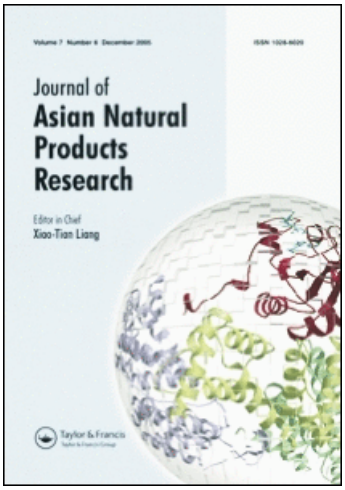

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Microbial transformation of neoandrographolide by *Aspergillus niger* (AS 3.739)

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Microbial transformation of neoandrographolide by *Aspergillus niger* (AS 3.739)

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The biotransformation of neoandrographolide (**1**) was investigated by using *Aspergillus niger* (AS 3.739). Five products were obtained and identified as 8(17),13-*ent*-labdadien-16,15-olid-19-oic acid (**2**), 19-hydroxy-8(17),13-*ent*-labdadien-16,15-olide (**3**), 18-hydroxy-8(17),13-*ent*-labdadien-16,15-olid-19-oic acid (**4**), 3α-hydroxy-8(17),13-*ent*-labdadien-16,15-olid-19-oic acid (**5**) and 8β,19-dihydroxy-*ent*-labd-13-en-16,15-olide (**6**) by spectroscopic and chemical means. Products **4**, **5** and **6** are new compounds.

Keywords: Neoandrographolide; Microbial transformation; *Aspergillus niger*; *ent*-Labdane diterpenoid

1. Introduction

Neoandrographolide is one of principal constituents of *ent*-labdane diterpenoid lactones isolated from the aerial parts of the famous herbal medicine, *Andrographis paniculata* Nees. Its chemical structure is 3-[2-[5-[(β-D-glucopyranosyloxy)methyl]decahydro-5,8a-dimethyl-2-methylene-1-naphthalenyl]ethyl]-2(5H)-furanone. Neoandrographolide has many bio-activities, such as anti-inflammatory [1], antiviral [2], anti-radical [3], hepatoprotective [4] and anti-human immunodeficiency virus (HIV) activities [5]; and the total diterpenoid lactones of *Andrographis paniculata* have been widely used in clinic for the treatment of fever, cold, inflammation, diarrhoea and other infectious diseases in China. Some studies on the structure modifications of the other principal diterpenoid of andrographolide from this plant through biological [6–8] and chemical methods [9,10] have been reported, and the two derivatives, sodium 14-deoxy-12(*R*)-sulfo-andrographolide (Lianbizhi) and monopotassium 14-deoxy-11,12-didehydroandrographolide-3,19-disuccinate (chuanhuning) have been developed into antibacterial and antiviral drugs in China. However, the biological or chemical modification of neoandrographolide has not been studied so far.

In this work, neoandrographolide was biotransformed by *Aspergillus niger* (AS 3.739) in potato medium. Five products were obtained, among which, three were identified as new compounds. The article mainly describes the isolation and identification of these three new products.

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2. Results and discussion

Compound **2**, white amorphous powder, was positive for the Legal and Kedde reactions, suggesting the presence of an α, β-unsaturated lactone [11]. The ^{1}H NMR and ^{13}C NMR spectral data were identical with those of 8(17),13-*ent*-labdadien-16,15-olid-19-oic acid from the aquatic plant *Potamogeton pectinatus* [12]. Therefore, **2** was identified as 8(17),13-*ent*-labdadien-16,15-olid-19-oic acid (see figure 1).

Compound **3**, colourless needles, mp 93–94°C, was positive for the Legal and Kedde reactions, suggesting the presence of an α, β-unsaturated lactone [11]. The ^{1}H NMR and ^{13}C NMR spectral data were identical with those of the aglycone of neoandrographolide [13]. Therefore, **3** was identified as 19-hydroxy-8(17),13-*ent*-labdadien-16,15-olide (see figure 1).

