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Microbial transformation of dehydroandrographolide by *Cunninghamella echinulata*

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ABSTRACT

The capabilities of 24 strains of filamentous fungi (from 11 genera) to transform dehydroandrographolide (**1**) were screened. The highly region-specific hydroxylation of **1** at C-9 by *Cunninghamella echinulata* AS 3.3400 was observed. Five products were obtained, and they were identified as 9β-hydroxydehydroandrographolide (**2**), 3-oxo-hydroxydehydroandrographolide (**3**), 3-oxo-9β-hydroxydehydroandrographolide (**4**), 7α-hydroxydehydroandrographolide (**5**) and 8β,17α-epoxydehydroandrographolide (**6**), respectively. Among them, products **2**, **4** and **5** were novel compounds.

1. Introduction

Andrographis paniculata is a common herb distributed in China, Southeast Asia and India [1]. Its stems and leaves are widely used to treat the acute hepatitis, inflammation and tumor. The principal active constituents of *A. paniculata* are the *ent*-labdane diterpenoids, such as dehydroandrographolide and andrographolide. They had a unique skeleton of *ent*-labdane diterpenoids, which have characteristic A/B *cis* junctures with γ-butyrolactone moiety and α-hydroxyl at C-3 position. They have anticancer [2], immunostimulant [3] and antiviral activities [4]. In recent reports, structure–activity relationships of several andrographolid derivatives in cytotoxic assays against various cancer cell lines were demonstrated [5,6].

Microbial transformation is a useful tool to modify the structures of bioactive substrates. And this approach has some advantages over organic synthesis such as high stereo- and region-selectivity. Some transformations such as hydroxylation at specific positions are difficult for chemical synthesis, but could be readily accomplished with microbial transformation [7–11].

In recent years, biotransformation of diterpenoid compounds has been frequently reported [12–15]. Readily isolated from plant extracts, these diterpenes often exhibit or are believed to have bioactivities, and are ideal substrates for biotransformation. In order to find more active compounds, we are especially interested in the various modified structures of *ent*-labdane diterpenoids such as dehydroandrographolide and andrographolide by microbial transformation. In the present paper, the biotransformation of dehydroandrographolide by *Cunninghamella echinulata* AS 3.3400 was carried out. Five products were obtained, and their structures were elucidated on the basis of extensive spectral data, including 2D-NMR. Among them, products **2**, **4** and **5** were new compounds. The high region-specific hydroxylation of compound **1** at C-9 position by C. *echinulata* AS 3.3400 was reported. In addition, the cytotoxicities of transformed products against Bel-7402 and Hela were also investigated.

2. Experimental

2.1. Apparatus

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. HRMS were obtained on a Bruker APEXII Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, USA) comprised a quaternary solvent delivery system, an auto-sampler, a column temperature controller and a diode-array detector (DAD) coupled with an analytical workstation.

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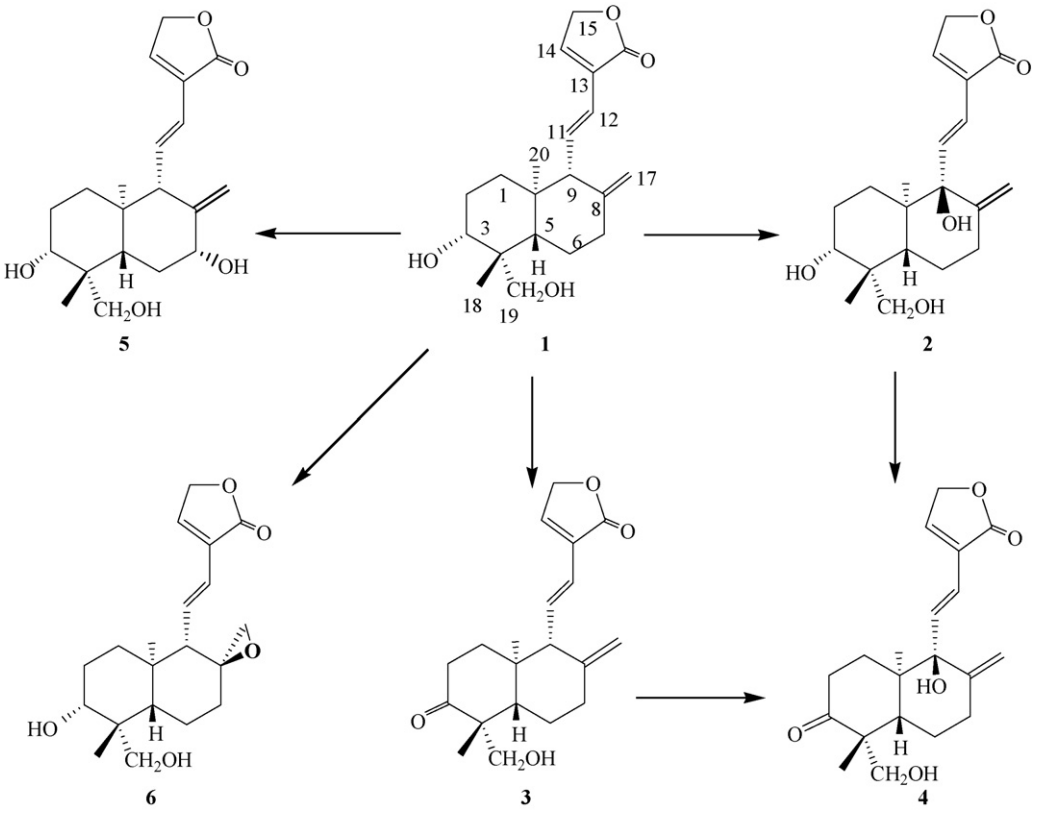

