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Microbial transformation of 14-deoxy-11, 12-didehydroandrographolide and 14-deoxyandrographolide and inhibitory effects on nitric oxide production of the transformation products

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ABSTRACT

Microbial transformations of 14-deoxy-11, 12-didehydroandrographolide (**a**) and 14-deoxyandrographolide (**b**) were performed by *Cunninghamella blakesleana* (AS 3.970), respectively. Sixteen metabolites were obtained and their structures were elucidated by spectroscopic data analyses. Among these metabolites, 3α, 12*S*, 19-trihydroxy-8(17), 9(11)-*ent*-labdadien-16, 15-olide (**a7**), 3-oxo-8α, 17β-epoxy-14-deoxyandrographolide (**b2**), 3α, 17, 19-trihydroxy-8, 13-*ent*-labdadien-16, 15-olide (**b6**), and 9β-hydroxy-14-deoxyandrographolide (**b9**) are new compounds. The configuration of C-12 in metabolite **a7** was determined as *S* by GIAO method. The proposed metabolic pathways of 14-deoxy-11, 12-didehydroandrographolide and 14-deoxyandrographolide by *C. blakesleana* were drawn. The inhibitory effects of these compounds on nitric oxide production in lipopolysaccaride-activated macrophages were evaluated and their preliminary structure-activity relationships (SAR) were discussed.

1. Introduction

Chuanxinlian is the dried aerial parts of *Andrographis paniculata* (Burm.f.) Nees, which is a famous traditional Chinese and Ayurvedic medicine used as an anti-inflammatory and antipyretic drug for treatment of fever, cold, laryngitis and diarrhea. The pharmacological researches show that diterpenoid lactones are major active components of *A. paniculata* [1]. 14-Deoxy-11, 12-didehydroandrographolide and 14-deoxyandrographolide (Figs. 1 and 2), two principal *ent*-labdane diterpenoid lactones of *A. paniculata*, exhibit anti-inflammatory [2], anti-hypertensive [3], immunoregulatory [4], and anti-tumor [5] properties.

Nitric oxide (NO) is an important cellular messenger molecule involved in many physiological and pathological processes within the mammalian body both beneficial and detrimental [6]. The chronic expression of NO is associated with various inflammatory conditions including multiple sclerosis, arthritis and ulcerative colitis [7]. Therefore, the *in vitro* inhibitory effects of diterpenoid lactones on NO production can provide scientific supports for the traditional use of the aerial parts of *A. paniculata* as a remedy for various inflammatory conditions.

Microbial transformation provides an important tool for structure modification of organic compounds, especially natural products with potent biological activities [8,9]. This method is known for its high stereo- and region-selectivity, ease of handling, low cost, and environmental-friendly nature [10,11].

Microbial transformation was first applied to the structural modification of neoandrographolide by our group [12,13]. Then, biotransformation of other principle diterpenoids of *A. paniculata* and cytotoxicity of the transformed products were studied by several other groups [14–18]. As our continuous investigation, the present work attempts to get more diterpenoid analogues by microbial biotransformation using different fungi in order to assess the structure-activity relationships of this class of constituents and tries to find some compounds with better anti-inflammatory activity.

This article describes the bioconversion of 14-deoxy-11, 12-didehydroandrographolide (**a**) and 14-deoxyandrographolide (**b**) by *Cunninghamella blakesleana* (AS 3.970). Sixteen bioconversion products, including four new compounds were isolated

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and identified. In addition, the inhibitory effects of these compounds on nitric oxide production in lipopolysaccaride-activated macrophages were evaluated.

2. Experimental

2.1. General experimental procedures

The NMR spectra were performed on Bruker ARX-600 spectrometer, using TMS as internal standard. Chemical shifts were expressed in δ (ppm) and coupling constants (J) were reported in Hertz (Hz). Optical rotation values were measured on a Perkin-Elmer 241 MC polarimeter. UV spectra were measured with a Shimadzu UV-1700 spectrophotometer. IR spectra were recorded with a Bruker IFS 55 spectrometer. HRESIMS spectra were obtained on Agilent 6210 TOF mass spectrometer, in m/z. Melting points were determined with an X-5 hot stage microscope melting point apparatus (uncorrected). Preparative HPLC separations were conducted using a Waters 600 chromatograph with an ODS column (C-18, 250 mm × 30 mm, 10 μm; YMC Co. Ltd., Japan) and Waters 490 UV detector. HPLC analyses were carried out on an ODS column (C-18, 250 mm × 4.6 mm, 5 μm; YMC Co. Ltd., Japan) using a Shimadzu LC-6A liquid chromatography instrument equipped with a Shimadzu SPD-6AV UV–vis spectrometric detector. Methanol was HPLC grade (Tianjin concord technology Co. Ltd., China) and water was double distilled in our laboratory. Column chromatography was performed on Silica gel (200–300 mesh) (Qingdao Marine Chemical Co. Ltd., China) and ODS (40–75 μm, Pharmacia Co., Ltd., USA). TLC was carried out on Silica gel GF_{254} plate and the spots were visualized by spraying with Legal and Kedde reagents. All the analytic reagents were analytical grade and purchased from Tianjin DaMao Chemical Company (Tianjin, China).

2.2. Substrates

Both 14-deoxy-11, 12-didehydroandrographolide and 14-deoxyandrographolide (>98%) were isolated from the aerial parts of *A. paniculata* (Burm.f.) Nees by ourselves, and were characterized by comparison of the NMR data with the references.

