Journal of Molecular Catalysis B: Enzymatic 68 (2011) 83–88

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Microbial transformation of neoandrographolide by *Mucor spinosus* (AS 3.2450)

Yixiong Wang[a,b], Lixia Chen[a,b,*], Feng Zhao[c], Zhihui Liu[a,b], Jianqiang Li[a,b], Feng Qiu[a,b,*]

[a] Department of Natural Products Chemistry, School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University 81#, No. 103 Road WenHua, Shenyang 110016, People's Republic of China
[b] Key Laboratory of Structure-Based Drug Design & Discovery, Ministry of Education, Shenyang Pharmaceutical University, Shenyang 110016, People's Republic of China
[c] School of Pharmacy, Yantai University, No. 32 Road QingQuan, Laishan District, Yantai 264005, People's Republic of China

ARTICLE INFO

Article history:
Received 21 June 2010
Received in revised form 28 August 2010
Accepted 28 September 2010
Available online 23 October 2010

Keywords:
Neoandrographolide
Microbial transformation
Mucor spinosus
Nitric oxide

ABSTRACT

Microbial transformation of neoandrographolide (**1**), was performed by *Mucor spinosus* (AS 3.2450). Ten metabolites were obtained and identified as 14-deoxyandrographolide (**2**), 17,19-dihydroxy-8,13-*ent*-labdadien-16,15-olide (**3**), 3,14-dideoxyandrographolide (**4**), 7β-hydroxy-3,14-dideoxyandrographolide (**5**), 17,19-dihydroxy-7,13-*ent*-labdadien-16,15-olide (**6**), 8(17),13-*ent*-labdadien-16,15-olid-19-oic acid (**7**), 8α,17β-epoxy-3,14-dideoxyandrographolide (**8**), 8β,17,19-trihydroxy-*ent*-labd-13-en-16, 15-olide (**9**), phlogantholide-A (**10**), 19-[(β-D-glucopyranosyl)oxy]-19-oxo-*ent*-labda-8(17),13-dien-16,15-olide (**11**) by spectroscopic and chemical means. Among them, products **3**, **5**, **6**, **8** and **9** were characterized as new compounds. The inhibitory effects of compounds **1**–**11** on nitric oxide production in lipopolysaccharide-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 Chinese herbal medicine used as an anti-inflammatory and antipyretic drug for treatment of fever, cold, laryngitis and diarrhea. The pharmacology researches show that diterpenoid lactones are the active component of *A. paniculata* (Burm.f.) Nees and possess lots of bioactive effects [1]. Neoandrographolide (Fig. 1) is one of the principal constituents of *ent*-labdane diterpenoid lactones and has many activities, such as anti-inflammatory [2–4], antiviral [5], anti-radical [6], hepatoprotective [7] and anti-human immunodeficiency virus (HIV) effects [8]. Some evidences [9,10] indicated that the *ent*-labdane diterpenoid bicyclo-skeleton and α,β-unsaturated lactone ring were the primary active structure of pyretolysis and anti-inflammatory. There were many studies on the structure modifications of andrographolide, one of the principal diterpenoids, through biological [11–13] and chemical methods [14,15], and the two derivatives, sodium 14-deoxy-12(*R*)-sulfoandrographolide (Lianbizhi) and monopotassium 14-deoxy-11,12-didehydroandrographolide-3,19-disuccinate (Chuanhuning) have been developed into antibacterial and antiviral drugs in China. However, few research of modification of neoandrographolide was reported previously.

Microbial transformation is an important tool for structure modification of organic compounds, especially natural products with complicated structures [16,17]. And this approach has some advantages over organic synthesis such as high stereo- and region-selectivity. Some transformation reactions such as hydroxylation at specific positions are difficult for chemical synthesis, but could be readily accomplished with microbial transformation [18,19]. In our previous study [20], we had reported five biotransformation products of neoandrographolide by *Aspergillus niger*. As an ongoing investigation, the present study attempts to get more types of diterpenoid derivatives by using of different fungi, and tries to find out some compounds with better activity than the substrate through pharmacological experiments.

In this work, twenty-seven kinds of fungi were screened for the bioconversion of neoandrographolide (**1**) in our experiments. Among them, *Mucor spinosus* (AS 3.2450) showed good ability to convert **1**, thus it was selected as biocatalyst for scaled-up biotransformation. Ten products were isolated from the fermentation broths. Metabolites **3**, **5**, **6**, **8** and **9** were identified as new compounds on the basis of their ^{1}H and ^{13}C NMR, DEPT, HSQC, HMBC, NOESY, and HRESIMS experiments. And the five known metabolites **2**, **4**, **7**, **10** and **11** were determined by comparison of the NMR data with those of the reported compounds [21–25]. The inhibitory effects of compounds **1**–**11** on nitric oxide production in lipopolysaccharide-activated macrophages were evaluated and their preliminary structure–activity relationships (SAR) were discussed.

