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Two dimeric lignans with an unusual α,β-unsaturated ketone motif from *Zanthoxylum podocarpum* and their inhibitory effects on nitric oxide production

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ABSTRACT

Two new dimeric lignans, zanthpodocarpins A (**1**) and B (**2**), and five known lignans, eudesmin (**3**), (1*R*,2*R*,5*R*,6*S*)-2-(3,4-dimethoxyphenyl)-6-(3,4-dihydroxyphenyl)-3,7-dioxabicyclo[3.3.0]octane (**4**), dimethoxysamin (**5**), *rel*-(1*R*,5*R*,6*S*)-6-(4-hydroxy-3-methoxyphenyl)-3,7-dioxabicyclo[3.3.0]octan-2-one (**6**), and magnone A (**7**), were isolated from the barks of *Zanthoxylum podocarpum*. Their structures were identified by using spectroscopic methods. Compounds **1** and **2** are rare dilignans bearing an unusual α,β-unsaturated ketone group from a natural source. Bioassay showed that compounds **1** and **2** could inhibit nitric oxide (NO) production in LPS stimulated RAW 264.7 cells with IC_{50} values of 5.31 μM and 12.15 μM, respectively.

Chronic inflammation is implicated in many diseases such as infection, allergy, tumefaction, rheumatoid arthritis, and cancer.[1,2] Search for anti-inflammatory agents will be a never-ending research topic in the future. It is now known that a variety of molecules are involved in the inflammation process, whereas, nitric oxide (NO) and prostaglandins (PG) are considered to be pivotal players. Thus, compounds having NO and PG production inhibitory effects may have anti-inflammatory activities.[3]

Despite a number of anti-inflammatory drugs have been synthesized in the past decades, there is still a continuous need for the discovery of new natural anti-inflammatory agents because their diverse structures are helpful for the chemical synthesis and may also possess novel mechanisms of action. Increasing evidence showed that rheumatic diseases are intimately related with inflammation and immune system.[2] Therefore, herbs with therapeutic effects on rheumatoid arthritis are rationally hypothesized to possess possible anti-inflammatory substances. *Zanthoxylum podocarpum* belongs to the genus *Zanthoxylum* of Rutaceae family, its bark has been widely used as a folk medicine in PR China mainly for the treatment of rheumatoid arthritis and swelling with unknown reasons.[4] Alkaloids, coumarins, amides and lignans are mainly represented in this genus. However, so far only very limited numbers of lignans and amides were found in *Z. podocarpum*,[5–7] and the specific components responsible for the therapeutic effects of this herb are still not well clarified. In the present study, we isolated a series of lignans (Fig. 1), including two new dilignans with a rare α,β-unsaturated ketone group, and evaluated their inhibitory activities against NO production in LPS stimulated RAW 264.7 cells.

Compound **1**[8] was obtained as white powders. The molecular formula was deduced as $C_{41}H_{42}O_{14}$ by its positive HR-ESI-MS at *m/z* 759.2651 ([M+H]$^+$, calcd for 759.2652). The IR spectrum showed absorption bands representative of hydroxy (3432 cm^{-1}), carbonyl (1726 and 1660 cm^{-1}), and aromatic groups (1608, 1592, 1510, 1465, 1446, 1414 cm^{-1}). The UV spectrum revealed absorptions at 242 and 283 nm. The ^{1}H NMR data (Table 1) implied three ABX systems at δ_H 6.80–7.56, indicative of the presence of three 1,3,4-trisubstituted benzene rings. Additionally, a signal at δ_H 5.53 (H-13′) was observed in the ^{1}H NMR spectrum. The ^{13}C NMR spectrum displayed 41 signals, apart from three methoxy, two methylenedioxy groups, the remained 36 carbon signals suggested that **1** might be a dilignan derivative. The diagnostic ^{13}C NMR signals at δ_C 87.6 (C-2), 54.5 (C-1), 71.0 (C-8), 81.7 (C-6), 49.8 (C-5), 69.5 (C-4), 84.5 (C-2′), 48.7 (C-1′), 72.3 (C-8′), 84.2 (C-6′), 54.4 (C-5′), and 72.4 (C-4′) were apparent a lignan with four tetrahydrofuran moieties. A close inspection of the NMR spectra of **1** found that its NMR data were similar to those of biplanispine A.[9] The two lignan parts of **1** were also linked via C-18–O–C-11′ supported by a weak HMBC correlation of H-19 (δ 7.25, d, J = 8.4 Hz)/C-11′ (δ 106.5) detected in acetone-d_6 with a $J_{H,C}$ value of 4 Hz (Fig. 2). 2D NMR experiments including COSY, HMQC,

