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Microbial transformation of three bufadienolides by *Penicillium aurantigriseum* and its application for metabolite identification in rat

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Abstract

Eighteen strains of filamentous fungi (from nine genera) were screened for their capabilities to transform three major bufadienolides, cinobufagin (**1**), bufalin (**2**) and resibufogenin (**3**), from the traditional Chinese medicine Chan Su. *Penicillium aurantigriseum* AS 3.4512 was found to be the most potent strain to transform these compounds into *in vivo* metabolites in rat. Ten transformation products were obtained with high yield and identified using chromatographic and spectral means. Among them, six products were found to be same with the *in vitro* metabolites transformed by rat liver microsome. Three compounds were the major *in vivo* metabolites of **1**, **2** and **3**, respectively. Compound **3a** was a firstly reported metabolite of **3** *in vivo*. All of these suggested the bioconversion of **1**, **2** and **3** by *P. aurantigriseum* were very similar with the metabolism of them *in vivo*, and these transformed products could be used as the reference standards to investigate the mammalian metabolism of bufadienolides.

Keywords: Biotransformation; Bufadienolides; Mammalian metabolism; *Penicillium aurantigriseum*

1. Introduction

Cinobufagin, bufalin and resibufogenin are the three typical bufadienolides from the traditional Chinese medicine Chan Su, which is reported to exhibit significant inhibitory activities against human hepatoma and prostate cancer cells [1–3]. However, most of them can adversely affect the myocardium and the most life-threatening toxicity includes ventricular ectopy, atrial arrhythmias and hyperkalemia [4,5]. Therefore, metabolic study of these toxic compounds are significant for evaluation of their pharmacological effects and safety.

Drug metabolism is regarded as a series of enzymatic transformation *in vivo* to form more polar products. These metabolites might lead to pharmacological or toxicological activities. So it is necessary to obtain these metabolites for investigation of drug metabolism. Traditionally, drug metabolism studies, to obtain some *in vivo* metabolites of drugs, have depended on the model systems such as small laboratory animal model, perfused tissue, and microsomal preparations. This method have a number of limitations, especially for the toxic drugs, such as the cost of experimental animals, ethical concerns and frequently few nanograms metabolites isolated, which are not enough to identify their structures and investigate their activities.

Now, biotransformation is becoming an important tool in structural modification and metabolism study of drugs. Microorganism possess the monooxygenase enzyme systems that have been proved to be similar to the mammalian hepatic monooxygenases. The use of microbial systems as models for drug metabolism has the advantages of saving the cost of large amounts of animals, identifying trace metabolites, avoidance of ethical concerns and obtaining enough amounts of metabolites to investigate their activities. Therefore, it has been commonly used as *in vitro* model for obtain the enough amount of *in vivo* metabolites of drug [6–13].

In this paper, the biotransformation of three major bufadienolides by *Penicillium aurantigriseum* AS 3.4512 was investigated. Ten transformed products were obtained and identified. All the

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metabolites could be used as chromatographic standards and tested for their biological activities with sufficient amounts. Six of them were same with the metabolites of rat liver microsome *in vitro*. Three compounds (**1d**, **2b** and **3a**) were same with the major *in vivo* metabolites of **1**, **2** and **3**, respectively. Compound **3a** was a firstly reported metabolite of **3** *in vivo*. All these features suggested that *P. aurantigriseum* AS 3.4512 could be used as the *in vitro* model with high yield to obtain enough amount of *in vivo* metabolites of bufadienolides.

2. Materials and methods

2.1. Apparatus

1D and 2D NMR (nuclear magnetic resonance) spectra were recorded in DMSO-d_6 using TMS as internal standard on a DRX-500 spectrometer (Bruker, Karlsruhe). A Finnigan LCQ Advantage ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) connected to Agilent 1100 HPLC system via an APCI interface was used.

2.2. Reagents

All solvents were AR grade (from Beijing Chemical Company). TLC (thin layer chromatography) analyses were performed on silica gel G (200–300 mesh), which were purchased from Qing Dao Haiyang Chemical Group Co., PR China. Cinobufagin (**1**), bufalin (**2**) and resibufogenin (**3**) were isolated from Chan Su by the author. Their purity was above 98% by HPLC analysis.