* Corresponding authors at: Department of Natural Products Chemistry, School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University 81#, No. 103 Road WenHua, Shenyang 110016, People's Republic of China. Tel.: +86 24 23986463; fax: +86 24 23993994.
E-mail addresses: syzyclx@163.com (L. Chen), fengqiu2000@tom.com (F. Qiu).

1381-1177/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2010.09.016

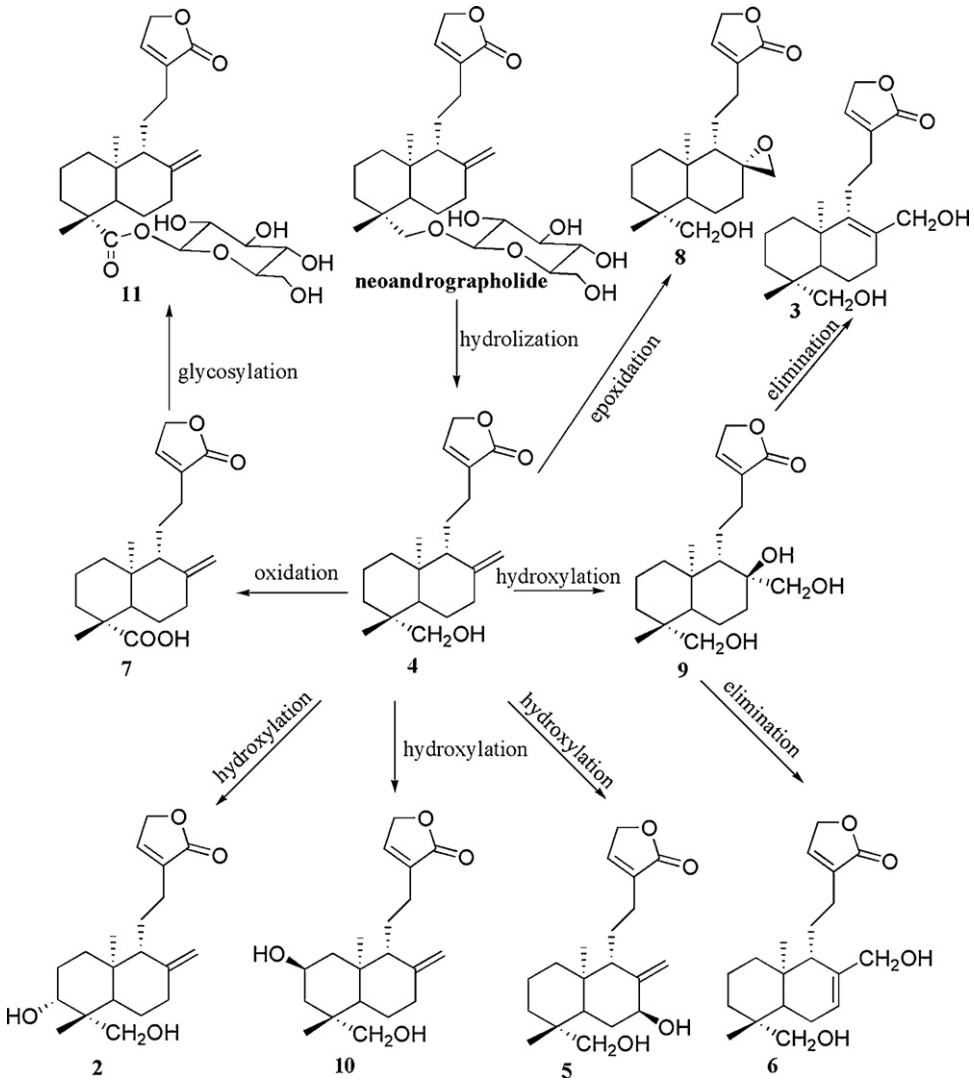

Fig. 1. Structures of products and their proposed biotransformation pathways.

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 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. 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 (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), Sephadex LH-20 (Pharmacia Co., Ltd., USA), 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. Substrate

Neoandrographolide (>98%) was isolated from the aerial parts of *A. paniculata* (Burm.f.) Nees by ourselves, and was characterized by comparison of the NMR data with the reference.

2.3. Microorganisms

M. spinosus (AS 3.2450) 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 mincing husked potato was boiled in 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.

