{**}p < 0.01$.

(20–100%) resulted in six fractions (VIIA–VIIF). Fraction VIIC was further purified by Rp-18 column with gradient aqueous MeOH (30–80%) to give compound **2** (5 mg, 0.000025% yield).

2.3.1. Myricananin A (1)

Colorless needles (MeOH); mp 161–162; $[\alpha]_D^{20}$ +28.3 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 295 (4.94) ,258 (4.12), 213 (4.65) nm; IR (KBr): ν_{max} 3440, 3425, 2934, 1628, 1508, 1414, 1243 cm^{-1}; ^{1}H and ^{13}C NMR data (Tables 1 and 2); ESIMS *m/z* 344 $[M]^+$; HRESIMS *m/z* 367.1523 $[M+Na]^+$ (calcd for $C_{20}H_{24}O_5Na$, 367.1521).

2.3.2. Myricananin B (2)

Colorless crystals (anhydrous EtOH); mp 210–212; $[\alpha]_D^{26}$ −41.7 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 297 (3.98), 258 (4.15), 212 (4.69), 195 (4.45) nm; IR (KBr) ν_{max} 3423, 2935, 1705, 1616, 1496, 1454, 1408, 1347, 1229, 1043 cm^{-1}; ^{1}H and ^{13}C NMR data (Tables 1 and 2); ESIMS *m/z* 374 $[M]^+$; HRESIMS *m/z* 397.1616 $[M+Na]^+$ (calcd for $C_{21}H_{26}O_6Na$, 397.1627).

2.3.3. Myricananin C (3)

Colorless crystals (MeOH); mp 192–193; $[\alpha]_D^{26}$ +31.7 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 298 (4.02), 250 (4.17), 214 (4.67) nm; IR (KBr) ν_{max} 3422, 2920, 1703, 1626, 1502, 1414, 1234, 1100 cm^{-1}; ^{1}H and ^{13}C NMR data (Tables 1 and 2); ESIMS *m/z* 326 $[M]^+$; HRESIMS *m/z* 349.1402 $[M+Na]^+$ (calcd for $C_{20}H_{22}O_4Na$, 349.1415).

2.3.4. Myricananin D (4)

Amorphous powders; mp 107–110; $[\alpha]_D^{26}$ +61.7 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 257 (4.66) , 221 (4.86), 196 (4.74) nm; IR (KBr) ν_{max} 3440, 2941, 1666, 1496, 1456, 1410, 1082 cm^{-1}; ^{1}H and ^{13}C NMR data (Tables 1 and 2); ESIMS *m/z* 370 $[M]^+$; HRESIMS *m/z* 371.1508 $[M+H]^+$ (calcd for $C_{21}H_{23}O_6$, 371.1494).

2.3.5. Myricananin E (5)

Amorphous powders; mp 129–131; $[\alpha]_D^{26}$ +45.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 259(4.19), 217(4.65) nm; IR (KBr) ν_{max} 3439, 2939, 1702, 1620, 1498, 1456, 1410, 1083 cm^{-1}; ^{1}H and ^{13}C NMR data (Tables 1 and 2); ESIMS *m/z* 402 $[M]^+$; HRESIMS *m/z* 425.1563 $[M+Na]^+$ (calcd for $C_{22}H_{26}O_7Na$, 425.1576).

2.3.6. Acetonide of myricananin A (6)

White powders; mp 114–116; $[\alpha]_D^{26}$ +10.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 293 (3.88), 255 (4.04), 211 (4.60), 195 (4.35), 192 (4.35) nm; IR (KBr) ν_{max} 3441, 3424, 2936, 1628, 1502, 1415, 1253, 1059, 1046 cm^{-1}; ^{1}H and ^{13}C NMR data (Table 3); ESIMS *m/z* 384 $[M]^+$; HRESIMS *m/z* 407.1837 $[M+Na]^+$ (calcd for $C_{23}H_{28}O_5Na$, 407.1834.

2.3.7. Acetonide of myricananin B (7)

White powders; mp 285–288; $[\alpha]_D^{26}$ −45.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 297 (4.05), 213 (4.79), 205 (4.80) nm; IR (KBr) ν_{max} 3448, 2935, 1616, 1495, 1259, 1203, 1061, 1039 cm^{-1}; ^{1}H and ^{13}C NMR data (Table 3); ESIMS *m/z* 414 $[M]^+$; HRESIMS *m/z* 437.1929 $[M+Na]^+$ (calcd for $C_{24}H_{30}O_6Na$, 437.1940.