2.3. Microorganisms

Alternaria alternata AS 3.577, *Alternaria alternata* AS 3.4578, *Aspergillus niger* AS 3.795, *Aspergillus candidus* CICC 2360, *Cunninghamella blakesleeana* Lendner AS 3.970, *Cunninghamella elegans* AS 3.1207, *Cunninghamella elegans* AS 3.2028, *Fusarium solani* AS 3.1829, *Mucor spinosus* AS 3.3450, *Mucor spinosus* AS 3.2450, *Mucor spinosus* AS 3.3447, *Mucor subtilissimus* AS 3.2454, *Mucor subtilissimus* AS 3.2456, *Mucor polymorphosporus* AS 3.3443, *Penicillium janthinellum* AS 3.510, *P. aurantigriseum* AS 3.4512, *Trichoderma viride* AS 3.2942 and *Rhizopus stolonifer* AS 3.2050 were purchased from China General Microbiological Culture Collection Center in Beijing, PR China.

2.4. Culture medium

All culture and bioconversion 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 water for 1 h, then solution was filtered and the filtrate were added with water to 1 L after addition of 20 g of glucose [14]. Microorganisms were maintained on agar slants of media recommended by the ATCC and were stored at 4 °C.

2.5. Culture and biotransformation procedures

Mycelia from the agar slants (1 cm^2) were transferred to 100 mL of culture medium in 250 mL Erlenmeyer flasks and cultured at 27 °C with 180 rpm for 36 h to make a stock inoculum. Then a 5 mL volume of the inoculum was added to a 250 mL flask contained 100 mL of potato medium. After 36 h of pre-culture, 2 mg of substrates in 0.2 mL acetone were added into each flask and these flasks were maintained under the fermentation conditions for 4 days.

Preparative scale biotransformation of cinobufagin (**1**), bufalin (**2**) and resibufogenin (**3**) by *P. aurantigriseum* AS 3.4512 were carried out in 1000 mL Erlenmeyer flasks. After 36 h of pre-culture, 10 mg of substrates with 0.5 mL acetone were added into 350 mL culture medium. In total, 400 mg of substrates were used for preparative biotransformation. The incubation was continued for four additional days. Other procedures were same with those in the screening scale biotransformation.

2.6. Extraction, purification and identification of biotransformation products

The culture was filtered and the filtrate was extracted with same volume of EtOAc for five times. The organic phase was collected and concentrated *in vacuo*. The residues were applied to a silica gel column and eluted with petroleum ether–acetone (in a gradient manner from 100:3 to 1:1, at a flow rate of 1.5 mL/min). All the products were identified on the basis of their spectral data.

2.7. Analysis methods

2.7.1. HPLC analysis

The samples were analyzed on an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA, USA) equipped with a YMC C-18 column (YMC, Japan), 4.6 mm × 250 mm (5 μm), and diode array detector at 296 nm. The mobile phase consisted of MeCN and water containing 0.3% (v/v) HOAc. The eluent was MeCN–H_2O (30:70 (v/v)), held for 5 min, gradient to (50:50 (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.

2.7.2. LC/MS analysis

High-pure nitrogen (N_2) was used as the nebulizing gas and ultrahigh pure helium (He) as the damping gas. The MS parameters were as follows: vaporizer temperature, 450 °C; sheath gas, 80 arbitrary units; discharge current, 5 μA; capillary temperature, 175 °C; capillary voltage, 35 V. The mass spectrometer was monitored in the positive ion mode and recorded in the range *m/z* 120–1000.

2.8. Bioassay

Human hepatoma cells (Bel-7402) was maintained in RPMI1640 medium (GIBCO/BRL, Maryland, USA) supplemented with 10% (v/v) fetal bovine serum and culture in 96 well microtiter plates for the assay. Appropriate dilutions of the

test compounds were added to the cultures. After incubation at 37 °C, 5% CO_2 for 72 h, the survival rates of the 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. The IC_{50} values (μmol/L) of some metabolites were 1.88 (**1a**), 2.63 (**1b**); 1.87 (**1c**); 63.9 (**1d**); 1.36 (**1e**); 4.20 (**2a**); 93.13 (**3a**) and 23.33 (**3b**).