Compound **4**, white amorphous powder, was positive for the Legal and Kedde reactions, suggesting the presence of an α, β-unsaturated lactone [11]. By TLC analyses, **4** was less polar than the substrate, indicating it might be an aglycone derivative without a glucose. The high-resolution ESI-MS showed the quasi-molecular ion $[M + Na]^+$ at *m/z* 371.1867, corresponding to the molecular formula $C_{20}H_{28}O_5$, which was further supported by the ^{1}H NMR and ^{13}C NMR spectral data (tables 1 and 2). The ^{1}H NMR and ^{13}C NMR data were closely similar to those of the known product **2** [12], except for two ^{1}H NMR signals at δ 4.38 (1H, d, $J = 10.2$ Hz) and 4.01 (1H, d, $J = 10.2$ Hz) attributable to a hydroxymethyl located at C-18 (δ 70.2) in **4** instead of a ^{1}H NMR signal at δ 0.88 (3H, s) attributable to a methyl located at C-18 (δ 13.2) in **2**. This was further corroborated by HMBC correlations of H_2-18 (δ 4.38, 4.01) with C-3 (δ 33.1), C-5 ((50.4) and C-19 (δ 178.7). In the NOESY spectrum, the existence of the correlation between H_2-18 (δ 4.38, 4.01) and H-5 (δ 1.86), and the absence of the correlation between H_2-18 (δ 4.38, 4.01) and 20-CH_3 (δ 0.97) demonstrated that 18-CH_2OH was of β-orientation, and consequently 19-COOH was of α-orientation. Based on the above evidence, **4** was determined to be 18-hydroxy-8(17),13-*ent*-labdadien-16,15-olid-19-oic acid, which has not previously been reported (see figure 1).

Compound **5**, white amorphous powder, was positive for the Legal and Kedde reactions, suggesting the presence of an α, β-unsaturated lactone [11]. By TLC analyses, **5** was also less polar than the substrate, indicating it might be an aglycone derivative without a glucose. The high-resolution ESI-MS showed the quasi-molecular ion $[M + Na]^+$ at *m/z* 371.1867, corresponding to the molecular formula $C_{20}H_{28}O_5$ in combination with the ^{1}H NMR and ^{13}C NMR spectral data (tables 1 and 2). The ^{1}H NMR and ^{13}C NMR data of **5** implied that it was an analogue of **2** [12], except for a ^{1}H NMR signal at δ 3.39 (1H, dd, $J = 12.0$, 3.6 Hz) attributable to a hydroxymethine located at C-3 (δ 78.3) in **5** instead of two ^{1}H NMR signals at δ 2.43 (1H, brd, $J = 13.2$ Hz) and 1.08 (1H, dt, $J = 13.2$, 3.6 Hz) ascribable to a methene located at C-3 (δ 38.8) in **2**, suggesting that C-3 was hydroxylated. This was confirmed by HMBC correlations of H-3 (δ 3.39) with C-2 (δ 29.8), C-4 (δ 49.9) and C-18 (δ 25.1). In the NOESY spectrum, the existence of the strong correlations of H-3 (δ 3.39) to H-5 (δ 1.27) and 18-CH_3 (δ 1.68), and the absence of the correlation of H-3 to 20-CH_3 (δ 0.91) were observed, suggesting that 3-OH was of α-orientation. Therefore, **5** was elucidated as 3α-hydroxy-8(17),13-*ent*-labdadien-16,15-olid-19-oic acid, which has not previously been reported (see figure 1).

Compound **6**, colourless plates, mp 166–167°C, was positive for the Legal and Kedde reactions, suggesting the presence of an α, β-unsaturated lactone [11]. By TLC analyses, **6** was also less polar than the substrate, indicating it might be an aglycone derivative without