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Table 1
NMR data of compounds **1** and **2** (500 MHz for ^{1}H and 125 MHz for ^{13}C, in $CDCl_3$)

	1		**2**	
	^{1}H	^{13}C	^{1}H	^{13}C
1	2.89 (m)	54.5 (d)	3.12 (m)	54.0 (d)
2	4.38 (d, 7.0)	87.6 (d)	4.74 (overlap)	85.6 (d)
4	α: 3.19 (m); β: 3.79 (m)	69.5 (t)	3.87 (overlap); 4.25 (dd, 7.0, 2.5)	71.5 (t)
5	3.33 (m)	49.8 (d)	3.07 (m)	54.3 (d)
6	4.85 (d, 6.0)	81.7 (d)	4.74 (overlap)	85.6 (d)
8	α: 3.83 (m); β: 4.10 (m)	71.0 (t)	3.85 (overlap); 4.28 (dd, 15.5, 2.5)	72.0 (t)
9		134.8 (s)		132.7 (s)
10	6.85 (overlap)	106.4 (d)	6.89 (overlap)	108.6 (d)
11		147.9 (s)		146.7 (s)
12		147.2 (s)		147.2 (s)
13	7.56 (d, 8.0)	108.1 (d)	6.88 (overlap)	114.2 (d)
14	6.80 (overlap)	119.5 (d)	6.81 (overlap)	118.9 (d)
15		137.1 (s)		139.9 (s)
16	6.97 (br s)	109.7 (d)	6.92 (d, 1.5)	109.8 (d)
17		152.7 (s)		152.9 (s)
18		140.7 (s)		141.4 (s)
19	7.17 (d, 8.0)	124.0 (d)	7.18 (d, 8.5)	124.0 (d)
20	6.80 (overlap)	117.7 (d)	6.82 (overlap)	117.9 (d)
1′	3.41 (m)	48.7 (d)	3.36 (m)	48.6 (d)
2′	4.63 (dd, 7.0, 3.0)	84.5 (d)	4.65 (dd, 6.5, 3.0)	84.6 (d)
4′	α: 4.18 (m); β: 3.69 (dd, 8.5, 6.0)	72.4 (t)	4.20 (m); 3.69 (dd, 8.5, 6.0)	72.4 (t)
5′	3.18 (m)	54.4 (d)	3.19 (m)	54.4 (d)
6′	4.72 (d, 4.7)	84.2 (d)	4.73 (overlap)	84.2 (d)
8′	3.83 (m); 4.12 (m)	72.3 (t)	3.87 (m); 4.11 (dd, 9.0, 7.5)	72.3 (t)
9′	3.25 (dd, 10.0, 2.5)	52.0 (d)	3.30 (dd, 10.0, 3.0)	51.9 (d)
10′	4.36 (dd, 10.0, 6.5)	72.9 (d)	4.37 (dd, 9.5, 6.0)	73.0 (d)
11′		105.1 (s)		105.1 (s)
12′		167.6 (s)		167.6 (s)
13′	5.53 (s)	102.9 (d)	5.53 (s)	102.8 (d)
14′		195.9 (s)		195.8 (s)
15′		133.3 (s)		133.4 (s)
16′	6.89 (br s)	109.1 (d)	6.90 (br s)	109.3 (d)
17′		149.0 (s)		149.1 (s)
18′		148.4 (s)		148.5 (s)
19′	6.83 (overlap)	110.8 (d)	6.83 (overlap)	111.0 (d)
20′	6.85 (overlap)	118.2 (d)	6.86 (overlap)	118.1 (d)
–OCH_2O-(11,12)	5.94 (s)	101.0 (t)	5.94 (s)	
OMe-11			3.89 (s)	55.9 (q)
OMe-17	3.79 (s)	55.6 (q)	3.78 (s)	55.6 (q)
–OCH_2O-(11′,12′)	5.44 (br s); 5.16 (br s)	100.8 (t)	5.46 (br s); 5.21 (br s)	100.7 (t)
OMe-17′	3.89 (s)	55.8 (q)	3.88 (s)	55.9 (q)
OMe-18′	3.86 (s)	55.9 (q)	3.86 (s)	55.9 (q)
OH-10′	3.98 (d, 6.5) in $CDCl_3$; 5.98 (d, overlap) in DMSO-d_6		5.60 (br s) in $CDCl_3$; 6.99 (d, 1.6) in DMSO-d_6	