2.4. X-ray crystallographic analysis

2.4.1. X-ray data of myricananin A (1)

Colorless prisms, crystal size, 0.393 × 0.143 × 0.051 mm; chemical formula, $C_{20}H_{24}O_5$; formula weight, 344; crystal system, monoclinic; space group, *P*2(1); unit cell dimensions, *a* = 11.1513(15) Å, *b* = 7.7807(11) Å, *c* = 22.470(3) Å; $\alpha = 90^o$, $\beta = 94.325(3)^o$, $\gamma = 90^o$, volume, 1944.1(5) Å^3; *Z* = 4; density, 1.286 mg cm^{-3}; absorption coefficient, 0.093 mm^{-1}; *F*(000) = 808.0; diffractometer used, CCD APEX diffractometer; radiation (λ) MoKα (0.71073) Å; reflections collected/unique, 10,384/3899 [$R_{(int)}$ = 0.0616; final *R* indices, *R* = 0.0465; wR_2 = 0.0763; good of fitness, *S* = 0.864; *T* = 293 K. Refinement method, full-matrix least-squares on F^2 (Crystallographic data for the structure **1** have been deposited at the Cambridge Crystallographic Data Centre, with Deposition No. CCDC 694116).

2.4.2. X-ray data of myricananin B (2)

Colorless prisms, crystal size, 0.422 × 0.347 × 0.251 mm; chemical formula, $C_{21}H_{26}O_6$; formula weight, 374; crystal system, orthorhombic; space group, *P*2(1)2(1)2(1); unit cell dimensions, *a* = 5.9174(5) Å, *b* = 18.9612(18) Å, *c* = 19.4293(18) Å; $\alpha = 90^o$, $\beta = 90^o$, $\gamma = 90^o$, volume, 2180(3) Å^3; *Z* = 4; density, 1.281 mg cm^{-3}; absorption coefficient, 0.094 mm^{-1}; *F*(000) = 904; diffractometer used, CCD APEX diffractometer; radiation (λ) MoKα (0.71073) Å; reflections number, 13,003; final *R* indices, *R* = 0.0430; wR_2 = 0.0967; good of fitness, *S* = 0.959; *T* = 293 K. Refinement method, full-matrix least-squares on F^2 (Crystallographic data for the structure **2** have been deposited at the Cambridge Crystallographic Data Centre, with Deposition No. CCDC 694115).

2.5. Assay for nitric oxide release inhibition

Nitric oxide (NO) release in macrophages stimulated by LPS was evaluated by the method described by Matsuda and co-workers.[13] In brief, 4% thioflycolated medium was injected intraperitoneally into male Balb/c mice. Four days later, peritoneal cells were collected by washing peritoneal cavities with 5 ml of ice-cold phosphate-buffered saline (PBS). The cells 100 μl per well were added to 96-well microplates at a concentration of 5 × 10^5/ml in 1640 medium containing 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were pre-cultured for 2 h at 37 °C with 95% air–5% CO_2. Then, the non-adherent cells were removed by washing with PBS. After washing, the culture medium was exchanged for fresh medium containing 10 μg/ml LPS and different concentrations of the test compounds for 20 h. NO production in the culture medium was determined by Gress assay. The cytotoxicity was assessed by the MTT colorimetric assay. The tested samples were dissolved in DMSO with final concentration of 0.1% in medium. *N*-monomethyl-L-arginine (L-NMMA) was used as a positive control. The inhibitory effect of the compounds was shown in percentage. The percentage inhibition was calculated as follows: percentage inhibition = $(A - B)/(A - C) \times 100\%$; where A was the average value of the control group, *B* was the sample group, *C* was the average value of the blank group.

2.6. Assay for detection of iNOS by Western blotting

The adherent macrophages were collected by the method described above.[13] Briefly, the adherent cells were grown in dish, and incubated in 200 μl of fresh medium supplemented with 10 μg/ml LPS and the test compounds were incubated for 12 h. After washing, the culture medium was exchanged for fresh medium containing 5% FCS, 20 μg/ml LPS and the tested compound for 12 h. Cells were lysed in cell lysis buffer (25 mmol Hepes, pH 7.5, 5 mmol EDTA, 1 mmol EGTA, 5 mmol magnesium chloride, 10 mmol sucrose, 5 mmol dithiothreitol (DTT), 1% 3-[-(3-chloramidopropyl)dimethylammonio]-1-propanesulfonic acid (Chaps), 10 μg/ml pepstatin, 10 μg/ml leupeptin and 1 mmol phenylmethylsulfonyl fluoride (PMSF)). Cells were then centrifuged at 10,000g for 10 min, the supernatants were collected and the protein concentration was measured by the BCA method. For SDS–PAGE, aliquots of 60 μg of protein from sample were subjected to electrophoresis in 8% separated gel, 5% laminose gel for 100 V 2 h. Following electrophoresis, the protein was transfer onto a nitrocellulose membrane, 50 V 1 h. The membrane was

washed by TBS 2 for 10 min with constant agitation. The membrane was blocked by milk in TBS for 1 h at room temperature (rt). Then the membranes were placed to mouse monoclonal IgG (dilution of 1:500) against iNOS at rt overnight. The membrane was washed twice for 5 min each by TBS-T, and washed twice for 5 min by milk at rt with constant agitation. The membrane was incubated with horseradish peroxidase-Ig (dilution of 1:5000) for 1 h at rt. The membrane was washed once for 5 min by TBS-T, then washed thrice for 5 min, each by TBS at rt with constant agitation. Detection was performed using an ECL kit.

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