Sesquiterpene Esters from the Fruits of *Celastrus orbiculatus*

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Three new β -dihydroagarofuran sesquiterpene esters, 6 α -acetoxy-9 β -benzoyloxy-1 β -cinnamoyloxy-8 β -butanoyloxy- β -dihydroagarofuran (**1**), 6 α -acetoxy-9 β -benzoyloxy-1 β -cinnamoyloxy-8 β -(2-methylbutanoyloxy)- β -dihydroagarofuran (**2**), and 6 α -acetoxy-1 β ,8 β -dibenzoyloxy-9 β -hydroxy- β -dihydroagarofuran (**6**), together with three known compounds (**3**–**5**) were isolated from the fruits of *Celastrus orbiculatus* THUNB. Their structures were elucidated on the basis of spectroscopic methods. Compound **4** showed moderate activity in inhibiting LPS-induced nitric oxide production in murine macrophage RAW264.7 cells with an IC₅₀ value of 43.7 μ M.

Key words *Celastrus orbiculatus*; sesquiterpene ester; β -dihydroagarofuran

Celastrus orbiculatus is a medicinal plant widely distributed in China, which acts as a tranquilizer.¹⁾ Some sesquiterpenes with antiinflammatory activities from *C. orbiculatus* were reported in the literature.²⁾ To find the active constituents responsible for antiinflammation, we investigated the constituents of the fruits. Three new β -dihydroagarofuran sesquiterpene esters, 6 α -acetoxy-9 β -benzoyloxy-1 β -cinnamoyloxy-8 β -butanoyloxy- β -dihydroagarofuran (**1**), 6 α -acetoxy-9 β -benzoyloxy-1 β -cinnamoyloxy-8 β -(2-methylbutanoyloxy)- β -dihydroagarofuran (**2**), and 6 α -acetoxy-1 β ,8 β -dibenzoyloxy-9 β -hydroxy- β -dihydroagarofuran (**6**) were isolated, along with three known compounds, 6 α -acetoxy-1 β ,8 β ,9 β -tribenzoyloxy- β -dihydroagarofuran (**3**), 1 β ,2 β -diacetoxy-6 α -benzoyloxy-9 α -cinnamoyloxy- β -dihydroagarofuran (**4**), and 1 β ,6 α -diacetoxy-8 β ,9 β -dibenzoyloxy- β -dihydroagarofuran (**5**). In this paper, we elucidate the structures of **1**, **2**, and **6** and the effects of compounds **1**–**6** on nitric oxide (NO) production in lipopolysaccharide-stimulated murine macrophage RAW264.7 cells. Compound **4** showed moderate inhibitory activity against LPS-induced NO production in murine macrophage RAW264.7 cells, with IC₅₀ values of 43.7 μ M with respect to aminoguanidine, an inducible NOS inhibitor (IC₅₀ 18.2 μ M).

Results and Discussion

The fruits of *C. orbiculatus* were extracted with 95% ethanol and partitioned successively with petroleum ether, CHCl₃, EtOAc, and *n*-BuOH. The petroleum ether fraction (160 g) was subjected to column chromatography on Silica gel and PHPLC to provide compounds **1**–**6**. The known compounds **3**, **4**, and **5** were identified by comparison of their physical and spectral data with those reported in the literature.^{3–5)}

ture.^{3–5)}

Compound **1** was obtained as a white powder. The HR-MS spectrum suggested a molecular formula of C₃₇H₄₄O₉ for **1**. The UV (MeOH) spectrum of **1** showed the presence of aromatic rings. The ¹H- and ¹³C-NMR spectra revealed the presence of one benzoyloxy group, one cinnamoyloxy group, one acetoxy group, and one butanoyloxy group. In addition, the ¹³C-NMR spectrum indicating the remaining parent compound consisted of 15 carbons: four methyl (δ 16.9, 12.1, 24.1, 30.6), two methylene carbons (δ 22.2, 26.6), six methine carbons (δ 33.8, 52.5, 71.2, 75.1, 74.3, 78.9), and three quaternary carbons (δ 48.9, 81.7, 91.0). These ¹³C-NMR data were very similar to those assigned to 1,6,8,9-tetra-substituted β -dihydroagarofuran.^{3,6)} Therefore compound **1** had four ester groups linked at C-1, C-6, C-8, and C-9.