HMBC, and ROESY measurements allowed unambiguous assignments of NMR data of **1**. The difference between compound **1** and biplanispine A was that a methylenedioxy group attached at one phenyl moiety was replaced by two methoxy groups. The HMBC correlations (Fig. 2) of protons at δ_H 3.86 and H-20′/C-18′ and protons at δ_H 3.89/C-17′ confirmed the positions of two methoxy groups and allowed the unambiguous assignments of their NMR data. The remained one methoxy moiety was located at C-17 supported by this OMe signal correlating with C-17 in the HMBC spectrum, and ROESY correlation of H-16/OMe. The relative configuration at tetrahydrofuran rings of western part of **1** was clarified by ^{13}C NMR chemical shift and a ROESY experiment. The chemical shift observed for C-9 at δ_C 134.8 was in accordance with that reported where the 3,4-methylenedioxyphenyl group was equatorial.[10,11] The ROESY spectrum showed correlations of H-2/Hβ-4 (δ 3.79), Hβ-8 (δ 4.10), indicating that these protons were on the same face of the ring and had β orientation, whereas those of H-6/Hα-4 (δ 3.19), H-5, and H-5/H-1 suggested that these protons were on the other side and had α orientation. The relative configuration of furofuran ring of eastern part in **1** was assigned according to a ROESY experiment and ^{1}H NMR data. The chemical shift of H-6′ at δ_H 4.72 was in accordance with those literatures reported where the benzolic proton (H-6′) was axial.[12,13] The ROESY correlations (Fig. 2) of Hβ-4′/H-2′, H-6, and Hα-4′/H-1′, H-5′ revealed that H-1′ and H-5′ were *cis*-oriented and had α orientation, H-5′ and H-6′, and H-1′ and H-2′ had *trans*-relationship. The relative configurations at C-9′ and C-10′ were determined by the large coupling constant of H-9′ and H-10′ (J = 10.0 Hz), indicating that these two protons are *trans* to each other. The unavailable ROESY data for methylenedioxy protons with other signals such as H-10′, H-9′, and OH-10′ (visible when collected in $CDCl_3$ or DMSO-d_6) made the determination of the relative configuration at C-11′ difficult. Unfortunately, an effort to cultivate qualified crystals of **1** or its acetate derivative was also not successful. Thus the relative configuration at C-11′ remained unresolved so far.