Table 1
^{1}H NMR (δ) spectroscopic data (600 MHz, pyridine-d_5, J in Hz) of metabolites 3, 5, 6, 8 and 9[a].

Position	3	5	6	8	9
1	1.94 m	1.77 m	1.93 br d (12.3)	1.78 br d (13.1)	1.77 br d (12.6)
	1.27 m	1.17 td (12.6, 3.4)	0.99 td (13.4, 3.5)	0.96 td (13.1, 3.6)	1.07 td (13.2, 3.6)
2	1.68 dt (13.7)	1.73 m	1.61 br d (13.7)	1.63 ddt (13.8, 3.0)	1.64 m
	1.47 dt (13.8)	1.47 dt (14.0, 3.2)	1.39 m	1.41 m	1.41 tt (13.8, 3.6)
3	2.22 m	2.26 o[b]	2.28 br t (11.8)	2.24 br d (13.8)	2.15 br d (13.2)
	1.01 m	1.08 td (13.7, 3.8)	0.95 td (13.3, 3.1)	1.04 br d (13.8)	1.01 td (13.2, 3.6)
4	–	–	–	–	–
5	1.38 br d (12.4)	2.26 br d (11.2)	1.42 m	1.19 m	1.23 br d (10.8)
6	1.97 m	2.30 o[b]	1.85 m	1.81 m	1.88 m
	1.59 m	1.78 m	1.70 m		1.52 m
7	2.66 m	4.63 br s	5.94 br s	1.86 m	2.65 br d (10.8)
	2.39 m			1.38 m	1.54 m
8	–	–	–	–	–
9	–	2.64 br d (11.1)	2.13 o[b]	1.36 br s	1.62 br s
10	–	–	–	–	–
11	2.58 o[b]	1.86 m	2.11 o[b]	1.46 m	2.12 m
	2.24 m	1.65 m		1.13 m	1.60 m
12	2.58 o[b]	2.56 br t(10.6)	3.01 t (11.9)	2.27 br t, 7.6	2.84 br t (10.8)
		2.18 m	2.55 m		2.55 br t (10.8)
13	–	–	–	–	–
14	7.18 br s	7.17 br s	7.21 o[b]	7.21 o[b]	7.16 br t (13.2)
15	4.74 br s	4.70 br s	4.73 br s	4.76 br s	4.71 br s
16	–	–	–	–	–
17	4.58 d (11.8)	5.15 br s	4.55 d (12.3)	2.81 d (4.2)	3.97 d (10.8)
	4.37 d (11.8)	4.83 br s	4.35 d (12.2)	2.30 d (4.2)	3.87 d (10.8)
18	1.25 s	1.30 s	1.19 s	1.24 s	1.21 s
19	4.05 d (10.7)	4.05 d (10.6)	4.09 d (10.4)	4.05 dd (10.6, 4.6)	3.93 d (10.2)
	3.69 d (10.7)	3.69 d (10.6)	3.73 d (10.4)	3.69 dd (10.6, 4.5)	3.67 d (10.2)
20	1.04 s	0.79 s	0.84 s	1.01 s	0.84 s

[a] Assignments based on HSQC and HMBC.
[b] "o" indicates overlapped.

2.5. Biotransformation of neoandrographolide (**1**) by M. spinosus (AS 3.2450)

Preparative scale biotransformation of neoandrographolide by *M. spinosus* (AS 3.2450) was carried out in 500 mL Erlenmeyer flasks containing 150 mL of potato medium. The flasks were placed on rotary shakers, operating at 180 rpm at 28 °C. After 48 h of culture, the substrate solution was added into the fermentation flasks, which was dissolved in ethanol with a concentration of 15 mg/mL, and total of 1 g of **1** was transformed by the strain. The fermentation was maintained under the same conditions for an additional 72 h. When the fermentation finished, the broths 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.

2.6. Isolation and purification of metabolites

The crude residues were subjected to column chromatography (CC) over silica gel and eluted with the mixtures of cyclohexane–acetoacetate (70:1, 3:1, 2:1, 1:1, 2:3, 0:100), then eluted with mixtures of acetoacetate–methanol (22:1, 0:100), which yielded eight fractions (1–8). Fraction 2 was submitted to ODS column chromatography eluted with MeOH–H_2O (9:1; 8:2; 7:3), and then purified by repeated RP-18 HPLC preparation to give **4** (30 mg) and **7** (22.6 mg). Fraction 4 was recrystallized from acetoacetate to afford **2** (50 mg). The mother liquor of **2** was applied to Sephadex LH-20 ($CHCl_3$–MeOH, 1:1), and the diterpenoid-containing fraction was further separated by RP-18 HPLC to afford **5** (21 mg), **6** (7.4 mg), and **8** (6.2 mg). Fraction 5 was subjected to ODS column chromatography eluted with MeOH–H_2O (8:2; 7:3), and then purified by RP-18 HPLC to yield 3 (17 mg). Fractions 4 and 7 were combined, evaporated, and chromatographed on a Sephadex LH-20 column ($CHCl_3$–MeOH, 1:1), and the diterpenoid-containing fraction was re-subjected to ODS column chromatography using MeOH–H_2O (7:3; 6:4; 5:5) as eluent, followed by RP-18 HPLC to afford **9** (8.4 mg), **10** (9 mg) and **11** (16 mg).

