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Microbial transformation of deoxyandrographolide by *Cunninghamella echinulata*

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ABSTRACT

The capabilities of twenty strains of filamentous fungi (from 9 genera) to transform deoxyandrographolide (1) were screened. And eleven derivatives of 1 transformed by *Cunninghamella echinulata* AS 3.3400 were isolated. Their chemical structures were identified by spectral methods including 2D NMR. Among them, 3-oxo-7α-hydroxy-14-deoxyandrographolide (2), 3-oxo-8β,17α-epoxy-14-deoxyandrographolide (3), 8α-formyl-14-deoxyandrographolide (4), 8β-methoxyl-17α-hydroxyl-14-deoxyandro-grapholide (5) and 3α,17,19-trihydroxyl-7,13-ent-labdadien-15,16-olide (6), are new compounds. And their structure–activity relationships (SAR) were also discussed.

1. Introduction

Andrographis paniculata, as a common herb medicine, is widely distributed in China [1,2]. Its leaves are used to treat various acute hepatitis, inflammations and tumors. The ent-labdane diterpenoids including andrographolide and deoxyandrographolide are the principal active constituents of this plant. They usually have characteristic A/B *cis* junctures and 3α-hydroxyl group with γ-butyrolactone moiety [3]. In recent years, the structure–activity relationships of various ent-labdane diterpenoids indicated that they had potent anticancer and anti-diabetes activities [4–6].

Microbial transformation is a useful tool to modify the structures of bioactive substrates by using microorganisms with the advantages as high stereo- and regioselectivity, mild reaction and avoiding protection and de-protection steps in chiral centers of natural products [7]. In our previous investigation [8–11], more than 30 novel products from natural terpenoids were obtained by using biotransformation approach. In recent years, biotransformation of diterpenoid compounds has been frequently reported as far as we know. The unique and inexpensive resource of diterpenoids could provide the structural skeletons for preparing hemi-synthesis precursor and medicinal leading compounds.

In this paper, 20 strains of filamentous fungi were screened for their capabilities to transform deoxyandrographolide (1), in order to find new chemical entities for improving its solubility and bioactivities. In total, 11 derivatives of deoxyandrographolide by *Cunninghamella echinulata* AS 3.3400 were isolated and identified by the extensive spectral methods including 2D NMR. Among them, five compounds (2–6) are novel (Fig. 1). The stereo-specific hydroxylation, epoxidation and dehydrogenation reactions of 1 were observed. In addition, the cytotoxicities of transformed products against MCF and A562 cells were also investigated.

2. Experimental

2.1. General experimental procedures

IR spectra were obtained on an Avatar 360 FT-TR spectrophotometer. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. NMR spectra were recorded on a Bruker DRX-500 spectrometer (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) in pyridine-d_6 with TMS as internal standard. ^{1}H and ^{13}C NMR spectra were measured at room temperature (22 °C). Chemical shifts are expressed on the δ scale and are referenced to tetramethylsilane (TMS) at $\delta = 0$ ppm for ^{1}H and ^{13}C. Coupling constant (J) is given in Hz. HR-MS was measured on a Bruker APEXII Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. The HPLC equipment was Ultimate 3000 HPLC system with pump, column compartment and photodiode array detector. The HPLC data were recorded by CHORMELEON chromatography management system. Silica gel was purchased from Qingdao Marine Chemical Group, China. Deoxyandrographolide (1) was isolated from *A. paniculata*