Fig. 1. Possible biotransformation pathway of 1 by *Cunninghamella echinulata* AS 3.3400.

2.2. Reagents

Thin layer chromatography (TLC) analyses were performed on silica gel G (200–300 mesh), which were purchased from Qing Dao Haiyang Chemical Group Co., PR China. All solvents were AR grade (from Shenyang Chemical Company). Dehydroandrographolide (**1**) was isolated from *A. paniculata* by the authors [1]. Its purity was 98% by HPLC analysis.

2.3. 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, *Mucor spinosus* AS 3.3450, *M. spinosus* AS 3.2450, *M. spinosus* AS 3.3447, *Mucor subtilissimus* AS 3.2454, *M. subtilissimus* AS 3.2456, *Mucor polymorphosporus* AS 3.3443, *Penicillium melinii* AS 3.4474, *Penicillium janthinellum* AS 3.510, *Rhizopus oryzae* AS 3.851, *Rhizopus cohnii* AS 3.2746, *Rhizopus stolonifer* AS 3.3463, *Rhizopus arrhizus* AS 3.2897, *Syncephalastrum racemosum* AS 3.264 and *Trichoderma viride* AS 3.2942 were purchased from China General Microbiological Culture Collection Center in Beijing, China.

2.4. Culture medium

All culture and biotransformation experiments using filamentous fungi were performed in potato medium. Potato medium was produced by the following procedure: 200 g of minced and husked potato were boiled in H_2O for 1 h, then solution was filtered and the filtrate were added with H_2O to 1 L after addition of 20 g of glucose [16]. The media were sterilized in an autoclave at 121 °C and 1.06 kg/cm^2 for 30 min. Microorganisms were maintained on agar slants of media and were stored at 4 °C.

2.5. Culture and biotransformation procedures

Mycelia from the agar slants (1 cm^2) were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of the potato medium and cultured at 28 °C with 180 rpm for 36 h to make a stock inoculum. Then 2 mL inoculum was added to 100 mL potato medium in a 250 mL flask. After pre-culture for 36 h, 2 mg 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 a rotary shaker. In addition, the culture controls were composed by the fermentation blanks in which the microorganisms were grown without adding the substrates. On the other hand, the substrate was added into the sterile medium without the microorganisms, and then incubated under the same fermentation conditions, which indicating whether the substrate was stable in the blank medium.

Preparative scale bioconversion of dehydroandrographolide (**1**) by *C. echinulata* AS 3.3400 was performed in 1000 mL culture flasks. After 36 h of pre-culture, 10 mg of substrates with 0.5 mL acetone were added into 400 mL conversion medium. In total, 500 mg of substrates were added in preparative biotransformation. Other procedures were same with those in the screening scale biotransformation.