Metabolite **3**: white powder; $[\alpha]_D^{25}$ −21.1 (*c* 0.83, MeOH); UV (MeOH) λ_{max} (log ε) 206 (4.14) nm; IR ν_{max}^{KBr} cm^{-1}: 3385, 2928, 1742, 1648, 1447, 1205, 1054, 1022; HRESIMS (positive) *m*/*z*: 357.2031 [M+Na]$^+$ (calcd. for $C_{20}H_{30}O_4Na$, 357.2036); ^{1}H NMR spectroscopic data (600 MHz, pyridine-d_5), see Table 1; ^{13}C NMR spectroscopic data (150 MHz, pyridine-d_5), see Table 2.

Metabolite **5**: white powder; $[\alpha]_D^{25}$ +13.1 (*c* 0.92, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.18) nm; IR ν_{max}^{KBr} cm^{-1}: 3375, 2928, 1742, 1648, 1446, 1204, 1054, 1021, 904; HRESIMS (positive) *m*/*z*:

Table 2
^{13}C NMR (δ) spectroscopic data (150 MHz, pyridine-d_5) of metabolites 3, 5, 6, 8 and 9[a].

Position	3	5	6	8	9
1	37.4	39.4	39.9	39.4	40.6
2	19.5	19.9	19.3	19.1	19.1
3	36.3	36.6	36.4	36.4	36.6
4	39.8	39.7	37.4	39.8	39.6
5	53.1	48.8	51.6	56.2	57.6
6	19.8	32.5	25.9	21.0	21.2
7	30.3	73.6	125.1	37.0	39.6
8	132.9	151.9	140.7	57.5	75.5
9	142.1	51.2	52.6	53.0	61.2
10	39.7	40.5	39.0	40.5	39.6
11	26.2	22.0	24.0	20.6	24.0
12	28.5	25.0	27.7	28.0	29.7
13	134.3	134.5	134.7	134.1	135.0
14	145.7	145.8	146.2	146.2	145.5
15	71.0	70.9	71.1	71.1	71.0
16	175.0	175.0	175.2	175.0	175.2
17	62.9	108.4	66.0	49.1	63.4
18	28.2	28.3	28.1	28.4	28.4
19	64.5	64.4	64.2	64.3	64.5
20	21.3	15.0	15.4	16.0	17.0

[a] Assignments based on HSQC and HMBC.

357.2035 [M+Na]$^+$ (calcd. for $C_{20}H_{30}O_4Na$, 357.2036); ^{1}H NMR spectroscopic data (600 MHz, pyridine-d_5), see Table 1; ^{13}C NMR spectroscopic data (150 MHz, pyridine-d_5), see Table 2.

Metabolite **6**: white powder; $[\alpha]_D^{25}$ −35.7 (*c* 0.33, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.41) nm; IR ν_{max}^{KBr} cm^{-1}: 3361, 2928, 1741, 1449, 1205, 1053, 1032; HRESIMS (positive) *m*/*z*: 357.2033 [M+Na]$^+$, (calcd. for $C_{20}H_{30}O_4Na$, 357.2036); ^{1}H NMR spectroscopic data (600 MHz, pyridine-d_5), see Table 1; ^{13}C NMR spectroscopic data (150 MHz, pyridine-d_5), see Table 2.

Metabolite **8**: white powder; $[\alpha]_D^{25}$ −37.7 (*c* 0.35, MeOH); UV (MeOH) λ_{max} (log ε) 209 (3.80) nm; IR ν_{max}^{KBr} cm^{-1}: 3405, 2926, 1746, 1448, 1205, 1054, 1031, 1019; HRESIMS (positive) *m*/*z*: 357.2031 [M+Na]$^+$, (calcd. for $C_{20}H_{30}O_4Na$, 357.2036); ^{1}H NMR spectroscopic data (600 MHz, pyridine-d_5), see Table 1; ^{13}C NMR spectroscopic data (150 MHz, pyridine-d_5), see Table 2.