Compound **2**[14] was obtained as white powders. The positive HR-ESI-MS of **2** at m/z 761.2813 $[M+H]^+$ ($C_{41}H_{45}O_{14}$, calcd for 761.2809) suggested its molecular formula to be $C_{41}H_{44}O_{14}$. The IR spectrum exhibited absorption bands attributive to hydroxyl (3425 cm^{-1}), carbonyl (1728 and 1658 cm^{-1}), and phenyl groups (1515 and 1463 cm^{-1}). The IR, ^{1}H and ^{13}C NMR data of **2** were very similar to those of **1**, except for a 3,4-methylenedioxy group at phenyl moiety being replaced by a 3-methoxy-4-hydroxyphenyl moiety. The HMBC correlation of proton at δ_H 3.89 (OMe)/C-11 and ROESY evidence of H-10/OMe (δ_H 3.89) suggested that this OMe was located at C-11. Further HMBC correlations of H-2/C-

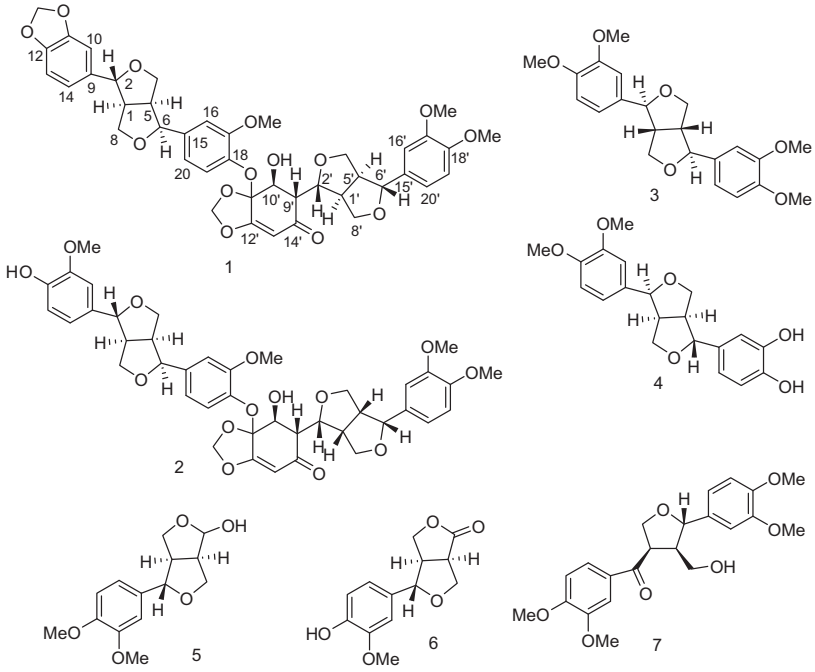

Figure 1. Chemical structures of compounds **1**–**7**.

14, in combination with the substituted pattern of this benzene ring confirmed the position of OH at C-12. In the same manner, the relative configuration of **2** was mainly achieved by ROESY correlations as shown (Fig. 2). It was noted that the relative configuration of furofuran ring of eastern part of **2** was different from that of **1**. The ROESY spectrum showed correlation of H-6′/H-1′, H-5′, H-2′/H-1′, H-5′, indicating these protons to be on the same side. Actually, lignans with diverse configuration have been characterized from *Zanthoxylum planispinum*.[9] The insufficiency of ROESY correlations for methylenedioxy with other proton signals and failure to obtain a qualified crystal made it impossible to assign the relative configuration at C-11′. Taken together, the structure of **2** was determined as shown.

The known compounds were isolated[15] and identified as eudesmin (**3**),[16] (1*R*,2*R*,5*R*,6*S*)-2-(3,4-dimethoxyphenyl)-6-(3,4-dihydroxyphenyl)-3,7-dioxabicyclo[3.3.0]octane (**4**),[17] dimethoxysamin (**5**),[18] *rel*-(1*R*,5*R*,6*S*)-6-(4-hydroxy-3-methoxyphenyl)-3,7-dioxabicyclo[3.3.0]octan-2-one (**6**),[19] and magnone A (**7**),[20] respectively, by comparison of their spectroscopic data with literatures.