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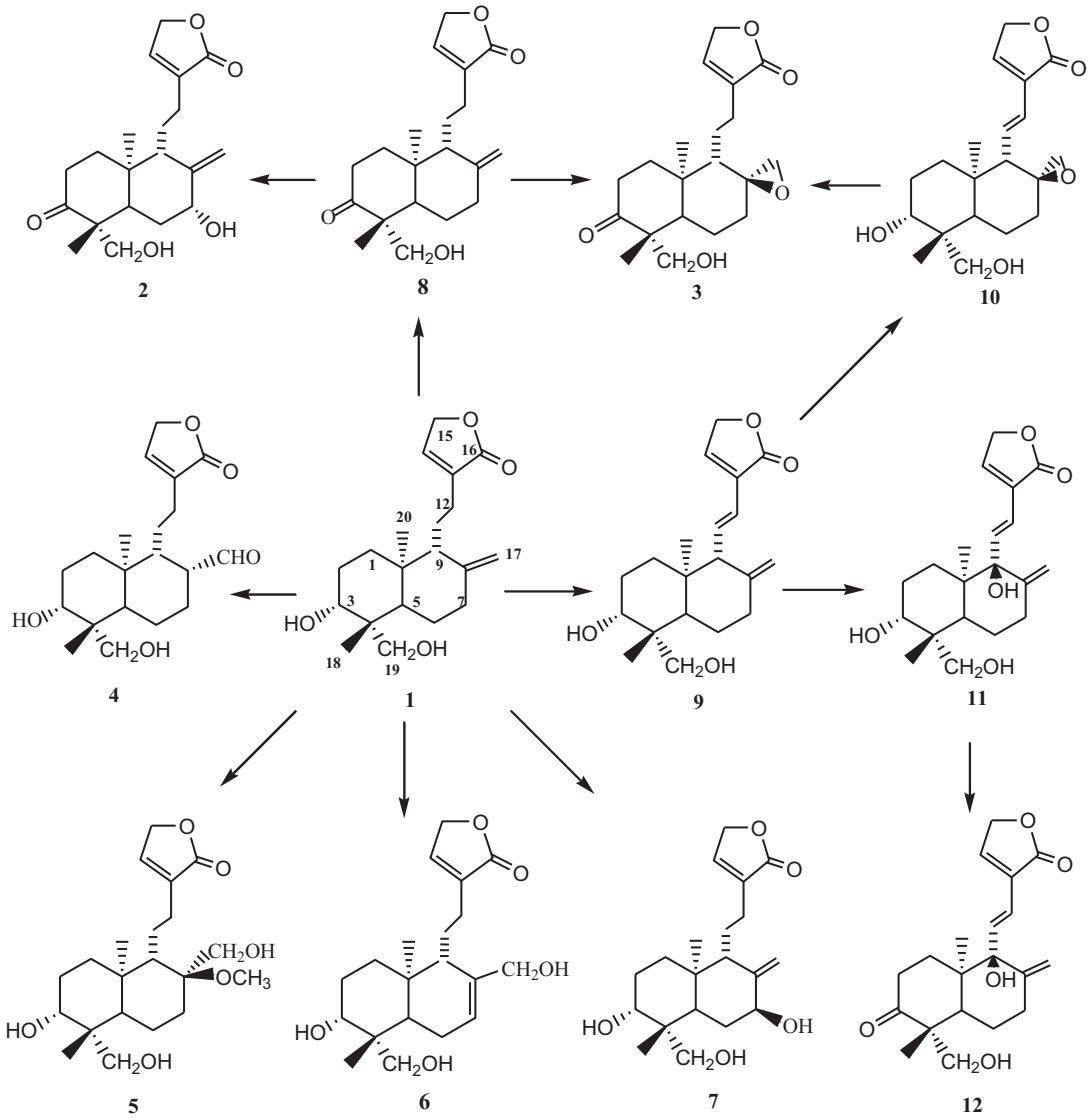

Fig. 1. A possible biotransformation pathway of 1 by *Cunninghamella echinulata*.

by the authors. And its purity was determined by HPLC with value of 98%.

2.2. Microorganisms

Absidia coerulea AS 3.3382, *Absidia ramose* AS 3.2892, *Absidia giauca* AS 3.3385, *Alternaria alternata* AS 3.577, *A. alternata* AS 3.4578, *Alternaria longipes* AS 3.2875, *Atachybotrys atra* AS 3.3734, *Curvularia lunata* AS 3.4381, *Cunninghamella blakesleana lender* AS 3.970, *C. echinulata* AS 3.3400, *Penicillium janthinellum* AS 3.510, *Rhizopus cohnii* AS 3.2746, *Rhizopus arrhizus* AS 3.2897, *Trichoderma viride* AS 3.2942, *Mucor spinosus* AS 3.3450, *M. spinosus* AS 3.2450, *M. spinosus* AS 3.3447, *Mucor subtilissimus* AS 3.2454 and *Mucor polymorphosporus* AS 3.3443 were purchased from Chinese General Microbiological Culture Collection Center in Beijing, China. Microorganisms were maintained on agar slants media and were stored at 4 °C.