Metabolite **9**: white powder; $[\alpha]_D^{25}$ +4.9 (*c* 0.45, MeOH); UV (MeOH) λ_{max} (log ε) 208 (3.95) nm; IR ν_{max}^{KBr} cm^{-1}: 3381, 2933, 1741, 1450, 1206, 1053, 1020; HRESIMS (positive) *m*/*z*: 375.2143 [M+Na]$^+$, (calcd. for $C_{20}H_{32}O_5Na$, 375.2142); ^{1}H NMR spectroscopic data (600 MHz, pyridine-d_5), see Table 1; ^{13}C NMR spectroscopic data (150 MHz, pyridine-d_5), see Table 2.

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 1×10^5 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 [26]. 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.

3. Results and discussion

Twenty-seven kinds of fungi were screened for the bioconversion of neoandrographolide (**1**) in our experiments. Among them, *M. spinosus* (AS 3.2450) showed good ability to convert **1**, thus it was selected as biocatalyst for scaled-up biotransformation. The inhibitory effects on nitric oxide production in lipopolysaccharide-activated macrophages of compounds **1**–**11** were evaluated and their structure–activity relationships (SAR) were discussed.

3.1. Identification of biotransformation products

Metabolite **3** was obtained as white powder and was positive for the Legal and Kedde reactions, suggesting the presence of an α,β-unsaturated lactone [27]. The HRESIMS exhibited the quasi-molecular ion [M+Na]$^+$ at *m*/*z* 357.2031, accordingly to its molecular formula of $C_{20}H_{30}O_4$ in combination with the ^{1}H and ^{13}C NMR spectroscopic data (Tables 1 and 2), indicating that **3** should be the aglycone derivative of **1**. Comparison of the ^{13}C NMR data of **3** with those of the known compound 8-methylandrograpanin [28] showed that the absence of one methyl signal at δ_C 19.7 in 8-methylandrograpanin, and the appearance of one hydroxymethyl signal at δ_C 62.9 in **3**. The HSQC spectrum exhibited that the proton signals at δ_H 4.37 and 4.58 correlated to the hydroxymethyl carbon signal at δ_C 62.9. The HMBC correlations of δ_H 4.37 and 4.58 with C-7 (δ_C 30.3), C-8 (δ_C 132.9) and C-9 (δ_C 142.1) confirmed that –OH group was linked to C-17 in **3**. The double bond located at C-8 and C-9 positions was determined by means of the HMBC correlations of CH_3-20 (δ_H 1.04), H_2-7 (δ_H 2.66, 2.39), H-11(δ_H 2.24), H-12 (δ_H 2.58), and H_2-17 (δ_H 4.58, 4.37) with C-9 (δ_C 142.1), and of H-6 (δ_H 1.97), H-7 (δ_H 2.66, 2.39), H_2-11 (δ_H 2.58, 2.24), and H_2-17 (δ_H 4.58, 4.37) with C-8 (δ_C 132.9), respectively. On the basis of the above evidence, **3** was identified as 17,19-dihydroxy-8,13-*ent*-labdadien-16,15-olide (Fig. 1).

Metabolite **5**, white powder, was positive for the Legal and Kedde reactions, suggesting the presence of an α,β-unsaturated lactone [27]. The molecular formula of **5** was established as $C_{20}H_{30}O_4$ according to the ion [M+Na]$^+$ at *m*/*z* 357.2035 [M+Na]$^+$ in HRESIMS, suggesting that **5** should be the aglycone derivative of **1** by combining the ^{1}H and ^{13}C NMR spectroscopic data (Tables 1 and 2). Comparison of the ^{13}C NMR and DEPT spectra of **5** with those of **4** [22] showed that the absence of one –CH_2 signal at δ_C 39.3 (C-7) in **4**, and the appearance of one –CH signal at δ_C 73.6 in **5**, suggesting the hydroxylation of C-7. This was further confirmed by the HMBC correlations of H-7 (δ_H 4.63) with C-5 (δ_C 48.8), C-9 (δ_C 51.2) and C-17 (δ_C 108.4). The ^{1}H NMR spectrum of **5** showed a broad singlet (brs) for H-7, which is different from the 7-epimer displaying double doublet (dd) [29], suggesting the β-orientation for 7-OH in **5**. In the NOESY spectrum, the presence of correlations of H-7 (δ_H 4.63) with 20-CH_3 (δ_H 0.79), H-6α (δ_H 2.30) and H-6β (δ_H 1.78), and the absence of correlation of H-7 (δ_H 4.63) with H-5 (δ_H 2.26) further confirmed the α-orientation of H-7 and the β-position of 7-OH. From these data, **5** was deduced to be 7β-hydroxy-3,14-dideoxyandrographolide (Fig. 1).