2.3. Microorganism

C. blakesleana (AS 3.970) was purchased from China General Microbiological Culture Collection Centre.

2.4. Medium

All culture and biotransformation experiments were performed in potato medium as following procedure: 200 g of minced husked

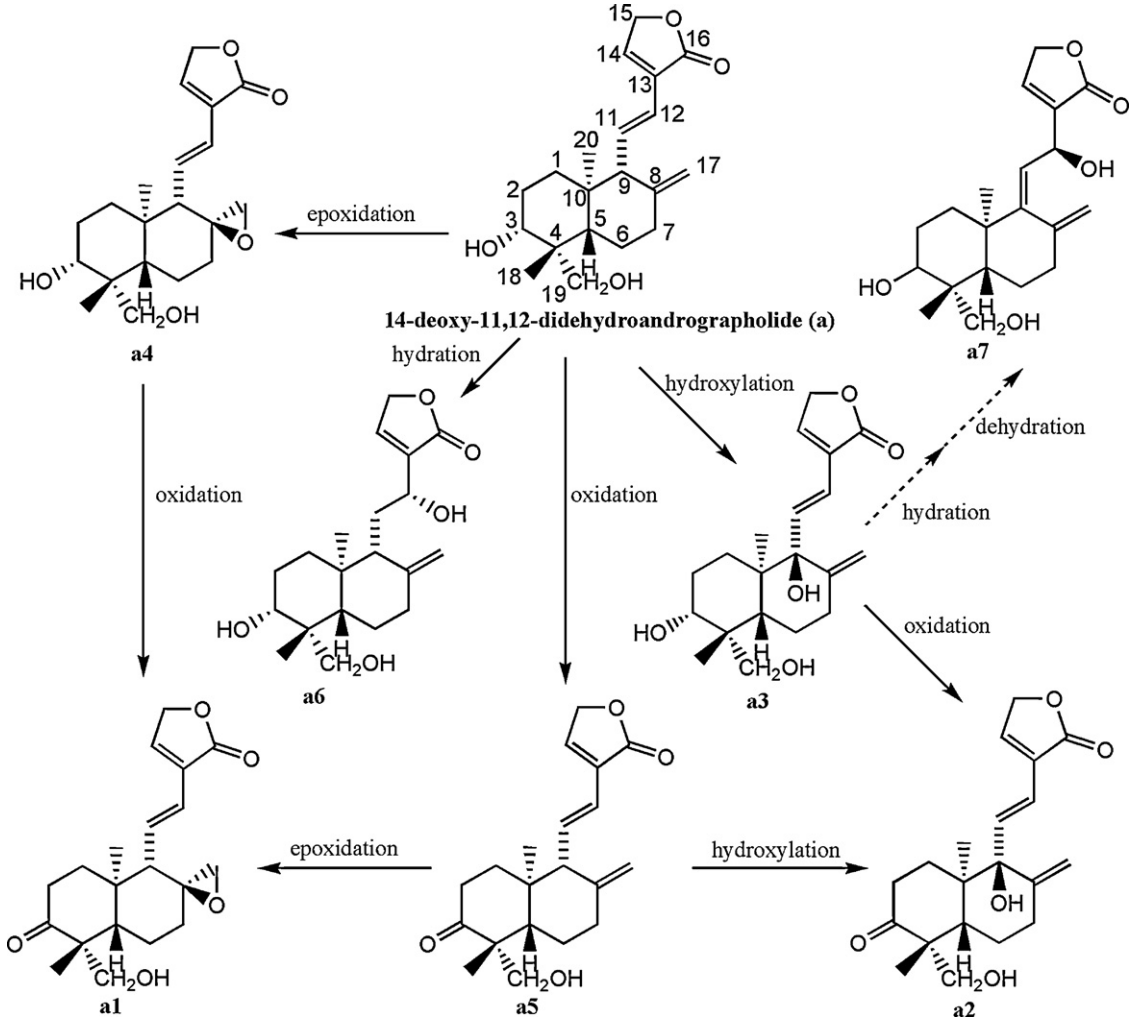

Fig. 1. Structures of biotransformed products of 14-deoxy-11, 12-didehydroandrographolide (**a**) and their proposed metabolic pathway.