2.3. Plant material and isolation of substrate

The aerial parts of *A. paniculata* were collected from Fujian Province, China. A voucher specimen was identified by Prof. Jingming Jia, and deposited at the Department of Pharmaceutical Science, Dalian Medical University, China. The crude plant was extracted by heating reflux with MeOH for three times. After removal of MeOH, the water suspension was partitioned with EtOAc. The EtOAc layer was concentrated and then subjected to silica gel column chromatography by eluting with $CHCl_3/CH_3OH$ (100:1–1:1), obtaining fractions I–VI. Fr III was re-subjected to CC of silica gel by *n*-hexane/AcOEt (2:1) to give deoxyandrographolide (1.2 g).

2.4. Culture medium

The cultures of microorganism were performed in potato medium which was made of the following composition (L): 20 g glucose and 200 g potato. The culture media were sterilized in an autoclave at 121 °C and 1.06 kg/cm^2 for 30 min [12].

2.5. Biotransformation procedures

Mycelia from agar slants were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of culture medium. And it was cultured at 28 °C in a rotary shaker (180 rpm) for 36 h to make a stock inoculum. Then 2 mL of inoculum was added to 100 mL potato medium in a 250 mL flask. After pre-culture for 36 h, 2 mg

Table 1
^{1}H NMR spectral data of compounds 2–6 (Pry-d_5, 500 MHz, δ in ppm, J in Hz).

No.	2	3	4	5	6
1a	1.96 m	2.01 m	1.64 m	1.15 m	1.92 m
b	1.58 m	1.53 m	0.98 m	1.75 m	1.12 m
2a	2.89 (dd 12.5, 2.5)	2.83 m	1.91 m	1.89 m	1.98 m
b	2.40 (dt, 10.0, 3.5)	2.38 m		2.05 m	2.03 m
3	–	–	3.56 m	3.62 m	3.62 m
5	3.59 (d, 5.0)	1.61 m	0.95 m	1.12 m	2.04 m
6a	2.15 m	1.78 m	1.91 m	1.92 m	2.16 m
b	1.91 m	1.74 m	1.47 m	1.52 m	2.23 m
7a	4.63 (d, 2.50)	1.46 m	1.41 m	1.43 m	5.92 (d, 5.0)
b		1.92 m	1.83 m	2.45 m	
8	–	–	2.56 m	–	–
9	2.61 m	1.56 m	1.44 m	1.43 m	1.34 (dd, 12.0, 5.0)
11a	1.78 m	1.18 m	1.65 m	2.08 m	1.69 m
b		1.45 m	1.66 m	1.91 m	1.83 m
12	2.16 m	2.37 m	2.40 m	2.56 m	2.50 m
13	2.63 m	2.53 m	2.42 m	2.47 m	3.01 m
14	7.15 brs	7.23 brs	7.26 brs	7.13 brs	7.22 (d, 1.5)
15	4.69 (dd, 3.5, 2.0)	4.69 (dd, 3.5, 2.0)	4.76 (d, 1.5)	4.72 (d, 1.5)	4.71 (d, 1.5)
16	4.58 (brd, 2.5)	4.58 (brd, 2.5)			
17a	5.18 brs	2.81 (d, 4.0)	10.10 (s)	4.42 (d, 4.0)	4.32 (dd, 12.0, 4.0)
b	4.89 brs	2.54 (d, 4.0)		3.80 (d, 4.0)	4.51 (dd, 12.0, 6.0)
18	1.51 (3H, s)	1.43 (3H, s)	1.47 (3H, s)	1.60 (3H, s)	1.48 (3H, s)
19	4.34 (dd, 11.0, 5.5)	4.25 (d, 11.0)	3.58 (brd, 11.0)	3.98 (d, 11.0)	3.77 (dd, 11.0, 5.5)
	3.84 (dd, 11.0, 5.5)	3.83 (d, 11.0)	4.38 (brd, 11.0)	3.95 (d, 11.0)	4.52 (dd, 11.0, 5.5)
20	1.03 (3H, s)	1.07 (3H, s)	0.73 (3H, s)	0.88 (3H, s)	0.81 (3H, s)

of substrates dissolving in 0.2 mL acetone were added into each flask and these flasks were under the fermentation conditions for 5 days. In addition, the substrate was added into the sterile medium without microorganisms, and then incubated under the same fermentation conditions for 5 days. No transformed products were observed, which suggests the substrate was stable in the blank medium. And the culture controls were composed by the fermentation blanks in which the microorganisms were grown without adding the substrates.