As aforementioned, rheumatic diseases are closely associated with inflammation and immune response, and NO is one of the crucial players in inflammation lesions. Since the bark of the title plant is mainly used to treat rheumatic diseases, metabolites isolated from this material were thus screened for anti-inflammatory activity by detecting inhibition of NO production in RAW 264.7 cells.[21] The results showed that compounds **1** and **2** exhibited NO production inhibitory effect with IC_{50} value of 5.31 μM and 12.15 μM, respectively, whereas other lignans were inactive on this assay. The difference for inhibition of NO production between these compounds implied that the presence of unsaturated ketone moiety might be necessary for the activity. In addition, it was noted that lignan dimmers discovered from nature are relatively rare,[9] dilignans possessing an α,β-unsaturated ketone motif are especially unusual. To our knowledge, previously only one example of this type of dilignans was isolated from *Z. planispinum*. This study not only provides a scientific rationale between the biological activity and the herbal remedies of this plant but also might

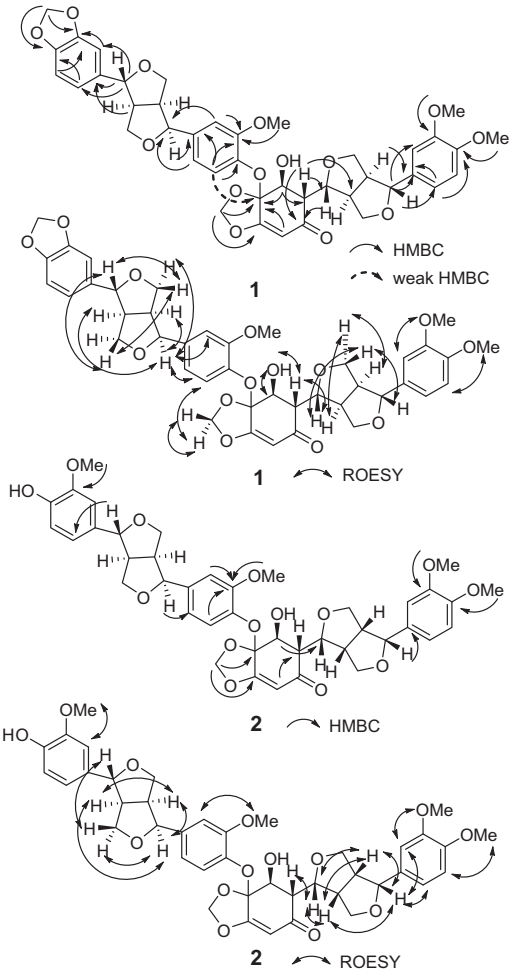

Figure 2. HMBC and ROESY correlations for compounds **1** and **2**.

disclose the importance of the α,β-unsaturated ketone in keeping the NO inhibitory activity.