Metabolite **6** was isolated as white powder and was positive for the Legal and Kedde reactions, suggesting the presence of an α,β-unsaturated lactone [27]. The HRESIMS showed the quasi-molecular ion [M+Na]$^+$ at *m*/*z* 357.2033, corresponding to the molecular formula of $C_{20}H_{30}O_4$ in combination with the ^{1}H and ^{13}C NMR spectroscopic data (Tables 1 and 2), indicating that **6** might be the aglycone derivative of **1**. Comparison of the ^{13}C NMR data of **6** with those of **4** [22] showed that the absence of characteristic exocyclic methylene signals (C-8 and C-17) at δ_C 148.8 and 107.3, whereas the appearance of two double bond resonances at δ_C 125.1 and 140.7, and one hydroxymethyl signal at δ_C 66.0 in **6**, indicating the rearrangement of the exocyclic double bond and hydroxylation of C-17. It was further corroborated by the HMBC correlations of H_2-17 (δ_H 4.55, 4.35) with C-7 (δ_C 125.1), C-8 (δ_C 140.7), and C-9 (δ_C 52.6); H-7 (δ_H 5.94) with C-5 (δ_C 51.6) and C-17 (δ_C 66.0); H-9 (δ_H 2.13) with C-7 (δ_C 125.1) and C-8 (δ_C 140.7); and H_2-6 (δ_H 1.85, 1.70) with C-8 (δ_C 140.7). On the basis of the above evidence, the structure of **6** was assigned as 17,19-dihydroxy-7,13-*ent*-labdadien-16,15-olide (Fig. 1).

Metabolite **8**, white powder, was positive for the Legal and Kedde reactions, suggesting the presence of an α,β-unsaturated lactone [27]. The molecular formula of $C_{20}H_{30}O_4$ was inferred from

positive HRESIMS analysis ([M+Na]$^+$ at m/z 357.2031) by combining the ^{1}H and ^{13}C NMR spectroscopic data (Tables 1 and 2), indicating that **8** might be the aglycone derivative of **1**. Careful comparison of the ^{13}C NMR and DEPT spectra of **8** with those of **4** [22] showed that the absence of characteristic exocyclic double bond resonances (C-8 and C-17) at δ_C 148.8 and 107.3 in **4**, whereas the presence of one quaternary carbon signal at δ_C 57.5 and one methylene signal at δ_C 49.1 in **8**. In the HSQC spectrum, the resonances at δ_H 2.30 and 2.81 were attributed to the carbon signal at δ_C 49.1. By comparison of the ^{1}H and ^{13}C NMR spectra of **8** with those of 8, 17-epoxy-14-deoxyandrographolide [30], many similar signals were observed, indicating that the exocyclic double bond was converted to an epoxy ring. This was further confirmed by the HMBC correlations of H_2-17 (δ_H 2.81, 2.30) with C-7 (δ_C 37.0), C-8 (δ_C 57.5) and C-9 (δ_C 53.0), and of H_2-6 (δ_H 1.81), H-9 (δ_H 1.36), and H_2-11 (δ_H 1.46, 1.13) with C-8 (δ_C 57.5). The configuration of the new chiral center at C-8 was determined by the presence of NOESY correlations of H_2-17 (δ_H 2.81, 2.30) with H-9 (δ_H 1.36), H-11b (δ_H 1.13) and H-7((δ_H 1.86), and the absence of correlations of H_2-17 (δ_H 2.81, 2.30) with 20-CH_3 (δ_H 1.01). On the basis of the above evidence, **8** was established as 8α,17β-epoxy-3,14-dideoxyandrographolide (Fig. 1).