Preparative scale biotransformation of deoxyandrographolide was carried out in 1000 mL Erlenmeyer flasks containing 400 mL of sterilized medium. The flasks were placed on rotary shaker operating at 180 rpm. The substrates were dissolved in acetone. After 48 h of pre-culture, 15 mg of substrates in 1 mL acetone were added into each flask with 400 mL culture medium. The incubation was continued for 5 days. In total, 400 mg of substrates were used for preparative biotransformation.

2.6. Extraction and isolation

The culture was filtered and the filtrate was extracted with same volume of EtOAc three times. The organic phase was collected and concentrated *in vacuo*. The residues (2.5 g) were applied to a ODS column and eluted with MeOH–H_2O in a gradient manner from 1:9 to 9:1. By HPLC analysis, No. 1–25 fractions were obtained. Fraction (No. 11) was subjected to semi-preparative RP-HPLC eluting with MeCN–water–TFA (17:83:0.03) to yield new products 1 (4 mg) and 2 (6 mg). And compounds 3 (5 mg) and 4 (6 mg) were obtained from fraction (No. 14) by HPLC eluting with MeCN–water–TFA (19:81:0.03). Fraction (No. 16) was separated by preparative HPLC and eluted with methanol–water–TFA (38:62:0.03) to give compounds 5 (7 mg) and 6 (5 mg). Fraction (No. 20) was separated by preparative HPLC and eluted with methanol–water–TFA (60:40:0.03) to yield compounds 7 (6 mg), 8 (9 mg) and 9 (13 mg). Fraction (No. 23) was separated by preparative HPLC and eluted with methanol–water–TFA (62:38:0.03) to give compounds 10 (9 mg), 11 (17 mg) and 12 (12 mg).

3-*Oxo*-7α-*hydroxy*-14-*deoxyandrographolide* (2): colorless solid (acetone); m.p. 154–155 °C; $[\alpha]_D^{22}$ +15.6° (*c* 0.10, MeOH); UV λ_{max} MeOH: 223 nm; IR (KBr) ν_{max} (cm^{-1}): 3460, 2980, 1690, 1050, 960. ^{1}H and ^{13}C NMR see Tables 1 and 2. HR-ESI-MS (*m*/*z*): 371.1836 [M+Na]$^+$ (calcd. for $C_{20}H_{28}O_5Na_1$; 371.1829).

3-*Oxo*-8β, 17α-*epoxy*-14-*deoxyandrographolide* (3): colorless solid (acetone); m.p. 154–155 °C; $[\alpha]_D^{22}$ +55.6° (*c* 0.10, MeOH); UV λ_{max} MeOH: 218 nm; IR (KBr) ν_{max} (cm^{-1}): 3440, 2980, 1715, 1080, 995. ^{1}H and ^{13}C NMR see Tables 1 and 2. HR-ESI-MS (*m*/*z*): 371.1828 [M+Na]$^+$ (calcd. for $C_{20}H_{28}O_5Na_1$; 371.1829).

8α-*Formyl*-14-*deoxyandrographolide* (4): colorless solid (acetone); m.p. 179–180 °C; $[\alpha]_D^{22}$ −21.6° (*c* 0.15, MeOH); UV λ_{max} MeOH: 220 nm; IR (KBr) ν_{max} (cm^{-1}): 3410, 2960, 1780, 1100, 995. ^{1}H and ^{13}C NMR see Tables 1 and 2. HR-ESI-MS (*m*/*z*): 373.2019 [M+Na]$^+$ (calcd. for $C_{20}H_{30}O_5Na_1$; 373.1985).