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8. Zanthpodocarpins A (**1**): white powders; $[\alpha]_D^{24.5}$ −287.0 (*c* 0.1, $CHCl_3$); UV λ_{max} ($CHCl_3$) nm (log ε): 283 (4.03), 242 (4.41); IR (KBr) ν_{max} cm^{-1}: 3432, 2958, 2924, 2854, 1726, 1660, 1608, 1592, 1510, 1465, 1446, 1414, 1329, 1263, 1187, 1073, 1034, 936, 809, 726; ^1H and ^{13}C NMR data, see Table 1; ESI-MS (positive) *m*/*z*: 759 $[M+H]^+$, 781 $[M+Na]^+$; HR-ESI-MS (positive) *m*/*z*: 759.2651 $[M+H]^+$ ($C_{41}H_{43}O_{14}$, calcd for 759.2652).
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14. Zanthpodocarpins B (**2**): white powders; $[\alpha]_D^{22.8}$ −107.2 (*c* 0.11, $CHCl_3$); UV λ_{max} ($CHCl_3$) nm (log ε): 280 (3.76), 240 (4.14); IR (KBr) ν_{max} cm^{-1}: 3425, 2956, 2925, 2854, 1729, 1658, 1515, 1463, 1268, 1124, 1028, 748; ^1H and ^{13}C NMR data, see Table 1; ESI-MS (positive) *m*/*z*: 761 $[M+H]^+$; HR-ESI-MS (positive) *m*/*z*: 761.2813 $[M+H]^+$ ($C_{41}H_{45}O_{14}$, calcd for 761.2809).
15. The air-dried and powdered barks of *Z. podocarpum* (10 kg) were soaked with 80% MeOH (2 × 30 L) at room temperature and concentrated in vacuo to afford a crude extract, which was suspended in water followed by successive partition with petroleum ether (3 × 3 L), EtOAc (3 × 3 L), and BuOH (3 × 3 L). The EtOAC extract (190 g) was subjected to column chromatography (CC) over silica gel (200–300 mesh, 3 kg) and eluted with a gradient of $CHCl_3$/MeOH (100:0, 98:2, 96:4, 94:6, 90:10, 85:15, 80:20, 70:30) to give fractions A–H. Fraction C (10 g) was submitted to CC over MCI gel CHP 20P eluting with gradient aqueous MeOH to yield three portions (C-1–C-3). Fraction C-1 (2.5 g) was passed through Sephadex LH-20 (MeOH) followed by preparative TLC (petroleum ether/^{i}PrOH, 15:2) to yield compound **4** (89 mg). Fraction C-2 (6 g) was separated by RP-18 (40–63 μM) eluting with gradient MeOH/H_2O (40–100%) to afford three parts (C-2a–C-2c), of which, fraction C-2a (800 mg) was submitted to preparative TLC (petroleum ether/EtOAc, 3:1) followed Sephadex LH-20 chromatography to yield **5** (148 mg). Fraction C-2b (600 mg) was purified by preparative TLC (petroleum ether/EtOAc, 1:1) followed by semi-preparative HPLC (Agilent 1200 liquid chromatography with a Zorbax SB-C18 column, 9.4 mm × 25 cm, i.d.) eluting with 40% aqueous MeOH to produce **6** (58 mg). Fraction C-2c (300 mg) was separated on RP-18 column (MeOH/H_2O, 60–100%) and then purified by preparative TLC (petroleum ether/EtOAc, 1:1) to afford **1** (38 mg). Fraction C-3 (4 g) was fractionated by silica gel CC (petroleum ether/EtOAc/^{i}PrOH, 10:10:1) to yield four sub-fractions (C-3a–C-3d). Fraction C-3a (300 mg) was subjected to preparative TLC developed with petroleum ether/EtOAc/^{i}PrOH (3:1:0.5) followed by Sephadex LH-20 chromatography to yield **2** (3 mg). Fraction C-3b (700 mg) was divided into three portions (C-3b-1–C-3b-3), of which fraction C-3b-2 (20 mg) was purified by semi-preparative HPLC eluting with 60% aqueous MeOH to produce **7** (8 mg). Fraction C-3c (350 mg) was purified by RP-18 column eluting with gradient aqueous MeOH (50–100%) to yield a nearly pure fraction, which was recrystallized in MeOH to afford **3** (78 mg).
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21. Inhibition of NO production in LPS stimulated RAW 264.7 macrophage cell line. The Murine monocytic RAW 264.7 macrophages were dispensed into 96-well plates (2×10^5 cells/well) containing RPMI 1640 medium (Hyclone, USA) with 10% FBS under a humidified atmosphere of 5% CO_2 at 37 °C. After 24 h pre-incubation, cells were treated with serial dilutions of the compounds **1**–**7** with the maximum concentration of 25 μM in the presence of 1 μg/mL LPS for 18 h. Each compound was dissolved in DMSO and further diluted in medium to produce different concentrations. NO production in each well was assessed by adding 100 μL of Griess reagents A and B to 100 μL of each supernatant from LPS or the compound-treated cells in triplicate. After 5 min incubation, the absorbance was measured at 570 nm with 2104 Envision Multilabel Plate Reader (Perkin–Elmer Life Sciences, Inc., Boston, MA, USA). Cytotoxicity was determined by the MTT assay. MG-132 was used as a positive control.