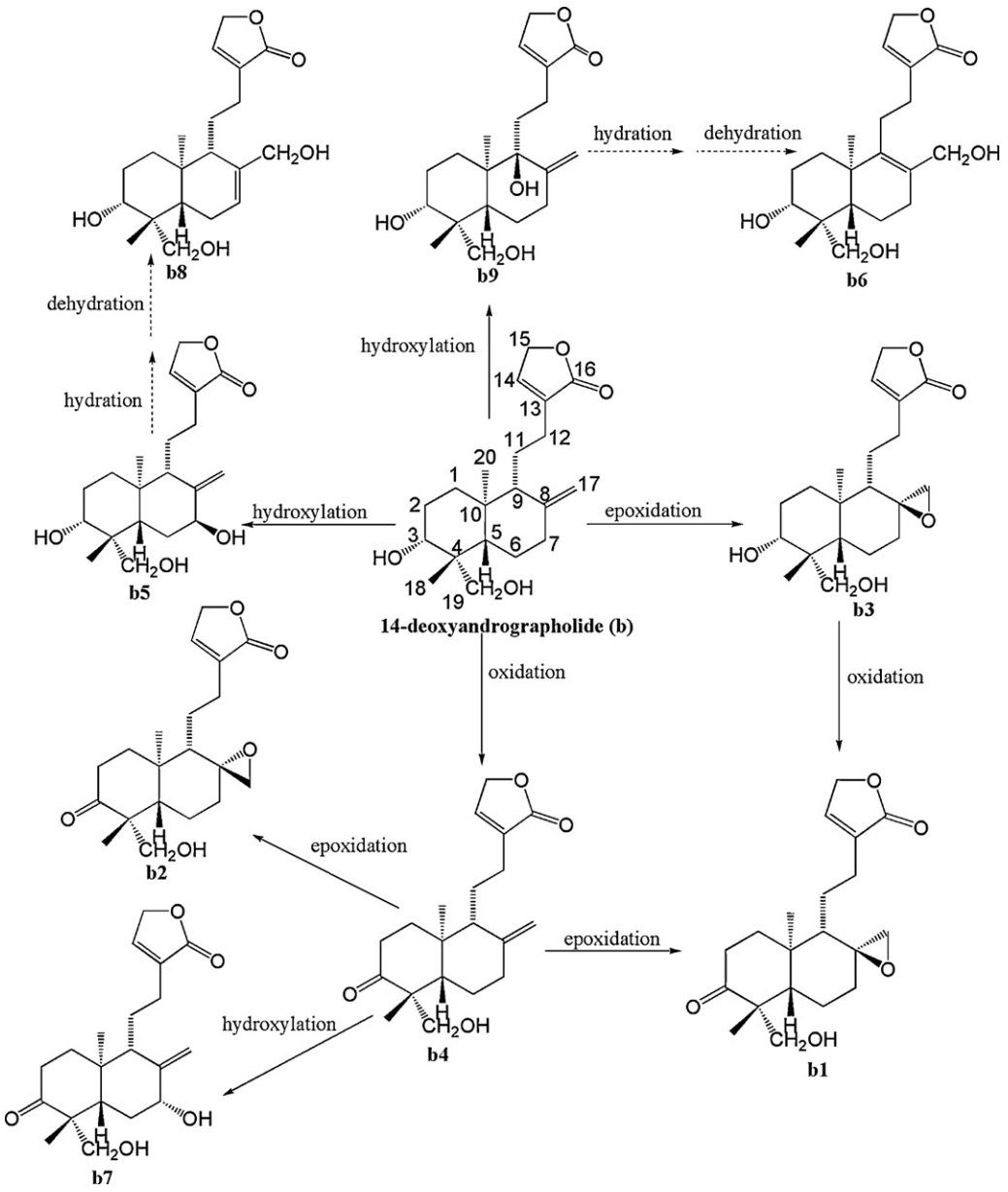

Fig. 2. Structures of biotransformed products of 14-deoxyandrographolide (**b**) and their proposed metabolic pathway.

potato was boiled in 1 L water for 1 h, then the extract was filtered and the filtrate was added with water to 1 L after addition of 20 g of glucose. The broth was autoclaved in individual Erlenmeyer flask at 121 °C and 15 psi for 20 min and cooled before incubation.

2.5. Biotransformations of 14-deoxy-11, 12-didehydroandrographolide (*a*) and 14-deoxyandrographolide (*b*) by C. blakesleana (AS 3.970)

Preparative scale biotransformations of 14-deoxy-11, 12-didehydroandrographolide (**a**) and 14-deoxyandrographolide (**b**) by *C. blakesleana* (AS 3.970) were carried out in 500 mL Erlenmeyer flasks containing 150 mL of potato medium (pH 6.5), respectively. The flasks were placed on rotary shakers, operating at 180 rpm at 28 °C. After 48 h of culture, 15 mg of substrates with 1 mL ethanol were added into the 150 mL culture mediums, respectively, and total of 400 mg of **a** and 600 mg of **b** were transformed by the strain. The following procedures are almost the same for the two substrates. The fermentations were maintained under the same conditions for an additional 96 h. Parallel control experiments were conducted which included an incubation of the fungus without substrate and an incubation of substrate in the medium without fungus. After 96 h, the culture medium was filtered and extracted with dichloromethane (12 L) in three portions. When the fermentations finished, the broths of substrate were filtered and the filtrates were extracted with the equal volume of ethyl acetate for three times, the mycelia were extracted with acetone by supersonic means. All extracts were evaporated to dryness under reduced pressure and combined together.

The biotransformation procedure of metabolites **a5** and **b4** (3 mg, respectively) was similar to that described above for the two substrates **a** and **b**. The culture medium was filtered and extracted with ethyl acetate for three times, and then was analyzed by HPLC and TLC. A time course study was carried out after every 12 h and the degree of transformation was analyzed by TLC and HPLC. The incubation continued for 96 h in the same condition.

2.6. Isolation and purification of metabolites

The crude transformation residues of 14-deoxy-11, 12-didehydroandrographolide (**a**) were subjected to column chromatography (CC) over silica gel and eluted with the mixtures of chloroform-acetone (15:1, 12:1, 10:1, 8:1, 6:1, 4:1, 2:1, 1:1), which yielded eight fractions (1–8). Fraction 1 was submitted to an ODS column chromatography eluted with MeOH–H_2O (9:1, 8:2, 7:3), and then purified by RP-18 HPLC preparation to give **a5** (20 mg, 5% yield). Fraction 2 was performed on an ODS column chromatography eluted with MeOH–H_2O (9:1, 8:2, 7:3), and then purified by repeated RP-18 HPLC to yield **a1** (7 mg, 1.75% yield) and **a2** (12 mg, 3% yield). Fraction 4 was applied to RP-18 HPLC to afford **a4** (25 mg, 6.25% yield). Fraction 5 was subjected to an ODS column chromatography eluted with MeOH–H_2O (8:2, 7:3, 6:4), and then purified by RP-18 HPLC to give **a3** (19 mg, 4.75% yield). Fraction 7 was chromatographed on an ODS column using MeOH–H_2O (7:3, 6:4, 5:5) as eluent, followed by RP-18 HPLC to afford **a6** (16 mg, 4% yield) and **a7** (12 mg, 3% yield).