The ester group distribution in **1** was determined from the HMBC spectrum, which showed cross peaks between H-6 [δ 5.93 (1H, s)] and the carbonyl (δ 169.9) of acetoxy, H-1 [δ 5.37 (1H, dd, J =11.1, 4.9 Hz)] and the carbonyl (δ 166.1) of cinnamoyloxy, H-8 [δ 5.54 (1H, dd, J =5.2, 4.2 Hz)] and the carbonyl (δ 172.4) of butanoyloxy, and H-9 [δ 5.61 (1H, d, J =5.2 Hz)] and the carbonyl (δ 164.9) of benzoyloxy, respectively. These results showed that the acetoxy was situated at C-6, while cinnamoyloxy, butanoyloxy, and benzoyloxy were at C-1, C-8, and C-9, respectively. Generally, H-1 and H-6 have axial stereochemistry in this class of compounds.^{6,7)} The NOESY spectrum showed cross peaks between H_{ax}-1 and H-9, and H-8 and H-9, which suggests the orientation of axial H-9 and equatorial H-8.^{3,5–7)} Thus the structure of **1** was elucidated to be 6 α -acetoxy-9 β -benzoyloxy-1 β -cinnamoyloxy-8 β -butanoyloxy- β -dihydroagarofuran.

Compound **2** was C₃₈H₄₆O₉ based on the HR-MS spectrum. The UV, ¹H- and ¹³C-NMR spectra showed the presence of one acetoxy, one cinnamoyloxy, one 2-methylbutanoyloxy, and one benzoyloxy groups. The ¹H- and ¹³C-NMR spectra of **1** and **2** were very similar except for the ester moieties, which indicates that the position of the ester function was at C-1, C-6, C-8, and C-9. The HMBC spectrum showed cross peaks between H-1 [δ 5.38 (1H, dd, J =10.8, 5.3 Hz)] and the carbonyl (δ 166.1) of cinnamoyloxy, H-6 [δ 5.94 (1H, s)] and the carbonyl (δ 169.8) of acetoxy, H-8 [δ 5.56 (1H, dd, J =5.2, 4.2 Hz)] and the carbonyl (δ 175.2) of 2-methylbutanoyloxy, and H-9 [δ 5.63 (1H, d, J =5.2 Hz)] and the carbonyl (δ 164.8) of benzoyloxy, which

- 1** R₁ = OCin, R₂ = Butanoyloxy, R₃ = OBz
- 2** R₁ = OCin, R₂ = 2-Methylbutanoyloxy, R₃ = OBz
- 3** R₁ = R₂ = R₃ = OBz
- 5** R₁ = OAc, R₂=R₃ = OBz
- 6** R₁ = R₂ = OBz, R₃ = OH

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Table 1. NMR Data for Compounds **1**, **2**, and **6** in CDCl₃ (δ ppm)

No.	1		2		6	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	78.9	5.37 dd (11.1, 4.9)	78.9	5.38 dd (10.8, 5.3)	79.6	5.56 dd (10.8, 5.0)
2	22.2	1.75 m	22.3	1.77 m	22.3	1.79 m
3	26.6	2.25 m 1.48 m	26.6	2.20 m 1.50 m	26.7	2.20 m 1.51 m
4	33.8	2.25 m	33.9	2.26 m	33.8	
5	91.0		91.1		91.0	
6	75.1	5.93 s	75.0	5.94 s	75.1	6.04 s
7	52.5	2.50 d (4.2)	52.6	2.49 d (4.2)	52.5	2.53 d (4.2)
8	71.2	5.54 dd (5.2, 4.2)	70.9	5.56 dd (5.2, 4.2)	74.1	5.59 dd (5.0, 4.2)
9	74.3	5.61 d (5.2)	74.6	5.63 d (5.2)	73.2	4.42 d (5.0)
10	48.9		48.9		49.0	
11	81.7		81.7		81.3	
12	16.9	1.07 d (7.5)	16.9	1.08 d (7.3)	16.8	1.08 d (7.4)
13	12.1	1.57 s	12.2	1.58 s	11.3	1.46 s
14	24.1	1.58 s	24.2	1.60 s	24.1	1.49 s
15	30.6	1.42 s	30.7	1.43 s	30.7	1.43 s

Figures in parentheses are coupling constants in Hz. All signals were assigned using 1D and 2D NMR.

means that acetoxy, cinnamoyloxy, 2-methylbutanoyloxy, benzoyloxy were situated at C-6, C-1, C-8, and C-9, respectively. In the NOESY spectrum, cross peaks between H-1 and H-8, and H-9 and H-8 suggested H-9 axial, and H-8 equatorial orientations.^{3,6–8)} Thus compound **2** was identified as 6 α -acetoxy-9 β -benzoyloxy-1 β -cinnamoyloxy-8 β -(2-methylbutanoyloxy)- β -dihydroagarofuran.