Metabolite **9**, was obtained as white powder and was positive for the Legal and Kedde reactions, suggesting the presence of an α,β-unsaturated lactone [27]. The HRESIMS showed the quasi-molecular ion [M+Na]$^+$ at m/z 375.2143, accordingly to the molecular formula of $C_{20}H_{32}O_5$, indicating that **9** might be the aglycone derivative of **1**, which was further supported by the ^{1}H and ^{13}C NMR spectroscopic data (Tables 1 and 2). By comparison, the NMR data of **9** and the known compound 8β, 19-dihydroxy-*ent*-labd-13-en-16,15-olide [20] are closely similar, except for the presence of one hydroxymethyl signal at δ_C 63.4 in **9**, and the absence of one methyl signal at δ_C 24.5 in the reference compound. It was implied that an additional –OH group was present at C-17 in **9**, which was further confirmed by the HMBC correlations of H_2-17 (δ_H 3.97, 3.87) with C-7 (δ_C 39.6), C-8 (δ_C 75.5) and C-9 (δ_C 61.2), and of H_2-7 (δ_H 2.65, 1.54), H-9 (δ_H 1.62), and H_2-11 (δ_H 2.12, 1.60) with C-8 (δ_C 57.5). The configuration of the new chiral center at C-8 was determined on the basis of the presence of NOESY correlations of H_2-17 (δ_H 3.97, 3.87) with 20-CH_3 (δ_H 0.84), H-7((δ_H 1.56) and H-11a (δ_H 2.15), and the absence of correlations of H_2-17 (δ_H 3.97, 3.87) with H-9 (δ_H 1.62). Thus, **9** was designated as 8β,17,19-trihydroxy-*ent*-labd-13-en-16,15-olide (Fig. 1).

In addition to five new metabolites, five known ones, 14-deoxyandrographolide (**2**) [21], 3, 14-dideoxyandrographolide (**4**) [22], 8(17),13-*ent*-labdadien-16,15-olid-19-oic acid (7) [23], phlogantholide-A (**10**) [24], 19-[(β-D-glucopyranosyl)oxy]-19-oxo-*ent*-labda-8(17),13-dien-16,15-olide (**11**) [25] were also isolated and 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

Compounds **1**–**11** were examined for their inhibitory effects on NO production induced by LPS in macrophages (Table 3). Cell viability in the present experiment was determined by the MTT method to find whether inhibition of NO production was due to cytotoxicity of test compounds (data not shown). As shown in Table 3, indomethacin (IC_{50} 12.84 ± 0.84 μM) was used as a positive control. Metabolites **4**–**7** showed strong inhibition of NO production induced by LPS similar to the substrate **1**. Metabolites **2**, **3**, **8**, and **9** exhibited moderate activities, which were weaker than indomethacin. While metabolites **10** and **11** showed no activity. From the structural features of the labdane skeleton, it was found that the positions of hydroxyl and double bond were important for the activity. Metabolite **5** with 7-OH in the ring B exhibited stronger activity than **2** and **10** with 3-OH and 2-OH in the ring A, respectively. Metabolites **4** and **7** with *exo*-methylene and **6** with double bond between C-7 and C-8 in the B ring were more effective than **8** and **9** with no double bond in the B ring, and **3** with double bond between C-8 and C-9. For the compounds with a glucose on C-19, the carbonylation of C-19 led to a marked decrease of the NO inhibitory effect (e.g., **1** and **11**). Whereas, the carbonylation of C-19 could not influence conspicuously the activity for the aglycones (e.g., **4** and **7**).

Table 3
Inhibitory effect of compounds **1**–**11** on NO production induced by LPS in macrophages[a].

Compound	IC_{50} ± SD (μM)	Compound	IC_{50} ± SD (μM)
1	10.40 ± 0.88	**7**	13.55 ± 1.91
2	31.67 ± 3.02	**8**	35.13 ± 2.74
3	53.69 ± 4.84	**9**	67.39 ± 3.51
4	9.10 ± 1.02	**10**	>100
5	15.28 ± 1.61	**11**	>100
6	11.65 ± 1.24	Indomethacin[b]	12.84 ± 0.84

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

4. Conclusion

Mucor spinosus (AS 3.2450) has good ability to transform neoandrographolide. By careful analysis of the structure of these metabolites, it is not hard to find that there are many kinds of enzymatic reactions happened during the biotransformation, such as enzymatic hydrolization, hydroxylation, oxidation, glycosylation, epoxidation and elimination. It might be related to the physical and chemical properties of neoandrographolide and the diversity of the enzymes in the fungi. Based on the structures of the isolated metabolites, the possible biotransformation pathways were speculated as shown in Fig. 1. Some metabolites showed inhibitory effects on nitric oxide production induced by LPS in macrophages similar to the substrate. Their preliminary structure–activity relationships (SAR) results provided useful clues in the process of neoandrographolide development.

Acknowledgments

The authors thank Shenyang Planning Project of Science and Technology (No. F10-205-1-38) for the financial support. The authors are grateful to Ms. W. Li, Mr. Y. Sha, and Dr. W.M. Cheng of the Department of Instrumental Analysis, Shenyang Pharmaceutical University, for measuring the NMR and HRESIMS spectra.