8β-*Methoxyl*-17α-*hydroxyl*-14-*deoxyandrographolide* (5): colorless solid (acetone); m.p. 195–196 °C; $[\alpha]_D^{22}$ −18.3° (*c* 0.10, MeOH); UV λ_{max} MeOH: 218 nm; IR (KBr) ν_{max} (cm^{-1}): 3350, 2820, 1740, 1060. ^{1}H and ^{13}C NMR see Tables 1 and 2. HR-ESI-MS (*m*/*z*): 405.2264 [M+Na]$^+$ (calcd. for $C_{21}H_{34}O_6Na_1$; 405.2253).

3α,17,19-*Trihydroxy*-7,13-*ent*-*labdadien*-15,16-*olide* (6): colorless solid (acetone); m.p. 188–189 °C; $[\alpha]_D^{22}$ −10.6° (*c* 0.10, MeOH); UV λ_{max} MeOH: 220 nm; IR (KBr) ν_{max} (cm^{-1}): 3460, 2970, 1740, 1100, 990. ^{1}H and ^{13}C NMR see Tables 1 and 2. HR-ESI-MS (*m*/*z*): 373.2016 [M+Na]$^+$ (calcd. for $C_{20}H_{30}O_5Na_1$; 373.1985).

2.7. Bioassay

MCF (human breast cancer) and A562 cells (leukemic cell) were in RPMI 1640 medium (GIBCO/BRL, MD, USA) supplemented with 10% (v/v) fetal bovine serum and culture in 96 well microtiter plates. Appropriate dilutions of the test compounds were added to the cultures. After incubation with 5% CO_2 for 72 h at 37 °C, the survival rates of cancer cells were evaluated by MTT method. The activity was shown as the IC_{50} value, which is the concentration (μmol/L) of test compound to give 50% inhibition of cell growth.

3. Results and discussion

3.1. Preliminary screening for biotransformation

Twenty strains of filamentous fungi (from 9 genera) were initially screened for their abilities to transform the substrate. Among

Table 2
^{13}C NMR spectral data of compounds 1–12 (Pry-d_5, 125 MHz, δ in ppm).

	1	2	3	4	5	6	7	8	9	10	11	12
1	37.2	38.8	38.5	37.6	38.6	38.1	37.7	38.7	38.7	37.0	33.4	32.0
2	29.0	36.9	36.1	28.9	29.6	29.0	29.6	28.9	28.9	40.0	37.0	29.3
3	79.9	216.2	214.2	80.2	80.3	80.8	80.6	80.1	80.1	214.1	214.9	80.7
4	43.3	55.0	55.2	43.6	43.6	42.8	43.3	43.4	43.4	55.5	55.1	43.7
5	55.4	50.0	56.8	55.9	56.5	52.5	48.3	54.8	54.8	56.8	48.3	46.5
6	24.6	33.2	21.7	24.0	28.8	23.7	32.4	23.6	23.6	24.7	24.6	23.8
7	38.6	73.2	36.5	24.9	39.3	124.8	73.4	37.0	37.0	37.0	33.6	33.6
8	147.9	151.1	59.0	47.4	80.8	140.5	151.5	149.3	149.3	149.2	152.1	152.5
9	56.5	50.4	52.4	53.8	60.9	51.3	51.0	61.8	61.8	61.5	79.9	79.9
10	39.4	40.2	40.5	38.7	39.2	37.0	40.1	39.0	39.0	39.4	43.1	42.2
11	22.3	22.0	20.9	19.8	21.0	25.8	22.3	135.6	135.6	135.7	140.0	140.5
12	25.0	25.0	27.8	27.4	24.0	27.7	25.0	121.6	121.6	122.8	119.9	119.6
13	134.1	134.3	134.2	134.1	134.9	134.7	134.5	128.9	128.9	128.8	129.3	129.5
14	145.4	145.9	145.9	146.2	145.5	146.0	145.8	145.0	145.0	145.8	145.8	145.4
15	70.6	71.0	70.9	71.1	70.9	71.0	71.0	70.3	70.3	70.7	70.8	70.6
16	174.6	175.0	174.9	174.6	175.2	175.1	175.0	172.8	172.8	172.8	173.4	173.1
17	107.2	109.0	50.8	205.0	63.3	65.7	108.7	108.8	108.8	109.9	112.0	111.3
18	23.7	21.1	21.7	24.0	24.0	24.2	24.0	23.6	23.6	21.6	22.1	24.4
19	64.2	65.9	65.6	64.6	64.7	64.6	64.8	64.2	64.2	65.8	66.2	64.9
20	15.3	14.6	15.2	16.4	16.9	15.3	15.0	16.0	16.0	15.8	18.6	19.2