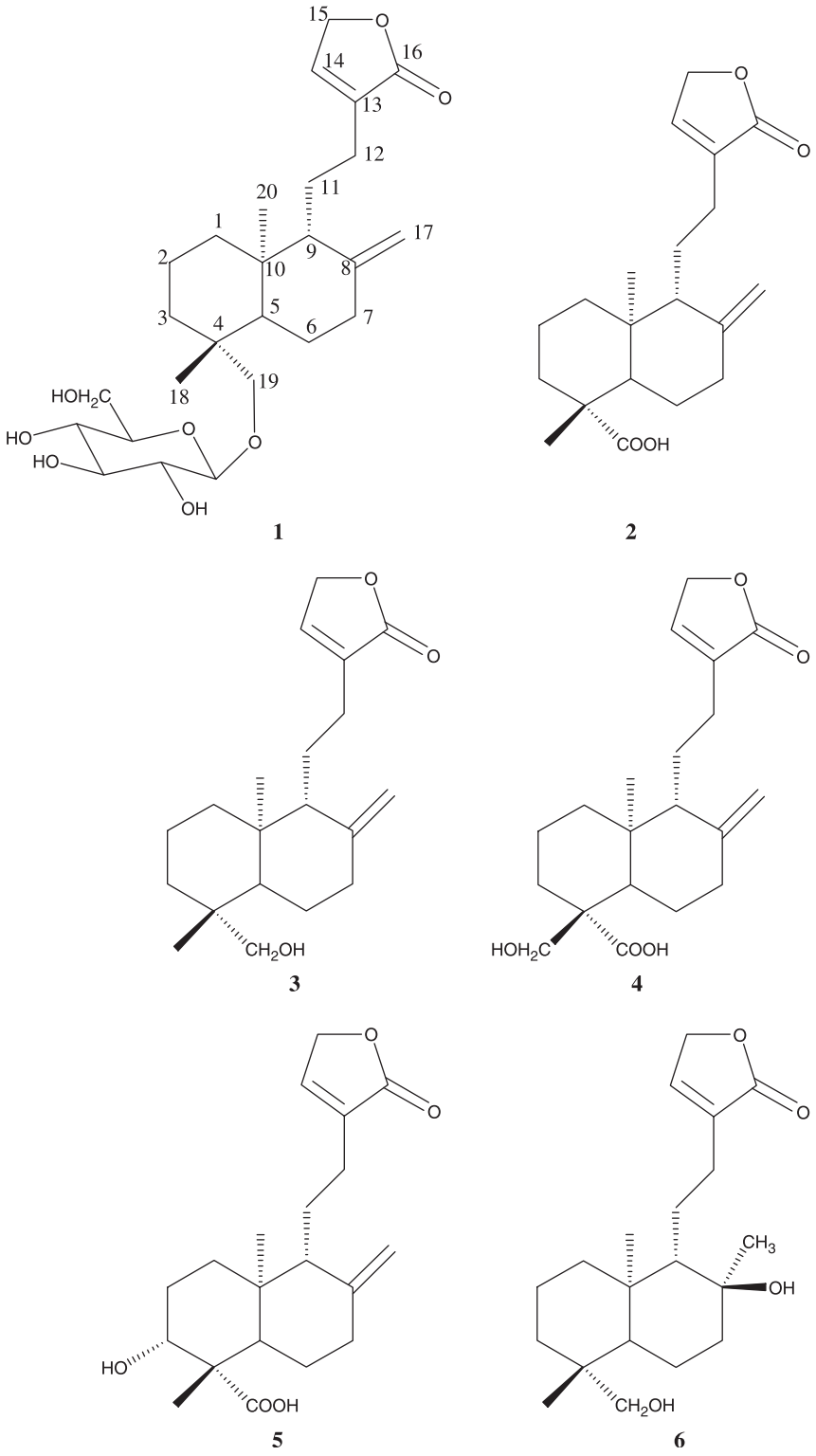

Figure 1. Structures of neoandrographolide and its transformation products.

Table 1. ^{1}H NMR (600 MHz) spectral data of compounds **4**–**6** (in pyridine-d_5, δ ppm, J in Hz).

No.	***4***	***5***	***6***
1	1.88 (1H, brd, $J = 13.2$ Hz) 1.15 (1H, dt, $J = 13.2$, 3.4 Hz)	1.84 (1H, brd, $J = 12.6$ Hz) 1.19 (1H, brt, $J = 13.2$ Hz)	1.76 (1H, brd, $J = 12.6$ Hz) 1.01 (1H, dt, $J = 13.2$, 3.0 Hz)
2	2.42 (1H, m) 1.70 (1H, brd, $J = 12.6$ Hz)	2.56 (1H, o) 1.98 (1H, o)	1.63 (1H, brd, $J = 13.2$ Hz) 1.37 (1H, brd, $J = 13.2$ Hz)
3	2.89 (1H, brd, $J = 12.6$ Hz) 1.63 (1H, dt, $J = 13.2$, 3.6 Hz)	3.39 (1H, dd, $J = 12.0$, 3.6 Hz)	2.13 (1H, brd, $J = 13.2$ Hz) 0.97 (1H, dt, $J = 13.2$, 3.0 Hz)
4	–	–	–
5	1.86 (1H, brd, $J = 12.6$ Hz)	1.27 (1H, brd, $J = 12.0$ Hz)	1.16 (1H, brd, $J = 12.6$ Hz)
6	2.30 (1H, o) 2.28 (1H, o)	2.26 (1H, m) 2.16 (1H, brd, $J = 12.0$ Hz)	1.82 (1H, brd, $J = 12.6$ Hz) 1.47 (1H, m)
7	2.45 (1H, brd, $J = 12.0$ Hz) 2.02 (1H, m)	2.45 (1H, brd, $J = 12.0$ Hz) 1.98 (1H, o)	2.04 (1H, brd, $J = 12.6$ Hz) 1.73 (1H, dt, $J = 12.6$, 3.0 Hz)
8	–	–	–
9	1.77 (1H, brd, $J = 10.8$ Hz)	1.67 (1H, brd, $J = 10.0$ Hz)	1.44 (1H, t, $J = 3.6$ Hz)
10	–	–	–
11	1.83 (1H, m) 1.67 (1H, m)	1.80 (1H, brt, $J = 10.8$ Hz) 1.65 (1H, o)	1.97 (1H, m) 1.65 (1H, m)
12	2.55 (1H, brt, $J = 12.0$ Hz) 2.20 (1H, m)	2.56 (1H, o) 2.21 (1H, m)	2.84 (1H, brt, $J = 10.2$ Hz) 2.53 (1H, brd, $J = 10.2$ Hz)
13	–	–	–
14	7.18 (1H, s)	7.19 (1H, s)	7.14 (1H, s)
15	4.74 (2H, s)	4.75 (2H, s)	4.67 (2H, s)
16	–	–	–
17	4.96 (1H, s) 4.74 (1H, s)	4.96 (1H, s) 4.75 (1H, s)	1.32 (3H, s)
18	4.38 (1H, d, $J = 10.2$ Hz) 4.01 (1H, d, $J = 10.2$ Hz)	1.68 (3H, s)	1.21 (3H, s)
19	–	–	3.95 (1H, d, $J = 10.8$ Hz) 3.68 (1H, d, $J = 10.8$ Hz)
20	0.97 (3H, s)	0.91 (3H, s)	0.86 (3H, s)