The crude transformation residues of 14-deoxyandrographolide (**b**) were subjected to column chromatography (CC) over silica gel and eluted with the mixtures of chloroform-acetone (20:1, 15:1, 10:1, 8:1, 6:1, 4:1, 2:1, 1:1), which yielded nine fractions (1–9). Fraction 1 was submitted to an ODS column chromatography eluted with MeOH–H_2O (9:1, 8:2, 7:3), and then purified by RP-18 HPLC preparation to give **b4** (25 mg, 4.17% yield). Fraction 3 was performed on an ODS column chromatography eluted with MeOH–H_2O (8:2, 7:3), and then purified by RP-18 HPLC to yield **b1** (7 mg, 1.17% yield) and **b2** (11 mg, 1.83% yield). Fraction 4 was applied to repeated RP-18 HPLC to afford **b3** (30 mg, 5% yield). Fraction 7 was chromatographed on an ODS column using MeOH–H_2O (7:3, 6:4, 5:5) as eluent, followed by RP-18 HPLC to afford **b7** (6 mg, 1% yield), **b8** (16 mg, 2.67% yield) and **b9** (23 mg, 3.83% yield). Fraction 8 was subjected to repeated RP-18 HPLC to give **b6** (15 mg, 2.5% yield). From fraction 9, **b5** (130 mg, 21.7% yield) was gotten through recrystallization using methanol.

2.6.1. Metabolite *a7*

Colorless needle crystals (MeOH); mp 218–219 °C; $[\alpha]_D^{25}$ + 146.5 (*c* 0.83, MeOH); UV (MeOH) λ_{max} (log ε) 211 (4.10) nm; IR ν_{max}^{KBr} cm^{-1}: 3453, 2939, 1757, 1636, 1401, 1198, 1080, 1040, 920; HRESIMS (positive) *m/z*: 371.1825 $[M+Na]^+$ (calcd. for $C_{20}H_{28}O_5Na$, 371.1829); ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5), see Tables 1 and 4.

2.6.2. Metabolite *b2*

Colorless plate crystals (MeOH); mp 144–145 °C; $[\alpha]_D^{25}$ − 24.4 (*c* 0.16, MeOH); UV (MeOH) λ_{max} (log ε) 215 (4.21) nm; IR ν_{max}^{KBr} cm^{-1}: 3457, 2952, 2856, 1752, 1705, 1651, 1452, 1395, 1052; HRESIMS (positive) *m/z*: 371.1828 $[M+Na]^+$ (calcd. for $C_{20}H_{28}O_5Na$, 371.1829); ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5), see Tables 2 and 4.

2.6.3. Metabolite *b6*

Colorless cubic crystals (MeOH); mp 231–232 °C; $[\alpha]_D^{25}$ − 46.3 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 213 (3.89) nm; IR ν_{max}^{KBr} cm^{-1}: 3282, 2971, 2933, 2892, 2861, 1757, 1079, 1058, 1036, 994; HRESIMS (positive) *m/z*: 373.1981 $[M+Na]^+$ (calcd. for $C_{20}H_{30}O_5Na$, 373.1985); ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5), see Tables 3 and 4.

2.6.4. Metabolite *b9*

White powder (MeOH); mp 203–204 °C; $[\alpha]_D^{25}$ − 38.1 (*c* 0.16, MeOH); UV (MeOH) λ_{max} (log ε) 216 (3.96) nm; IR ν_{max}^{KBr} cm^{-1}: 3443, 2947, 1731, 1639, 1451, 1401, 1083, 1062, 1035, 911; HRESIMS (positive) *m/z*: 373.1981 $[M+Na]^+$ (calcd. for $C_{20}H_{30}O_5Na$, 373.1985); ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5), see Tables 3 and 4.

Table 1
^1H NMR (δ) spectroscopic data (600 MHz, pyridine-d_5, *J* in Hz) of **a**, **a6** and **a7**.

Position	a	a6	a7
1	1.52 m	1.96 m	1.63 m
	1.15 td (12.8, 4.0)	1.38td (13.5, 3.2)	1.67 td (13.1, 3.6)
2	2.04 td (12.8,4.0)	2.06 m	2.08 td (13.0, 3.2)
	1.99 m	1.99 o	2.05 m
3	3.64 br d (10.8)	3.67o	3.57 br d (11.0)
4	–	–	–
5	1.22 dd (12.8, 2.4)	1.27br d (12.6)	1.18 dd (12.8, 2.5)
6	1.93 m	1.81 br d (12.0)	1.77 m
	1.41 qd (13.0, 4.2)	1.32 m	1.51 qd (13.0, 4.2)
7	2.40 m	2.37 br d (12.8)	2.36 br d (13.0)
	1.78 m	1.99 o	1.89 td (13.0, 5.1)
8	–	–	–
9	2.38 brd (10.4)	2.00o	–
10	–	–	–
11	7.17 dd (16.0, 10.4)	2.40 m	5.76 d (9.1)
		2.23 m	
12	6.26 d (16.0)	5.05 dd (9.6, 5.1)	5.89 d (9.1)
13	–	–	–
14	7.32 t (2.0)	7.68 br s	7.68 br s
15	4.78 br s	4.83 br s	4.81 br s
16	–	–	–
17	4.86 d (1.6)	4.98 br s	5.12 br s
	4.75 d (1.6)	4.95 br s	5.08 br s
18	1.52 s	1.47 s	1.45 s
19	4.47 d (10.8)	4.48 d (10.8)	4.51 d (11.0)
	3.67 d (10.8)	3.67 o	3.07 d (11.0)
20	0.89 s	0.77 s	1.02 s

Assignments based on HSQC and HMBC. "o" indicates overlapped.