Compound **6**, C₃₁H₃₆O₈, contained one hydroxyl, one acetoxy, and two benzoyloxy groups as shown by its ¹H-, ¹³C-NMR, and MS spectra. The chemical shifts and coupling pattern assignable to H-6, H-7, H-8 and H-9 in the ¹H- and ¹³C-NMR spectra of **6** were very similar to those of the known compounds 6 α -acetoxy-8 β ,9 β -dibenzoyloxy-1 β -hydroxy- β -dihydroagarofuran, and 6 α -acetoxy-1 β ,9 β -dibenzoyloxy-8 β -hydroxy- β -dihydroagarofuran reported in the literature.^{3,9)} The substituted group distribution in **6** was confirmed from the HMBC spectrum, which indicated cross peaks between H-6 [δ 6.04 (1H, s)] and the carbonyl (δ 169.9) of acetoxy, H-1 [δ 5.56 (1H, dd, J =10.8, 5.0 Hz)] and the carbonyl (δ 166.2) of benzoyloxy, and H-8 [δ 5.59 (1H, dd, J =5.0, 4.2 Hz)] and the carbonyl (δ 166.2) of benzoyloxy, respectively. Therefore one acetoxy situated at C-6 and two benzoyloxy groups and hydroxy were at C-1, C-8, and C-9, respectively. In the NOESY spectrum, cross peaks between H-1 and H-9, and H-8 and H-9 suggested that compound **6** has the same relative stereochemistry as compounds **1** and **2**. Thus compound **6** was elucidated to be 6 α -acetoxy-1 β ,8 β -dibenzoyloxy-9 β -hydroxy- β -dihydroagarofuran.

Compounds **1**–**6** were examined for their dose–response effects against LPS-induced NO production. The excess production of NO by iNOS in macrophages and endothelial cells is responsible for the inflammatory response and implicated in the pathogenesis of several inflammatory diseases such as septic shock, rheumatoid arthritis, graft rejection, and diabetes.¹⁰⁾ The effects of compounds **1**–**6** were investigated on NO production in LPS-stimulated RAW264.7 cells with re-

spect to aminoguanidine, an iNOS inhibitor. Compound **4** showed moderate inhibitory activity against LPS-induced NO production in murine macrophage RAW264.7 cells, with an IC₅₀ value of 43.7 μ M, which is comparable to that of aminoguanidine (IC₅₀ 18.2 μ M). However, compounds **1**, **2**, **3**, **5**, and **6** showed very weak activity. Cell viability measured using the MTT assay showed that compound **4** had no significant cytotoxicity against RAW264.7 cells at effective concentrations for the inhibition of NO production (data not shown).

Experimental

Melting points were measured on a Yamaco hot-stage and are uncorrected. All NMR spectra were recorded on Bruker-ARX-300 spectrometer, using TMS as an internal standard. UV spectra were recorded on a Shimadzu UV-260 UV–Vis spectrometer. ESI-MS were measured on a VG-70SE mass spectrometer. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. Silica for chromatography was produced by Qingdao Ocean Chemical Group Co., China. The HPLC system used a Shimadzu CTO-6A equipped with a Shimadzu SPD-6A UV detector (Shimadzu Shim-pack PREP-ODS, i.d. 2.5 $\times$ 21.6 cm).

Plant Material The plant material was collected in Shenyang, Liaoning province, and was identified by Professor Guo Yunzheng (Shenyang Pharmaceutical University).

Extraction and Isolation Dried fruits (10 kg) of *C. orbiculatus* were extracted with 95% ethanol. The extract was concentrated and then extracted successively with petroleum ether, CHCl₃, EtOAc and *n*-BuOH. The petroleum ether fraction (160 g) was subjected to column chromatography on silica gel and eluted with petroleum ether–acetone (100:0–1:1) to provide seven fractions. Fraction 3 (4.7 g) was subjected by column chromatography on HPLC to yield compounds **1** (7 mg, 90 min), **2** (5 mg, 110 min), **4** (4 mg, 73 min), and **5** (4 mg, 51 min) using MeOH–H₂O (78:22) as eluent, and fraction 4 (5.1 g) was subjected to column chromatography on HPLC to yield compounds **3** (4 mg, 41 min) and **6** (5 mg, 64 min) using MeOH–H₂O (75:25) as eluent.