the microorganisms screened, *C. echinulata* AS 3.3400, *C. lunata* AS 3.4381, *C. blakesleana lender* AS 3.970 and *Rhizopus oryzae* AS 3.851 were found to have good capabilities to convert 1 into more polar metabolites. By the HPLC-PDA analysis, *C. echinulata* AS 3.3400 was found to be the most potent strain to transform the substrate. Therefore, it was selected for a preparative biotransformation.

3.2. Identification of biotransformation products

Eleven compounds were isolated after incubation of deoxyandrographolide with *C. echinulata* for 6 days. Their structures were identified as 3-oxo-7α-hydroxy-14-deoxyandrographolide (2), 3-oxo-8β,17α-epoxy-14-deoxy andrographolide (3), 1β-hydroxy-14-deoxyandrographolide (4), 8β-methoxyl-17α-hydroxyl-14-deoxy-andrographolide (5), 3α,17,19-trihydroxy-7, 13-ent-labdadien-15,16-olide (6), 7β-hydroxy-14-deoxyandrographolide (7), 3-oxo-14-deoxyandrographolide (8), dehydroandrographolide (9), 8β, 17α-epoxy-dehydroandrographolide (10), 9β-hydroxy-dehydroandrographolide (11) and 3-oxo-9β-hydroxydehydroandrographolide (12), respectively. Among them, 2–6 are novel compounds. All ^{1}H and ^{13}C NMR spectral data of known compounds 7–12 agreed with spectral data reported in the literatures [13–15]. The spectral data of transformed products are compiled in Tables 1 and 2, respectively.

Compound 2 was colorless powder, $[\alpha]_D^{22}$ +15.6° (*c* 0.10, MeOH). It showed a molecular formula of $C_{20}H_{28}O_5$ by HR-ESI-MS ([M+Na]$^+$, found *m*/*z* = 371.1836, calcd. 371.1829). Similar with that of 1, the butyrolactone moiety was also observed, due to a positive Legal reaction. Compared with deoxyandrographolide, the additional ketone carbon (δ 216.2) and bearing-oxygen carbon (δ 73.2) signals were observed. The HMQC spectrum indicated that the new oxygen-bearing carbon (δ 73.2) should be a tertiary carbon. In HMBC spectrum, the proton signal of δ 4.63 correlated with the carbon signals of C-5 (δ 50.0), C-6 (δ 33.2) and C-8 (δ 151.1), which suggests that compound 2 possessed 7-hydroxyl group. In addition, the carbon signal of δ 216.2 had the HMBC correlations of H-1, H-2, H-5 and H-18, suggesting a carbonyl group should be located at C-3. In NOE experiment, the proton signal at δ 4.63 (H-7) had the NOE enhancements with the signals at δ 2.61 (H-9) and δ 3.59 (H-5). Meanwhile, H-5 had the NOE effects with H-9 and H-18. These evidence indicated that HO-7 should be in α configuration. On the basis of above analysis, compound 2 was identified as 3-oxo-7α-hydroxy-14-deoxyandrographolide. All the ^{1}H and ^{13}C NMR spectral data were unambiguously assigned by 2D NMR spectra.