2.7. Bioassay for NO production

Mouse monocyte-macrophage RAW 264.7 cells (ATCC TIB-71) were purchased from the Chinese Academy of Science. RPMI 1640 medium, penicillin, streptomycin, and fetal bovine serum were purchased from Invitrogen (New York). Lipopolysaccharide (LPS), dimethylsufoxide (DMSO), 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT), and hydrocortisone were obtained from Sigma Co. RAW 264.7 cells were suspended in RPMI 1640 medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% heat-inactivated fetal bovine serum. The cells were harvested with trypsin and diluted to a suspension in fresh medium. The cells were seeded in 96-well plates with 1 × 105 cells/well and allowed to adhere for 2 h at 37 °C in 5% CO_2 in air. Then, the cells were treated with 1 μg/mL of LPS for 24 h with or without various concentrations of test compounds. DMSO was used as a solvent for the test compounds, which were applied at a final concentration of 0.2% (v/v) in cell culture supernatants. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using Griess reagent [19]. Briefly, 100 μL of the supernatant from incubates was mixed with an equal volume of Griess reagent (0.1% *N*-[1-naphthyl]ethylenediamine and 1% sulfanilamide in 5% H_3PO_4). Cytotoxicity was determined by the MTT colorimetric assay, after 24 h incubation with test compounds. The concentration of NO_2^- was calculated by a working line from 0, 1, 2, 5, 10, 20, 50, and 100 μM sodium nitrite solutions, and the inhibitory rate on NO production induced by LPS was calculated by the NO_2^- levels as follows:

$$\text{Inhibitory rate}\,(\%) = 100 \times \frac{[NO_2^-]_{LPS} - [NO_2^-]_{LPS+sample}}{[NO_2^-]_{LPS} - [NO_2^-]_{untreated}}$$

Experiments were performed in triplicate, and data are expressed as the mean ± SD of three independent experiments.

Table 2
^{1}H NMR (δ) spectroscopic data (600 MHz, pyridine-d_5, J in Hz) of **b**, **b1**and **b2**.

Position	**b**	**b1**	**b2**
1	1.70 m	2.00 ddd (13.0, 5.6, 2.9)	2.04 ddd (13.0, 5.5, 3.2)
	1.13 td (12.8, 5.2)	1.53 td (13.9, 4.7)	1.44 o
2	2.01 td (12.8, 3.6)	2.87 td (14.5, 5.8)	2.88 td (14.5, 5.7)
	1.96 m	2.39 dt (14.8, 3.7)	2.38 ddd (14.5, 4.0, 3.2)
3	3.63o	–	–
4	–	–	–
5	1.20 dd (12.8, 2.4)	1.63 dd (12.4, 2.8)	1.62 dd (12.4, 2.2)
6	1.81 m	1.79 m	1.99 td (13.0, 3.7)
	1.33qd (12.9, 4.1)	1.74 qd (12.9, 3.6)	1.76 ddd (10.5, 4.3, 2.4)
7	2.37 dt (12.9, 4.1)	1.90 dt (12.6, 2.9)	1.88 td (13.4, 4.5)
	1.93 m	1.43 dt (12.6, 2.9)	1.39 o
8	–	–	–
9	1.63 t (4.8)	1.58 t (4.2)	1.42 o
10	–	–	–
11	1.78 m	1.48 o	1.46 o
	1.65 m	1.20 m	1.20 m
12	2.50 m	2.54 d (4.2)	2.29 o
	2.18 m	2.37 d (4.2)	
13	–	–	–
14	7.19 t (1.5)	7.23 br s	7.25 br s
15	4.73d (1.5)	4.72 br s	4.77 d (1.6)
16	–	–	–
17	4.92 br s	2.84 d (4.4)	2.87 d (4.2)
	4.74 br s	2.57 d (4.4)	2.36 d (4.2)
18	1.49 s	1.46 s	1.48 s
19	4.45 d (10.0)	4.31 d (11.0)	4.35 d (11.0)
	3.63 o	3.88 d (11.0)	3.88 d (11.0)
20	0.69 s	1.09 s	1.28 s

Assignments based on HSQC and HMBC. "o" indicates overlapped.

3. Results and discussion

3.1. Identification of biotransformation products

Metabolite **a7** was obtained as colorless needle crystals (MeOH), and was positive for the Legal and Kedde reactions, suggesting the presence of an α, β-unsaturated lactone [20]. The HRESIMS exhibited the quasi-molecular ion peak $[M+Na]^+$ at m/z 371.1825, accordingly to its molecular formula of $C_{20}H_{28}O_5$ in combination with the ^{1}H and ^{13}C NMR spectroscopic data (Tables 1 and 4). Comparison of the ^{13}C NMR data of **a7** with those of the substrate (**a**) [21] showed that the absence of $\Delta^{11,12}$ double bond at δ_C 135.6 and 121.9 in **a**, and the appearance of two sp^2-hybrid signals at δ_C 153.8 and 120.6 and one oxygenated carbon signal at δ_C 64.2 in **a7**. In the ^{1}H NMR spectrum of **a7**, the *trans*-olefinic protons at δ_H 7.17 and 6.26 disappeared comparing to the substrate

Table 3
^{1}H NMR (δ) spectroscopic data (600 MHz, pyridine-d_5, J in Hz) of **b6**, **b8** and **b9**.