Compound **1**: White powder (EtOAc), mp 181–183 °C. UV λ_{max} (MeOH): 223.0 nm. [α]_D²⁰ = –7.0° (c =0.75, MeOH). ESI-MS: m/z 633 [M+H]⁺, HR-MS: m/z 633.3070 [M+H]⁺ (Calcd for C₃₇H₄₅O₉, 633.3064). ¹H-NMR (CDCl₃, 300 MHz) see Table 1, acetoxy [2.12 (3H, s)]. Benzoyloxy and cinnamoyloxy [5.73 (1H, d, J =15.9 Hz), 6.91–7.91 (11H, m)], butanoyloxy [0.88 (3H, t, J =14.7, 7.5 Hz), 1.66 (2H, m), 2.37 (2H, t,

$J=7.5$ Hz). ^{13}C -NMR (CDCl_3 , 75 MHz) see Table 1, acetoxy (21.3, 169.9), benzoyloxy (164.9, 132.6, 129.8, 129.5, 128.2), cinnamoyloxy (166.1, 144.2, 134.2, 129.9, 128.3, 127.8, 118.1), butanoyloxy (172.4, 36.4, 18.3, 13.7).

Compound 2: White powder (EtOAc), mp 231–233 °C. UV λ_{max} (MeOH): 223.2 nm. $[\alpha]_{\text{D}}^{20} = -8.9^\circ$ ($c=0.40$, MeOH). ESI-MS: m/z 647 $[\text{M}+\text{H}]^+$, HR-MS: m/z 647.3220 $[\text{M}+\text{H}]^+$ (Calcd for $\text{C}_{38}\text{H}_{47}\text{O}_9$, 647.3227). ^1H -NMR (300 MHz, in CDCl_3) see Table 1, acetate [2.15 (3H, s)]; 2-methylbutanoyloxy [0.79 (3H, t), 1.16 (3H, d, $J=7.0$ Hz), 1.49 (2H, m), 2.41 (1H, m)]; benzoyloxy and cinnamoyloxy [5.73 (1H, d, $J=15.9$ Hz), 6.92–7.92 (11H, m)]; ^{13}C -NMR (75 MHz, in CDCl_3) see Table 1, acetoxy (21.3, 169.8); 2-methylbutanoyloxy (175.2, 41.6, 26.6, 11.6, 16.4); benzoyloxy (164.8, 132.6, 129.8, 129.6, 128.2), cinnamoyloxy (166.1, 144.2, 134.2, 129.8, 128.3, 127.9, 118.1).

Compound 6: White powder (EtOAc), mp 217–219 °C. UV λ_{max} (MeOH): 227.0 nm. $[\alpha]_{\text{D}}^{20} = -286^\circ$ ($c=0.70$, MeOH). ESI-MS: m/z 537 $[\text{M}+\text{H}]^+$, HR-MS: m/z 537.2436 $[\text{M}+\text{H}]^+$ (Calcd for $\text{C}_{31}\text{H}_{37}\text{O}_8$, 537.2488). ^1H -NMR (CDCl_3 , 300 MHz) see Table 1, acetoxy [2.09 (3H, s)], benzoyloxy [7.35–8.14 (10H, m)], ^{13}C -NMR (CDCl_3 , 75 MHz) see Table 1, acetoxy (21.2, 169.9), benzoyloxy (166.2, 166.2, 133.1, 132.7, 129.7, 129.4, 128.5, 128.3).

Determination of NO Production RAW264.7 cells were transferred to 96-well plates at a density of 1×10^5 cells/well. After 3-h incubation, the cells were stimulated with LPS (1 $\mu\text{g}/\text{ml}$) for 24 h in the presence or absence of the compounds tested. As a parameter of NO synthesis, nitrite concentration was measured in the supernatant of RAW264.7 cells with the Griess reaction.¹¹⁾ Briefly, 100 μl of cell culture supernatant was reacted with 100 μl of Griess reagent [1:1 mixture of 0.1% *N*-(1-naphthyl)ethylenediamine in H_2O and 1% sulfanilamide in 5% phosphoric acid] in a 96-well plate, and absorbance was read with a microplate reader (Molecular Devices Co., Menlo Park, CA, U.S.A.) at 570 nm. The nitrite concentration in the super-

natants was calculated by comparison with a sodium nitrite standard curve.

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