Compound 3 was isolated as colorless powder, $[\alpha]_D^{22}$ +55.6° (*c* 0.10, MeOH). Its HR-MS provided a *quasi*-molecular ion [M+Na]$^+$ at *m*/*z* 371.1828, suggesting the molecular formula of $C_{20}H_{28}O_5$. In ^{1}H NMR spectrum, two quaternary methyl signals at δ 1.07 and δ 1.43 were observed, respectively. In DEPT and HMQC spectra, eight CH_2 groups (δ 20.9, 21.7, 27.8, 36.1, 36.5, 38.5, 50.8 and 65.6), and the presence of a new oxygen-bearing CH_2 at δ 50.8 were observed, and two olefin protons disappeared, all of which suggesting an epoxy ring would be substituted in the molecule of compound 3. Similar with compound 2, the presence of a new carbonyl group was also observed. In HMBC spectrum, the proton signal of δ 2.81 had the long-range correlations with carbon signals at δ 59.0 (C-8), δ 52.4 (C-9) and δ 36.1 (C-7). And the carbon signal of δ 59.0 had HMBC correlations with H_a-11 (δ 1.18), H-9 (δ 1.56) and H_b-7 (1.92), all of which suggesting epoxidation of C-8, 17 in the chemical structure of 3. In addition, comparing with 1, the carbon signals at δ 29.0 (C-2) and δ 42.8 (C-4) shifted upfield to δ 36.1 and δ 55.2, respectively. The proton signals of H-2, H-18 and H-19 correlated with the carbon signal of δ 214.2, proving that a ketone group should be at C-3. In NOESY experiment, the proton signal of δ 2.81 had NOE enhancements with H-11 and H-20, suggesting H-17 should be in α configuration. On the basis of the above analysis, compound 3 was identified as 3-oxo-8β, 17α-epoxy-14-deoxyandrographolide. All the ^{1}H and ^{13}C NMR spectral data were unambiguously assigned by 2D NMR technology.

Compound 4 was obtained as colorless solid in acetone. Its HR-MS provided a *quasi*-molecular ion [M+Na]$^+$ at *m*/*z* 373.2019, suggesting the molecular formula of $C_{20}H_{30}O_5$. By comparing with the spectrum of 1, the ^{13}C NMR and DEPT spectra of 4 exhibited a new formyl group at δ 205.3, disappearance of two olefin carbons at δ 147.9 and δ 107.2. In HMBC spectrum, the protons of H-9 and H-7 had the long-rage correlations with the aldehyde carbon of δ 205.3. And ^{1}H–^{1}H-COSY spectrum, H-8 had correlations with H-9 and H-7. These evidence indicated that an aldehyde group should be located at C-17 position. And the NOESY cross signal of H-17 with H-11 revealed that 17-CHO was in α configuration. In addition, the NOE enhancement of H-3 with Me-18, implied the configuration of 3-OH should be α. On the basis of above analysis, compound 4 was identified as 8α-formyl-14-deoxyandrographolide. All the ^{1}H and ^{13}C NMR spectral data were unambiguously assigned by 2D NMR spectra.

Compound 5 was obtained as a white powder, $[\alpha]_D^{22}$ −35.2° (*c* 0.08, MeOH). The IR spectrum showed the presence of hydroxyl group (3435 cm^{-1}) and an α, β-unsaturated γ-lactone (1740,

Table 3
Cytotoxic activities of the metabolites against human cancer cell lines (IC_{50}, μmol/L).

	1	2	3	4	5	6	7	8	9	10	11	12
MCF	69.5	>100	>100	18.7	63.7	40.3	83.1	>100	78.3	>100	32.5	36.9
A562	87.3	>100	>100	38.6	93.8	95.3	>100	>100	>100	>100	75.6	68.2

1636 cm^{-1}). Its HR-ESI-MS provided a *quasi*-molecular ion peak at *m/z* 405.2264 $[M+Na]^+$, suggesting the molecular formula of $C_{21}H_{34}O_6$ (calcd. 405.2253). By comparing the NMR data with those of 14-deoxyandrographolide, three additional oxygen-bearing carbons at δ 63.3 (CH_2), δ 49.6 (OMe) and δ 80.8 were observed, respectively. In HMBC spectrum, the protons (δ 4.42) had the long-rage correlations with C-8 (δ 80.8) and C-9 (δ 60.9), respectively. The protons of methoxyl group (δ 3.32) correlated with C-8. In addition, in ^1H–^1H COSY spectrum, the proton signal (δ 1.91) correlated with H-12 (δ 2.56 and 2.47) and H-9 (δ 1.43), respectively. And H-9 had the HMBC correlations with C-8, C-10 and C-11. All evidence suggested that CH_2OH and OMe groups were substituted at C-8. The carbon at δ 80.3 correlated with H-2, H-18 and H-19 in HMBC spectrum, suggesting that a hydroxyl group was introduced at C-3. The NOESY enhancement of H-17 with H-11 revealed that 8-CH_2OH was in α configuration. And the NOE enhancements of 8-OMe with H-9 suggested 8-OMe should be in β configuration. In addition, the NOE correlations of H-3 with H-5 and Me-18, implied the configuration of 3-OH should be α. On the basis of above analysis, compound 5 was identified as 8β-methoxyl-17α-hydroxyl-14-deoxyandrographolide. All the ^1H and ^{13}C NMR spectral data were unambiguously assigned by 2D NMR spectra.