Position	**b6**	**b8**	**b9**
1	1.96 br d (13.0)	1.97 td (13.9, 3.2)	2.25 td (12.7, 5.5)
	1.40 td (13.0, 3.8)	1.14 td (13.3, 3.3)	1.64 br d (12.9)
2	2.09 td (13.0, 2.5)	2.04 br d (13.3)	2.20 td (11.6, 3.9)
	2.02 o	1.95 dt (13.2, 3.2)	2.11 o
3	3.67 o	3.63 br d (10.7)	3.81 dd (10.5, 5.5)
4	–	–	–
5	1.34 br d (12.1)	1.37 dd (11.9, 4.9)	2.60 dd (13.0, 2.7)
6	1.91 m	2.15 dt (15.4, 4.1)	1.89 m
	1.55 m	2.05 m	1.44 qd (13.1, 4.4)
7	2.66 td (17.6, 5.7)	5.94 d (4.9)	2.87 td (12.8, 5.1)
	2.36 ddd (18.1,11.5, 6.8)		2.44 m
8	–	–	–
9	–	2.07 m	–
10	–	–	–
11	2.58 o	1.86 m	2.11 o
	2.25 m	1.71 m	2.11 o
12	2.58 br t (6.7)	3.01 m	2.85 m
		2.54 m	2.71 m
13	–	–	–
14	7.21 br s	7.25 br t (4.9)	7.18 br t (1.3)
15	4.76 br s	4.74 br s	4.74 dd (4.1, 1.3)
16	–	–	–
17	4.60 d (11.8)	4.54 d (12.4)	5.09 br s
	4.40 d (11.8)	4.35 d (12.4)	5.06 br s
18	1.56 s	1.50 s	1.56 s
19	4.53 d (10.6)	4.58 d (10.9)	4.64 d (10.8)
	3.70 d (10.6)	3.81 d (10.9)	3.75 d (10.8)
20	1.05 s	0.83 s	0.96 s

Assignments based on HSQC and HMBC. "o" indicates overlapped.

Table 4
^{13}C NMR (δ) spectroscopic data (150 MHz, pyridine-d_5) of **a**, **a6**, **a7**, **b**, **b1**, **b2**, **b6**, **b8** and **b9**.

Position	a	a6	a7	b	b1	b2	b6	b8	b9
1	38.7	37.8	36.0	37.2	38.6	38.7	35.5	38.1	31.0
2	28.9	29.5	29.4	29.0	36.2	36.2	29.4	29.1	29.5
3	80.1	80.4	80.6	79.9	214.1	214.3	80.5	80.9	80.5
4	43.4	43.7	44.0	43.3	55.3	55.4	43.8	42.9	43.7
5	54.8	56.0	53.7	55.4	56.9	57.0	52.5	51.3	46.9
6	23.6	25.1	24.1	24.6	23.3	21.9	19.9	24.2	25.0
7	37.0	39.1	37.9	38.6	37.1	36.6	30.5	124.8	34.5
8	149.3	149.8	145.8	147.9	59.1	57.2	133.2	140.6	151.2
9	61.8	53.0	153.8	56.5	53.5	52.2	141.7	52.5	79.3
10	39.0	40.2	41.2	39.4	40.6	40.1	39.6	37.0	44.1
11	135.6	32.5	120.6	22.3	20.9	20.9	26.4	25.9	29.6
12	121.9	66.5	64.2	25.0	27.9	27.9	28.6	27.8	22.1
13	128.9	138.7	138.4	134.1	134.3	133.9	134.3	134.7	135.2
14	145.0	147.4	147.8	145.4	146.0	146.5	145.8	146.2	145.6
15	70.3	71.3	71.1	70.6	71.1	71.2	71.2	71.1	71.2
16	172.8	174.1	173.6	174.6	175.1	175.1	175.1	175.2	175.4
17	108.8	107.6	114.3	107.2	50.9	49.1	62.8	65.8	110.4
18	23.6	24.1	24.1	23.7	21.8	21.6	24.1	23.8	24.5
19	64.2	64.7	64.8	64.2	65.7	65.7	64.9	64.7	65.0
20	16.0	16.0	22.4	15.3	15.3	15.4	21.4	15.3	18.1

Assignments based on HSQC and HMBC.

(**a**), and there were two resonances at δ_H 5.76 (1H, d, J = 9.1 Hz) and 5.89 (1H, d, J = 9.1 Hz), which connected with the carbon signals at δ_C 120.6 and 64.2, respectively, on the basis of the HSQC spectrum. The above NMR data of **a7** indicated the rearrangement of $\Delta^{11,12}$ double bond to $\Delta^{9,11}$ and the hydroxylation at C-12. The HMBC correlations of H-14 (δ_H 7.68) with C-12 (δ_C 64.2); H-11 (δ_H 5.76) with C-10 (δ_C 41.2), C-12 (δ_C 64.2), C-13 (δ_C 138.4), and C-8 (δ_C 145.8); H-12 (δ_H 5.89) with C-11 (δ_C 120.6), C-13 (δ_C 138.4), C-14 (δ_C 147.8), C-9 (δ_C 153.8), and C-16 (δ_C 173.6); and of CH_3-20 (δ_H 1.02), H-5 (δ_H 1.18), H-7a (δ_H 2.36), and H_2-17 (δ_H 5.12, 5.08) with C-9 (δ_C 153.8) further confirmed that the hydroxyl group was linked to C-12 and the double bond located at C-9 and C-11 in **a7**. For the C-12 chirality of **a7**, the ^{13}C NMR values were calculated at the B3LYP/6-311+G(2d,p)//3LYP/6-31G(d) using GIAO method after low energy conformation search via Hperchem package [22]. The magnetic shielding values were finally converted into chemical shifts [23,24]. The relative errors between the computed and experimental data are summarized in Table S1 and Fig. 2S in the Supplementary Information. Based on the evidence of the relative error magnitudes, the favorable configuration on C-12 for **a7** should be 12*S*. Therefore, **a7** was identified as 3α, 12*S*, 19-trihydroxy-8(17), 9(11)-*ent*-labdadien-16, 15-olide (Fig. 1).