2.6. Purification and identification of new biotransformation products

The culture was filtered and the filtrate was extracted with same volume of EtOAc for four times. The organic phase was collected and concentrated at 30 °C *in vacuo*. The extraction were applied to ODS column (Ø3 cm by 80 cm) and eluted with MeOH–H_2O (in a gradient manner from 10:90 to 90:10, at a flow rate of 1.0 mL/min). Fractions were concentrated at 30 °C *in vacuo* and monitored by HPLC-DAD, finally five transformed products were isolated (Fig. 1), including **2**

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

	H				
	2	**3**	**4**	**5**	**6**
1a	1.38 m	2.42	1.69 m	1.26 m	1.08 m
b	2.20 m	2.82	2.30 m	1.53 m	1.53 m
2a	1.99 m	1.47	2.45 m	1.93 m	1.91 m
b	2.10 m	1.76	2.92 m	2.00 m	2.03 m
3	3.80 m	–	–	3.74 m	3.64 m
5	2.59 m	1.60 m	3.02 dd (3.5, 14.5)	2.22 m	1.17 m
6a	1.51 m	1.66 m	1.79 m	1.76 m	1.59 m
b	1.84 m	1.73 m	1.80 m	2.24 m	1.83 m
7a	2.28 m	2.05 m	2.52 m	4.62 brs	1.25 m
b	2.84 m	2.34 m	2.81 m		1.44 m
9	–	2.44 d (10.0)	–	3.26 d (10.0)	2.25 d (10.0)
11	7.70 d (16.0)	7.20 d (16.0)	7.77 d (16.0)	7.20 dd (10.0, 16.0)	6.78 dd (10.0, 16.0)
12	7.00 d (16.0)	6.24 d (16.0)	7.02 d (16.0)	6.24 d (16.0)	6.30 d (16.0)
14	7.30 s	7.32 brs	7.30 brs	7.25 brs	7.21 d (13.5)
15	4.75 brs	4.81 brs	4.76 brs	4.77 brs	4.71 brs
17a	4.92 s	4.80 s	4.97 s	4.85 s	2.53 brs
b	5.00 s	4.89 s	5.08 s	5.08 s	2.88 brs
18	1.57 s	1.41 s	1.48 s	1.62 s	1.52 s
19	3.72 d (11.0)	3.79 d (11.0)	3.88 d (11.0)	3.71 d (11.0)	3.71 d (11.0)
	4.59 d (11.0)	4.26 d (11.0)	4.37 d (11.0)	4.53 d (11.0)	4.49 d (11.0)
20	1.11 s	1.18 s	1.44 s	0.94 s	0.99 s

(325 mg, 65% yield), **3** (6 mg, 1.2% yield), **4** (10 mg, 2% yield), **5** (9 mg, 1.8% yield) and **6** (4 mg, 0.8% yield). All the ^{1}H NMR and ^{13}C NMR spectral data of new compounds **2**, **4** and **5** were unambiguously assigned by 2D-NMR spectra.

9β-Hydroxydehydroandrographolide (**2**): colorless solid (acetone). m.p. 152–153 °C, $[\alpha]_D^{22}$ −10.7° (*c* 0.20, MeOH); UV (MeOH): λ_{max} = 254 nm. IR (KBr): ν_{max} = 3410, 1770, 1630, 896 cm^{-1}. ^{1}H NMR and ^{13}C NMR see Tables 1 and 2; HR-ESI-MS: m/z = 371.1859 $[M+Na]^+$ (calcd. for $C_{20}H_{28}O_5Na_1$; 371.1834).

3-Oxo-9β-hydroxydehydroandrographolide (**4**): colorless solid (acetone). m.p. 152–153 °C, $[\alpha]_D^{22}$ −19.1° (*c* 0.13, MeOH); UV (MeOH): λ_{max} = 254 nm. IR (KBr): ν_{max} = 3320, 1725, 1667, 910 cm^{-1}. ^{1}H NMR and ^{13}C NMR see Tables 1 and 2; HR-ESI-MS: m/z = 369.1696 $[M+Na]^+$ (calcd. for $C_{20}H_{26}O_5Na_1$; 369.1678).

7α-Hydroxydehydroandrographolide (**5**): colorless solid (acetone). m.p. 152–153 °C, $[\alpha]_D^{22}$ −33.7° (*c* 0.12, MeOH); UV (MeOH): λ_{max} = 258 nm. IR (KBr): ν_{max} = 3419, 1750, 1644, 896 cm^{-1}. ^{1}H NMR and ^{13}C NMR see Tables 1 and 2; HR-ESI-MS: m/z = 371.1873 $[M+Na]^+$ (calcd. for $C_{20}H_{28}O_5Na_1$; 371.1834).