Compound 6 was obtained as colorless powder, $[\alpha]_D^{22}$ −10.6° (*c* 0.10, MeOH). Its HR-MS provided a *quasi*-molecular ion $[M+Na]^+$ at *m/z* 373.2016, suggesting a molecular formula of $C_{20}H_{30}O_5$. The Legal reaction of 6 was positive, suggesting α-alkylidene γ-butyrolactone moiety in chemical structure. Compared to that of deoxyandrographolide, the ^{13}C NMR spectrum of 6 exhibited the disappearance of an exocyclic double bond, and the presence of an additional oxygen-bearing carbon signal at δ 65.7 (CH_2). In HMBC spectrum, the proton signals of δ 4.32 and 4.51 (CH_2) had the long-range correlations with the carbon signals of C-8 (δ 140.5), C-7 (δ 124.8) and C-9 (δ 51.3), suggesting a hydroxymethyl group should be at C-8. In addition, the proton signal of δ 5.92 had HMBC correlations with the carbon signals of C-8 (δ 140.5), C-6 (δ 23.7) and C-5 (δ 52.5). And H-9 (δ 1.34) had HMBC correlations with C-8, C-10, C-11 and C-20. All these evidence suggested that a double bond should be substituted at C-7 and C-8 in its molecule. In NOESY spectrum, Me-20 had the NOE enhancements with H-11 (δ 1.83) and H-19 (δ 3.77 and 4.52), suggesting that CH_2OH-19 should be in α configuration. And NOE effect of Me-18 with H-3 implied that the configuration of 3-OH should be in α. On the basis of the above analysis, compound 6 was identified as 3α,17,19-trihydroxy-7,13-ent-labdadien-15,16-olide. All the ^1H and ^{13}C NMR spectral data were unambiguously assigned by 2D NMR spectra.

The various types of transformed reactions were observed such as dehydrogenation, epoxide and hydroxylation in transformation of 1 by *C. echinulata* AS 3.3400 (Fig. 1). The main reaction sites of dehydrogenation were at C-3, C-11 and C-12 positions, respectively. The epoxide happened at C_8–C_{17} double bond of ent-labdane skeleton. In addition, the hydroxylation sites were usually observed at α-position of olefinic bond such as products 2, 6–7 and 11–12. The 3-OH dehydrogenation from 1 to 8 was similar to the result reported before [7], and then product 8 could be metabolized by microorganisms to yield the series of 3-ketone derivatives of 1. In addition, the transformed reactions from 9 to 12 were also observed in biotransformation pathway of dehydroandrographolide by *C. echinulata* AS 3.3400 [13].

In order to prove the character of enzymes involved in the bioconversion of 1 by *C. echinulata* AS 3.3400, the microorganisms were grown for 2 days in the presence of 1-aminobenzotriazole, a cytochrome P-450 inhibitor, and then administrating 1. As a result, after 3 days of incubation, substrate was only transformed to dehydroandrographolide (9) with the low yield. And the other oxidized metabolites were not observed at all, which indicates a series of oxidation reactions catalyzed by the cytochrome P-450 monooxygenases of *C. echinulata* [16].

The *in vitro* cytotoxicities of metabolites against MCF and A562 cells were determined by the MTT bioassay. The results are given in Table 3. Our results indicated that 3-ketone group would decrease the cytotoxic activity, which was also observed before [7]. And the hydroxylation of C-17 (compounds 5 and 6) did not change the cytotoxicities of 1, but the formyl group at C-8 (compound 4) would slightly enhance bioactivities against MCF and A562 cells. In addition, the presence of ester functional group is essential for improving the activities of andrographolide [14,17], while our investigation suggested that the hydroxylation was the main biotransformation reaction, and providing more functional hydroxyl groups for semi-synthesis of ester derivatives.

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