Metabolite **b2**, colorless plate crystals (MeOH), was positive for the Legal and Kedde reactions, suggesting the presence of an α, β-unsaturated lactone [20]. The molecular formula $C_{20}H_{28}O_5$ was drawn from its HRESIMS which gave the ion peak $[M+Na]^+$ at m/z 371.1828, and its ^{1}H and ^{13}C NMR spectroscopic data (Tables 2 and 4). The ^{13}C NMR spectra of **b2** and **b1** [18] were very similar, except the signals at C-6, C-7, C-8, C-9, C-10, and C-17 shifted upfield to δ_C 21.9, 36.6, 57.2, 52.2, 40.1, and 49.1, respectively. The HMBC correlations of H_2-17 (δ_H 2.87, 2.36) with C-7 (δ_C 36.6), C-8 (δ_C 57.2), and C-9 (δ_C 52.2) confirmed the existence of 8, 17-epoxy group. Therefore, **b2** could be the 8-epimer of **b1**. This was further corroborated by the obvious NOESY correlations between H_2-17 (δ_H 2.87, 2.36) and H-7β (δ_H 1.88), H-9 (δ_H 1.42), and H-11b (δ_H 1.20), and the absence of correlations between H_2-17 (δ_H 2.87, 2.36) and CH_3-20 (δ_H 1.28). On the basis of the above evidence, **b2** was established as 3-oxo-8α, 17β-epoxy-14-deoxyandrographolide (Fig. 2).

Metabolite **b6** was obtained as colorless cubic crystals (MeOH), and was positive for the Legal and Kedde reactions, suggesting the presence of an α, β-unsaturated lactone [20]. The HRESIMS exhibited the quasi-molecular ion peak $[M+Na]^+$ at m/z 373.1981, accordingly to its molecular formula of $C_{20}H_{30}O_5$ by combining the ^{1}H and ^{13}C NMR spectroscopic data (Tables 3 and 4). Comparison of the ^{13}C NMR data of **b6** with those of the known isodeoxyandrographolide [25] showed that the absence of one methyl signal at δ_C 20.4 in isodeoxyandrographolide, and the appearance of one hydroxymethyl signal at δ_C 62.8 in **b6**. The HSQC spectrum exhibited that the proton resonances at δ_H 4.60 (1H, d, J = 11.8 Hz) and 4.40 (1H, d, J = 11.8 Hz) correlated to the hydroxymethyl carbon signal at δ_C 62.8. The HMBC correlations of the proton resonances at δ_H 4.60 and 4.40 with C-7 (δ_C 30.5), C-8 (δ_C 133.2) and C-9 (δ_C 141.7) confirmed that the hydroxyl group was linked to C-17 in **b6**. The location of $\Delta^{8,9}$ double bond was determined by means of the HMBC correlations of CH_3-20 (δ_H 1.05), H-1b (δ_H 1.40), H_2-7 (δ_H 2.66, 2.36), H_2-11 (δ_H 2.58, 2.25), H_2-12 (δ_H 2.58), and H_2-17 (δ_H 4.60, 4.40) with C-9 (δ_C 141.7); and of H-6a (δ_H 1.91), H_2-7 (δ_H 2.66, 2.36), H_2-11 (δ_H 2.58, 2.25), and H_2-17 (δ_H 4.60, 4.40) with C-8 (δ_C 133.2). On the basis of the above evidence, **b6** was identified as 3α, 17, 19-trihydroxy-8, 13-*ent*-labdadien-16, 15-olide (Fig. 2).

Metabolite **b9**, white powder, was positive for the Legal and Kedde reactions, suggesting the presence of an α, β-unsaturated lactone [20]. The molecular formula of $C_{20}H_{30}O_5$ was inferred from positive HRESIMS analysis ($[M+Na]^+$ at m/z 373.1981) in combination with the ^{1}H and ^{13}C NMR spectroscopic data (Tables 3 and 4). By comparing the ^{13}C NMR data of **b9** with those of 14-deoxyandrographolide (**b**) [21], the carbon signal at δ_C 79.3 was an additional bearing-oxygen carbon, suggesting that **b9** might be a hydroxylation derivative of the substrate (**b**). The HMBC spectrum exhibited that the carbon signal at δ_C 79.5 correlated with H_2-17 (δ_H 5.09, 5.06), H-7b (δ_H 2.24), H_2-11 (δ_H 2.11) and CH_3-20 (δ_H 0.96), suggesting that the hydroxyl group was located at C-9. In the NOESY spectrum, the NOE enhancement between H_2-11 (δ_H 2.11) and CH_3-20 (δ_H 0.96) proved that 9-OH should be β-oriented [17]. Therefore, metabolite **b9** was characterized as 9β-hydroxy-14-deoxyandrographolide (Fig. 2).