Table 2
^{13}C NMR spectral data of compounds **1–6** (Pry-d_5, 125 MHz, δ in ppm).

	C					
	1	**2**	**3**	**4**	**5**	**6**
1	38.7	31.6	36.7	33.0	39.5	38.6
2	28.9	28.9	39.6	36.7	29.0	28.4
3	80.1	80.3	213.7	214.6	80.3	80.0
4	43.4	43.3	55.1	54.8	43.0	43.3
5	54.8	46.0	56.4	47.9	47.4	54.5
6	23.6	23.4	24.2	24.2	31.4	21.9
7	37.0	33.2	36.6	33.2	72.3	36.2
8	149.3	152.1	148.8	151.7	152.3	58.4
9	61.8	79.5	61.2	79.4	56.7	59.5
10	39.0	42.8	39.0	42.7	39.5	39.4
11	135.6	140.0	135.2	139.6	135.4	131.4
12	121.6	119.2	122.4	119.5	122.1	124.6
13	128.9	129.0	128.8	128.9	128.9	128.7
14	145.0	145.1	145.4	145.5	145.0	145.3
15	70.3	70.3	70.3	70.3	70.3	70.2
16	172.8	173.1	172.8	173.0	172.8	172.7
17	108.8	110.9	109.5	111.6	110.2	50.5
18	23.6	23.9	21.3	21.7	23.6	23.7
19	64.2	64.5	65.4	65.8	64.4	64.2
20	16.0	18.8	15.5	18.3	15.4	16.1

2.7. Analysis methods

The analysis of samples were performed on an Agilent 1100 series HPLC equipped with a Alltech C-18 column (USA), 4.6 mm × 250 mm (5 μm), and diode array detector at 254 nm. The mixture of MeOH and water were used as the mobile phase. The eluent was MeOH–H_2O (25:75, v/v), held for 5 min, gradient to (60:40, v/v) in 25 min, and then held for 10 min, and then gradient to (90:10, v/v) in 10 min, then held for 10 min. The flow rate was 0.8 mL/min and column temperature was 30 °C. Concentrations of compound **1** and **2** in the reaction mixture were determined based on corresponding UV absorption peak areas at 254 nm and calibration curves prepared with standards.

2.8. Bioassay

Human hepatoma cells (Bel-7402) and human cervical carcinoma dells (Hela) were in RPMI1640 medium (GIBCO/BRL, Maryland, 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 the cancer cells were evaluated by MTT method [17]. 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-four strains of filamentous fungi (from **11** genera) were initially screened by HPLC chromatography for their ability to transform the substrate (**1**). Among the cultures screened, *A. alternata* AS 3.577, *C. lunata* AS 3.4381, *C. blakesleana lender* AS 3.970, *C. echinulata* AS 3.3400 and *R. oryzae* AS 3.851 were found to be able to convert **1** into more polar metabolites. By the HPLC analysis, *C. echinulata* AS 3.3400 was found to be the most potent strain to transform the substrate in high yield. Therefore it was selected for a preparative biotransformation (Fig. 2).

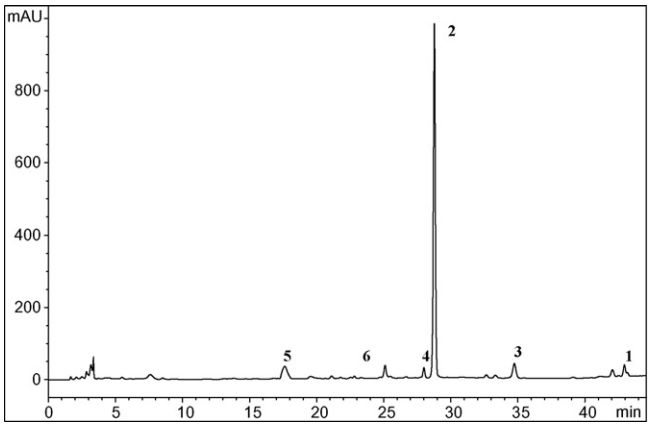

Fig. 2. HPLC chromatogram of the transformed products of 1 by *C. echinulata* AS 3.3400.