"o" indicates overlapped.

a glucose. The high-resolution ESI-MS showed the quasi-molecular ion $[M + Na]^+$ at m/z 359.2154, corresponding to the molecular formula $C_{20}H_{32}O_4$ by combining the ^{1}H NMR and ^{13}C NMR spectral data (tables 1 and 2). Comparison of the ^{13}C NMR data of **6** with those of **3** [13] showed that the absence of two signals of *exo*-methylene at δ 148.3 and 106.9 in **3**, and the appearance of one methyl signal at δ 24.5 and one tertiary carbon signal linked with hydroxyl group at δ 73.1 in **6**. Correspondingly, the ^{1}H NMR spectrum exhibited the absence of two signals of *exo*-methylene at δ 4.93 and 4.76 in **3** and the appearance of one tertiary methyl signal at δ 1.32 (3H, s) in **6**. This was further confirmed by HMBC correlations of 17-CH_3 (δ 1.32) with C-7 (δ 45.6), C-8 (δ 73.1) and C-9 (δ 62.1). The NOESY spectrum showed correlations of 17-CH_3 (δ 1.32) to H-7e (δ 2.04), H-6a (δ 1.47) and 20-CH_3 (δ 0.86) suggesting that 17-CH_3 was of α-orientation and consequently 8-OH was of β-orientation. On the basis of the above evidence, **6** was identified as 8β,19-dihydroxy-*ent*-labd-13-en-16,15-olide, which has not previously been reported (see figure 1).

Table 2. ^{13}C NMR (150 MHz) spectral data of compounds **4**–**6** (in pyridine-d_5, δ ppm).

No.	***4***	***5***	***6***
1	39.4	38.1	40.3
2	20.4	29.8	18.7
3	33.1	78.3	36.2
4	51.1	49.9	39.2
5	50.4	55.8	57.2
6	26.8	26.7	21.3
7	39.0	38.9	45.6
8	148.5	148.2	73.1
9	56.2	55.9	62.1
10	40.7	40.4	39.3
11	22.3	22.5	24.1
12	25.1	25.1	29.4
13	134.2	134.2	134.7
14	145.4	145.5	145.0
15	70.7	70.7	70.6
16	174.7	174.7	174.8
17	106.8	107.0	24.5
18	70.2	25.1	28.0
19	178.7	180.8	64.2
20	13.5	13.3	16.3

3. Experimental

3.1 General experimental procedures

The NMR spectrum was recorded on a Bruker ARX-600 spectrometer (600 MHz for ^{1}H and 150 MHz for ^{13}C) in pyridine-d_5 with TMS as internal standard. Chemical shifts were expressed in δ (ppm) and coupling constants (J) were reported in Hertz (Hz). IR spectra were recorded with a Bruker IFS 55 spectrometer. High-resolution ESI-MS spectra were obtained on a Bruker APEX II mass spectrometer. Preparative HPLC was performed with an ODS column (C-18, 250 × 20 mm, Inertsil Pak) in a Waters 600 liquid chromatograph apparatus equipped with a Waters 490 UV detector. Methanol was HPLC grade and water was double distilled in our laboratory. Silica gel 60 (Qingdao Haiyang Chemical Co. Ltd, China) was used as column chromatography stationary phases. TLC was carried out on Silica gel 60 and the spots were visualized by spraying with Legal and Kedde reagent. All the analytic reagents were analytical grade and purchased from Shenyang Chemical Company (Shenyang, China).