References

[1] S.K. Mishra, N.S. Sangwan, R.S. Sangwan, Pharmacog. Rev. 1 (2007) 283–298.
[2] J. Batkhuu, K. Hattori, F. Takano, S. Fushiya, K.I. Oshiman, Y. Fujimiya, Biol. Pharm. Bull. 25 (2002) 1169–1174.
[3] J. Liu, Z.T. Wang, L.L. Ji, B.X. Ge, Mol. Cell. Biochem. 298 (2007) 49–57.
[4] J. Liu, Z.T. Wang, L.L. Ji, Am. J. Chin. Med. 35 (2007) 317–328.
[5] C. Wiart, K. Kumar, M.Y. Yusof, H. Hamimah, Z.M. Fauzi, M. Sulaiman, Phytother. Res. 19 (2004) 1069–1070.
[6] R.E. Kamdem, S. Sang, C.T. Ho, J. Agric. Food Chem. 50 (2002) 4662–4665.
[7] A. Kapil, I.B. Koul, S.K. Banerjee, B.D. Gupta, Biochem. Pharmacol. 46 (1993) 182–185.
[8] A. Basak, S. Cooper, A.G. Roberge, U.K. Banik, M. Chretien, N.G. Seidah, Biochem. J. 15 (1999) 107–113.
[9] W.L. Deng, R.J. Nie, J.Y. Liu, Zhongguoyaoxuezazhi 17 (1982) 3–6.
[10] W.L. Deng, J.Y. Liu, R.J. Nie, Zhongguozhongyaozazhi 10 (1985) 38–42.
[11] X.J. He, J.K. Li, H. Gao, F. Qiu, K. Hu, X.M. Cui, X.S. Yao, Tetrahedron 59 (2003) 6603–6607.
[12] X.J. He, J.K. Li, H. Gao, F. Qiu, K. Hu, X.M. Cui, X.S. Yao, Chem. Pharm. Bull. 51 (2003) 586–589.
[13] L. Cui, F. Qiu, X.S. Yao, Drug Metab. Dispos. 33 (2005) 555–562.

[14] Z.M. Meng, Acta Pharmacol. Sin. 16 (1981) 571–575.
[15] Z.M. Zou, Zhongcaoyao 8 (1978) 343–345.
[16] A. Wendy, W.A. Loughlin, Bioresour. Technol. 9 (2000) 49–62.
[17] S. Riva, Curr. Opin. Chem. Biol. 5 (2001) 106–111.
[18] H. Zhang, F. Qiu, X.S. Yao, G.X. Qu, J. Asian Nat. Prod. Res. 9 (2007) 311–319.
[19] H. Zhang, N. Kang, F. Qiu, G.X. Qu, X.S. Yao, Chem. Pharm. Bull. 55 (2007) 451–454.
[20] L.X. Chen, F. Qiu, G.X. Qu, X.S. Yao, J. Asian Nat. Prod. Res. 9 (2007) 463–469.
[21] T. Matsuda, M. Kuroyanagi, S. Sugiyama, K. Umehara, A. Ueno, K. Nishi, Chem. Pharm. Bull. 42 (1994) 1216–1225.
[22] T. Fujita, R. Fujitani, Y. Takeda, Y. Takaishi, T. Yamada, Chem. Pharm. Bull. 32 (1984) 2117–2125.
[23] P. Waridel, J.L. Wolfender, J.B. Lachavanne, K. Hostettmann, Phytochemistry 64 (2003) 1309–1317.
[24] A.K. Barua, M.K. Chowdhury, S. Biswas, C. Dasgupta, S.K. Banerjee, S.K. Saha, A. Patra, A.K. Mitra, Phytochemistry 24 (1985) 2037–2039.
[25] L.X. Chen, H. Wei, F. Qiu, G.X. Qu, X.S. Yao, Helv. Chim. Acta 89 (2006) 2654–2664.
[26] V.M. Dirrsch, H. Stuppner, A.M. Vollmar, Planta Med. 64 (1998) 423–426.
[27] L.J. Haynes, K. Peach, M.V. Tracey (Eds.), Modern Methods in Plant Analysis, vol. II, Springer-Verlag, Berlin, 1955, p. 583.
[28] G.C. Wang, Y.M. Hu, X.Q. Zhang, W.C. Ye, Zhongguoyaokedaxuexuebao 36 (2005) 405–407.
[29] L.X. Chen, H.J. Zhu, R. Wang, K.L. Zhou, Y.K. Jing, F. Qiu, J. Nat. Prod. 71 (2008) 852–855.
[30] Y.H. Shen, R.T. Li, W.L. Xiao, G. Xu, Z.W. Lin, Q.S. Zhao, H.D. Sun, J. Nat. Prod. 69 (2006) 319–322.