3.2. Identification of biotransformation products

Compound **2** was given as white powder (MeOH). Its HR-ESI-MS gave a quasi-molecular ion $[M+Na]^+$ at m/z 371.1859, suggesting the molecular formula of $C_{20}H_{28}O_5$. In ^{1}H NMR spectrum, five alkene protons at δ 7.00 (d, 16.0 Hz), δ 7.30 (s), δ 7.70 (d, 16.0 Hz), δ 4.92 (s) and δ 5.00 (s) were observed, respectively. In ^{13}C NMR spectrum, four bearing-oxygen carbons of δ 64.5, 70.3, 79.5 and 80.3 were observed. By comparing with **1**, the carbon signal at δ 79.5 was an additional bearing-oxygen carbon, suggesting that **2** was a hydroxylated product. The HMBC spectrum indicated that the carbon signal of δ 79.5 correlated with H-11 (δ 7.70), H-12 (δ 7.00), H-17 (δ 4.92, 5.00), H-5 (δ 2.59), H-7 (δ 2.28) and Me-20 (δ 1.11), which suggesting that the hydroxylation group was located at C-9. In addition, the proton of H-11 (δ 7.70) had NOE enhancement with Me-20 (δ 1.11) in NOESY spectrum. This evidence proved that HO-9 should be in β-configuration. Therefore, metabolite **2** was characterized as 9β-hydroxyl-dehydroandrographolide.

Compound **4** showed a molecular formula of $C_{20}H_{26}O_5$ by HR-ESI-MS ($[M+Na]^+$, found m/z = 369.1696, calcd. 369.1678). In ^{13}C NMR spectrum, an additional oxo carbon signal (δ 214.6) was observed. And DEPT spectrum showed that the new bearing-oxygen carbon (δ 79.4) was a quaternary carbon. In HMBC spectrum, this carbon signal had long-rang couplings with H-11 (δ 7.77), H-12 (δ 7.02), H-17 (δ 4.97 and 5.08) and Me-20 (δ 1.44), suggesting the hydroxyl group should be at C-9. In addition, the HMBC correlations of the carbonyl carbon (δ 214.6) with H-19 (δ 4.37, 3.88), H-2 (δ 2.45, 2.92) and Me-18 (δ 1.48), implied introduction of an oxo group at C-3 position. The NOE enhancement between H-11 and Me-20 indicated that 9-OH should be β-configuration. From the above deductions, the structure of compound **4** could be elucidated as 3-oxo-9β-hydroxydehydroandrographolide.

Compound **5** showed a molecular formula of $C_{20}H_{28}O_5$ by HR-ESI-MS ($[M+Na]^+$, found m/z = 371.1873, calcd. 371.1834), suggesting that it might be a hydroxylated product of the substrate. A new tertiary carbon signal (δ 72.3) was observed in its ^{13}C NMR spectrum. The HMBC correlations of the proton (δ 4.62) with C-17 (δ 110.2), C-9 (δ 56.7) and C-5 (δ 47.4), indicated the introduction of hydroxyl group to be at C-7 position. The NOE enhancement between H-7 and H-9 suggested that 7-OH should be in α-configuration. On the basis of the above analysis, compound **5** was identified as 7α-hydroxydehydroandrographolide.

All the ^{1}H NMR and ^{13}C NMR spectral data of known compounds **3** and **6** were also unambiguously assigned by 2D-NMR spectra, and compared with spectral data of literature values [1].

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

	1	2	3	4	5	6
Bel-7402	80	12.5	>100	39	33.2	>100
Hela	65.5	4.3	>100	71.3	15.6	42.3

3.3. Cytotoxicity testing

The cytotoxicities of metabolites **2**–**6** against Bel-7402 and Hela cells were determined by the MTT bioassay (Table 3). Compared to **1**, **3** and **6** had lower cytotoxic activity against both cell lines (compound **3**) or Bel-7402 cell line (compound **6**), indicating that C8–C17 and C3 OH regions are important for the activity of **1**. Similar effect of modification at C8–C17 was reported previously [6]. New metabolites **2** and **5** exhibited better cytotoxicity against both cell lines than that of **1**, suggesting hydroxylation at C-9 or C-7 would enhance the activity.