2.9. Animals and sample preparation

Male Sprague–Dawley rats (200–220 g) were obtained from the Laboratory Animal Center of Peking University Health Science Center (Beijing, PR China). They were kept in environmentally controlled breeding room for 3 days before starting the experiments and fed with standard laboratory food and water *ad libitum* and fasted overnight before test. A 5 mL of ethyl acetate was added with 1 mL of rat serum, the supernatant was evaporated to dryness under a stream of nitrogen, after 10 min vortexing and 10 min centrifugation at 3000 rpm, the residue was dissolved in 200 μL methanol and stored at 4 °C until use.

3. Results and discussion

3.1. Preliminary screening for biotransformation

Eighteen strains of filamentous fungi (from nine genera) (see Section 2) were initially screened for their abilities to transform cinobufagin (**1**), bufalin (**2**) and resibufogenin (**3**) by TLC

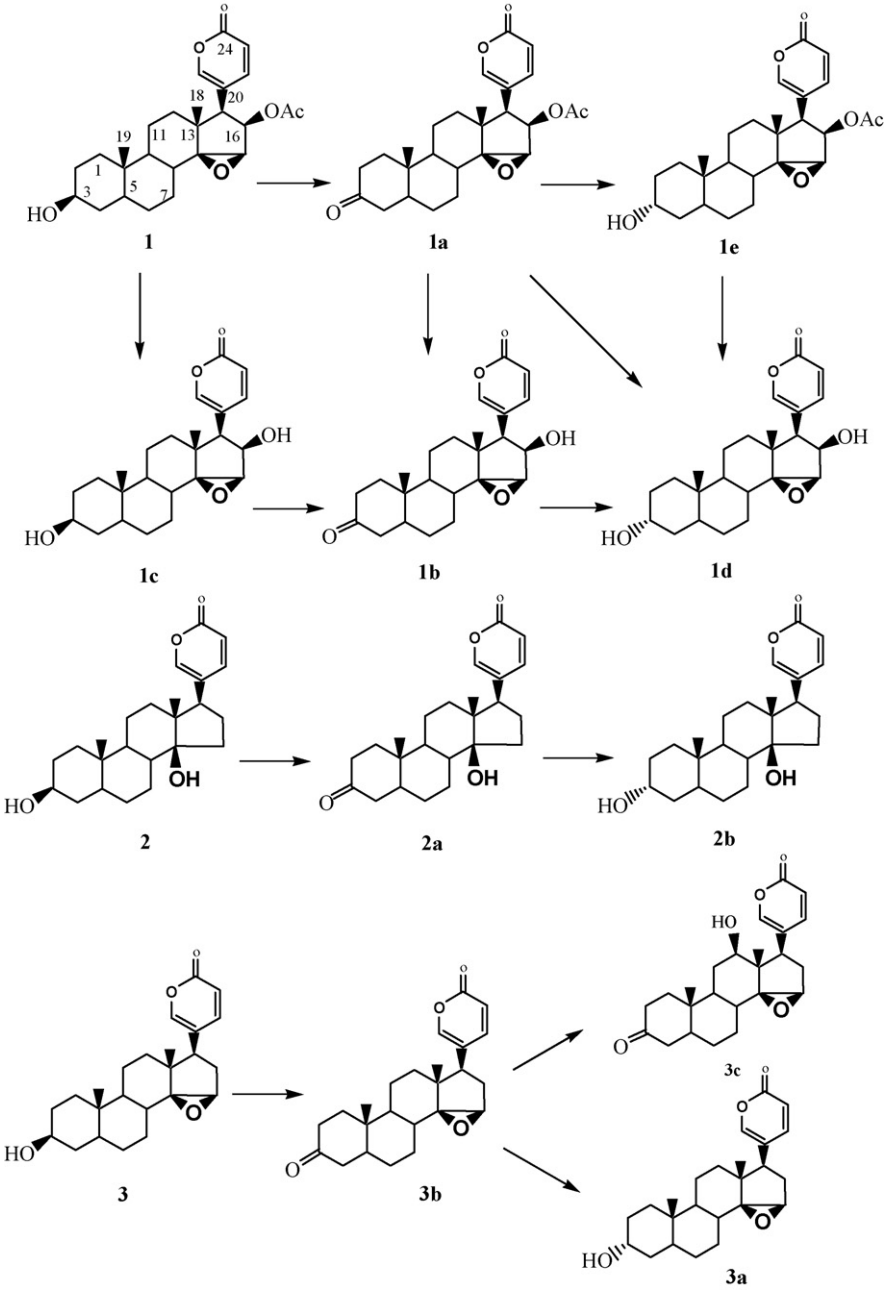

Fig. 1. A proposed biotransformation pathway of cinobufagin (**1**), bufalin (**2**) and resibufogenin (**3**) by *Penicillium aurantigriseum* AS 3.4512.

and HPLC chromatography. On TLC plate, the substrate and metabolites were visualized blue after spraying with 10% H_2SO_4 solution, followed by heating at 120 °C for 5 min. Among the cultures screened, the TLC and HPLC analysis showed that the R_f values, retention times and UV absorption of the metabolites by *P. aurantigriseum* AS 3.4512 were similar with these of *in vivo* metabolites in rats. Therefore it was selected for the preparative biotransformation of substrates.

3.2. Identification of biotransformation products

Incubation of cinobufagin (**1**), bufalin (**2**) and resibufogenin (**3**) with *P. aurantigriseum* AS 3.4512 for 6 days yielded **10** transformed products (Fig. 1): 3-ketocinobufagin (**1a**); 3-ketodeacetycinobufagin (**1b**); deacetycinobufagin (**1c**); 3-epideacetycinobufagin (**1d**); 3-epicinobufagin (**1e**); 3-ketobufalin (**2a**); 3-epibufalin (**2b**); 3-epiresibufogenin (**3a**); 3-ketoresibufogenin (**3b**); 12β-hydroxyl-3-ketoresibufogenin (**3c**), respectively, on the basis of their spectral data and compared with spectral data of literature values [15,16]. The ^{13}C NMR spectral data see Table 1.