3.2 Microorganisms

Aspergillus niger (AS 3.739) was purchased from China General Microbiological Culture Collection Centre.

3.3 Medium

All culture and biotransformation experiments were performed in potato medium, which was produced by the following procedure: 200 g of minced 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.

3.4 Culture and biotransformation procedures

Screening scale biotransformation of neoandrographolide by *A. niger* was carried out in 250-ml Erlenmeyer flasks containing 50 ml of potato medium. Microorganisms were transferred into the flasks from the slants. The flasks were placed on rotary shakers, operating at 180 rpm at 28°C. The substrate was dissolved in methanol with a concentration of 50 mg/ml. After 48 h of culture, 0.2 ml of the substrate solution was added into the fermentation flasks and these flasks were maintained under the same conditions for an additional 72 h. Culture controls consisted of fermentation blanks in which microorganisms were grown without substrate but with the same amount of methanol alone. Substrate controls consisted of sterile medium containing the same amount of substrate without microorganisms, and incubated under the same conditions. When the fermentation finished, the broths were filtered and the filtrates were extracted with the same volume of ethyl acetate three times. The cells were extracted with ethyl acetate by supersonic means. The extracts were evaporated to dryness under reduced pressure and the residues were dissolved in methanol. The solutions were spotted on silica gel plates which were developed by $CHCl_3$/MeOH (9:1), and visualized by spraying with Legal and Kedde reagent. TLC analyses revealed that *A. niger* could biotransform the substrate.

Preparative scale biotransformation of neoandrographolide by *A. niger* was carried out in 500-ml Erlenmeyer flasks containing 150 ml of potato medium. A total of 2 g of 1 was transformed by the strain. Other procedures were the same as screening scale biotransformation.

3.5 Isolation and characterization of biotransformed products

Twenty grams of yellow residue was obtained from the fermented broth of *A. niger*. The residue was chromatographed on silica gel column eluted with cyclohexane/ethyl acetate (9:1, 8:2, 7:3, 6:4) to give **2** (482 mg), **3** (239 mg), **4** (468 mg), **5** (15 mg) and **6** (23 mg). 540 mg of substrate remained after biotransformation.

3.5.1 18-Hydroxy-8(17),13-*ent*-labdadien-16,15-olid-19-oic acid (4). Obtained as white amorphous powder (MeOH); $[\alpha]_D^{23} - 58.2$ (*c* 0.25, MeOH); IR (KBr) ν_{max}: 3325, 2961, 1721, 1644, 1440, 1218, 908, 833 cm^{-1}; high-resolution positive ESI-MS *m/z*: 371.1867 $[M + Na]^+$ (calcd for $C_{20}H_{28}O_5Na$, 371.1834); ^1H NMR spectral data (600 MHz, pyridine-d_5), see table 1; ^{13}C NMR spectral data (150 MHz, pyridine-d_5), see table 2.

3.5.2 3α-Hydroxy-8(17),13-*ent*-labdadien-16,15-olid-19-oic acid (5). Obtained as white amorphous powder (MeOH); $[\alpha]_D^{23} - 28.7$ (*c* 0.23, MeOH); IR (KBr) ν_{max}: 3426, 2935, 1755, 1645, 1449, 1085, 904, 838 cm^{-1}; High-resolution positive ESI-MS *m/z*: 371.1867 $[M + Na]^+$ (calcd for $C_{20}H_{28}O_5Na$, 371.1834); ^1H NMR spectral data (600 MHz, pyridine-d_5), see table 1; ^{13}C NMR spectral data (150 MHz, pyridine-d_5), see table 2.

3.5.3 8β,19-Dihydroxy-*ent*-labd-13-en-16,15-olide (6). Obtained as colourless plates (MeOH), mp 166–167°C; $[\alpha]_D^{23} + 2.4$ (*c* 0.21, MeOH); IR (KBr) ν_{max}: 3493, 2928, 1727, 1459, 1344, 1090, 1021, 844 cm^{-1}; High-resolution positive ESI-MS *m/z*: 359.2154 $[M + Na]^+$ (calcd for $C_{20}H_{32}O_4Na$, 359.2198); ^1H NMR spectral data (600 MHz, pyridine-d_5), see table 1; ^{13}C NMR spectral data (150 MHz, pyridine-d_5), see table 2.

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