3.4. Biotransformation of *1* by C. echinulata AS 3.3400

The time course of bioconversion of **1** by *C. echinulata* AS 3.3400 was investigated. The result was shown in Fig. 3. After administration for 120 h, the substrate could be almost completely metabolized by microorganisms. The HPLC analysis showed that the major transformed product **2** reached the maximal concentration in 72% yield on 120 h, all of which suggesting that *C. echinulata* AS 3.3400 had good capability of hydroxylation at C-9 position of the substrate (**1**). As we all known, it was difficult to fulfill this reaction by the chemical synthesis, due to many by-reaction and low yield. So *C. echinulata* AS 3.3400 could be as a bioreactor to obtain the bioactive and new compound **2** effectively.

In order to investigate the effects of carbon double bonds in structure of **1** on the bioconversion, the biotransformation of transformed product **6** (8β,17α-epoxy-dehydroandrographolide), 14-deoxyandrographolide and andrographolide as the structural analogues of **1** [1], were carried out by *C. echinulata* AS 3.3400. Our results indicated that these compounds were not transformed at all within 5 days, suggesting the structural variation of double bonds at C_{11-12} or C_{8-17} would strongly inhibit the activity of hydroxylation enzymes. This evidence indicated that the double bonds at C_{11-12} and C_{8-17} in the structures of **1** were the helpful structural requirement for specificity of hydroxylation at C-9 by *C. echinulata* AS 3.3400. Meanwhile, compound **2** as the major metabolite of **1** by *C. echinulata* AS 3.3400, was an oxide product. In order to prove the character of oxygenase employed in the biotransformation of

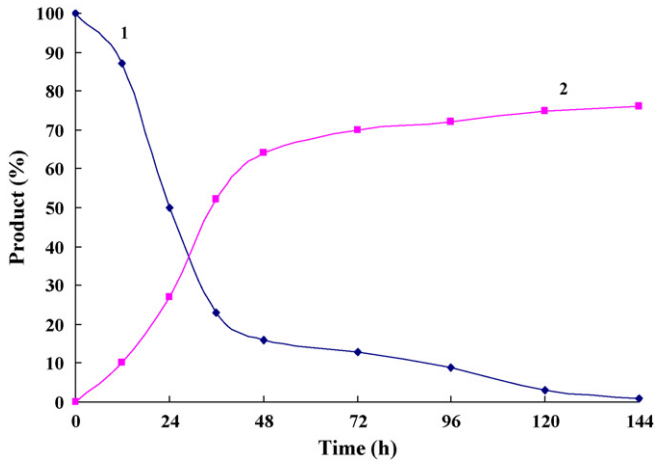

Fig. 3. Time course in biotransformation of 1 by *C. echinulata* AS 3.3400.

1 [18], *C. echinulata* AS 3.3400 was pre-cultured for 2 days after adding 1-aminobenotriazole, a cytochrome P-450 inhibitor [19,20], and then administrating the substrate. As a result, the biotransformation of **1** by microorganism was strongly inhibited, and only trace of **2** was observed by HPLC analysis, all of which suggesting hydroxylation reaction at C-9 position of **1** resulted from cytochrome P-450 monooxygenase of *C. echinulata* AS 3.3400.

In addition, the effect of PH values of conversion medium on the metabolism of **1** was also investigated. The optimum PH values were proved to be at 6–8 in 70% yield. When the values of PH were at 2–4 or 10–12, the transformation rates of **2** were decreased greatly at 7–18% yield by HPLC analysis.

4. Conclusion

The capabilities of 24 strains of filamentous fungi (from **11** genera) to transform dehydroandrographolide (**1**) were screened. The highly region-specific hydroxylation of **1** at C-9 by *C. echinulata* AS 3.3400 was observed in 72% yield. Five products were obtained, and they were identified as 9β-hydroxydehydroandrographolide (**2**), 3-oxo-hydroxydehydroandrographolide (**3**), 3-oxo-9β-hydroxydehydroandrographolide (**4**), 7α-hydroxydehydroandrographolide (**5**) and 8β,17α-epoxydehydroandrographolide (**6**), respectively. Among them, products **2** and **4-5** were novel compounds. The major product **2** had better cyctotoxicity than the substrate. This evidence indicated that biocatalytic hydroxylation by *C. echinulata* AS 3.3400 is a feasible and effective approach to directly obtain novel compound **2** with the advantage of high selectivity. Meanwhile, this major product could be as the hemisynthesis intermediate to produce more bioactive leading compounds in the future.

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