In addition to four new metabolites, twelve other known ones, 3-oxo-8β, 17α-epoxydehydroandrographolide (**a1**) [16], 3-oxo-9β-hydroxydehydroandrographolide (**a2**) [17], 9β-hydroxydehydroandrographolide (**a3**) [17], 8β, 17α-epoxydehydroandrographolide (**a4**) [17], 3-oxo-dehydroandrographolide (**a5**) [17], 14-deoxy-12*R*-hydroxyandrographolide (**a6**) [26], 3-oxo-8β, 17α-epoxy-14-deoxyandrographolide (**b1**) [18], 8β, 17α-

Table 5
Inhibitory effects of compounds **a**, **a1-a7**, **b**, **b1-b9** on NO production induced by LPS in macrophages.[a]

Compound	$IC_{50} \pm SD$ (μM)	Compound	$IC_{50} \pm SD$ (μM)
a	94.12 ± 4.79	**b**	21.20 ± 1.66
a1	>100	**b1**	94.86 ± 6.85
a2	79.66 ± 5.88	**b2**	47.02 ± 1.32
a3	>100	**b3**	98.26 ± 7.12
a4	>100	**b4**	42.07 ± 3.81
a5	92.14 ± 5.37	**b5**	97.17 ± 6.33
a6	5.43 ± 0.65	**b6**	>100
a7	19.87 ± 0.88	**b7**	37.31 ± 2.80
Hydrocortisone[b]	40.64 ± 3.22	**b8**	44.44 ± 2.73
		b9	>100

[a] NO concentration of control group: 3.96 ± 0.29 μM. NO concentration of LPS-treated group: 34.78 ± 2.54 μM.
[b] Hydrocortisone was used as positive control.

epoxy-14-deoxyandrographolide (**b3**) [27], 3-oxo-14-deoxyandrographolide (**b4**) [27], 7*S*-hydroxy-14-deoxyandrographolide (**b5**) [28], 3-oxo-7*R*-hydroxy-14-deoxyandrographolide (**b7**) [18], 3α, 17, 19-trihydroxy-7, 13-*ent*-labdadien-16, 15-olide (**b8**) [18] were identified by comparison of their spectroscopic data with those reported in the literatures.

3.2. Inhibitory effects on NO production induced by LPS in macrophages

The substrates **a** and **b**, as well as their metabolites **a1**–**a7** and **b1**–**b9** were examined for their inhibitory effects on NO production induced by LPS in macrophages (Table 5). Compounds **a6**, **a7** and **b** showed strong inhibition of NO production induced by LPS. 14-Deoxy-12*R*-hydroxyandrographolide (**a6**) possessed the strongest effect, with an IC_{50} of 5.43 ± 0.65 μM. Metabolites **b2**, **b4**, **b7** and **b8** exhibited moderate activities, which were close to hydrocortisone and weaker than the substrate (**b**). While other compounds showed no activity. By comparison of the NO inhibitory effects of **a** and **b**, as well as their metabolites, it was found that the presence of $\Delta^{11,12}$ double bond on the side chain led to a marked decrease of the activity. The reason for the activity of **a7** being weaker than that of **a6** might be the presence of $\Delta^{10,11}$ double bond on the side chain or the difference of C-12 configuration. And the position of double bond in the B ring was important for the NO inhibitory effects. Metabolite **b8** with double bond between C-7 and C-8 was more effective than **b6** with double bond between C-8 and C-9. The position of hydroxyl could also influence the NO inhibitory effects. Metabolites **a6** and **a7** with 12-OH on the side chain exhibited stronger activity than **a2** and **a3** with 9-OH in the ring B. For the metabolites possessing an 8, 17-epoxy group, the orientation of the epoxy ring could affect the NO inhibitory effects. Metabolite **b2** with an 8α, 17β-epoxy presented stronger activity than metabolites **b1**, **b3**, **a1**, and **a4** with an 8β, 17α-epoxy.

4. Conclusion

C. blakesleana (AS 3.970) has good ability to transform 14-deoxy-11, 12-didehydroandrographolide and 14-deoxyandrographolide. The negative results of the control experiments (Section 2) in which both substrates were incubated with the medium alone indicated that the substrates were stable in the medium. By careful analysis of the structures of these metabolites, it is not hard to find that there are many kinds of enzymatic reactions happened during the biotransformation, such as hydroxylation, oxidation, epoxidation, hydration and dehydration. It might be related to the physical and chemical properties of the substrates and the diversity of the enzymes in the fungi. Time course analysis of the transformation of substrates **a** and **b** by *C. blakesleana* revealed that metabolites of hydroxylation and epoxidation (**a3**, **a4**, **a6** and **b3**, **b5**, **b9**) were the first products to be formed within 24 h of incubation. The C-3 oxidized metabolites of **a5** and **b4** were detected after 36 h of incubation. Metabolites **a1**, **a2**, **a7** and **b1**, **b2**, **b6**, **b7**, **b8** were found only after 48 h of incubation, and their contents increased with the extension of incubation time. Furthermore, incubation of metabolites **a5** and **b4** with *C. blakesleana* resulted in the production of metabolites **a1**, **a2**, and **b1**, **b2**, **b7**, respectively, suggesting that the epoxidation reaction caused by *C. blakesleana* was lack of stereoselectivity. The possible metabolic pathways for **a** and **b** with *C. blakesleana* were speculated as shown in Figs. 1 and 2.

Some metabolites showed stronger inhibitory effects on nitric oxide production induced by LPS in macrophages than the substrates and the positive drug. Their preliminary structure-activity relationship (SAR) results provided useful clues for the research and development of 14-deoxy-11, 12-didehydroandrographolide and 14-deoxyandrographolide as anti-inflammatory remedies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.06.012.

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