APCI–MS of **1b** provided a pseudo-molecular ion $[M+H]^+$ at *m/z* 339.3, suggesting the molecular formula of $C_{24}H_{30}O_5$. When compared to that of **1**, the ^{13}C NMR spectrum of **1b** showed the carbon signal at C-3 at δ 64.5 shifted downfield to δ 211.8, suggesting 3-OH should be changed to 3-ketone group. The carbon signals at 169.2 and 21.1 disappeared, suggesting that the acetyl group at 16-C was hydrolyzed. On the basis of above analysis, compound **1b** was identified as 3-ketodeacetycinobufagin.

APCI–MS of **1d** provided a pseudo-molecular ion $[M+H]^+$ at *m/z* 401.4, suggesting the molecular formula of $C_{24}H_{32}O_5$. When compared to that of **1**, the ^{13}C NMR spectrum of **1d** showed the carbon signal at C-3 at δ 64.5 shifted downfield to δ 69.8, suggesting 3-OH should be α-configuration. The carbon signals at 169.2 and 21.1 disappeared, suggesting that the acetyl group at 16-C was hydrolyzed. On the basis of above analysis, compound **1d** was identified as 3-epideacetycinobufagin.

3.3. Cytotoxicity testing

The cytotoxicities of metabolites were assayed against human hepatoma cells (Bel-7402) as shown in Section 2.8. All of them exhibited weaker cytotoxicity than the substrates. It was also indicated that the substitution with 3α-hydroxy, 3-ketone or 16-deacetyl groups could significantly decreased the *in vitro* cytotoxicity of substrates, showing that the metabolism usually followed a detoxification pathway. But the pharmacological model on heart disease should be tested in the future, in order to evaluate their activities comprehensively.

3.4. Time course of biotransformation of *1*, *2* and *3*

The time course of the biotransformation of cinobufagin (**1**) by *P. aurantigriseum* AS 3.4512 was investigated. The result was illustrated in Fig. 2. The substrate was almost completely metabolized within 5 days after administration. The maximum concentrations of two major products **1b** and **1d** in the culture liquid, appeared on the 5th and 6th days, respectively, and

Table 1
^{13}C NMR spectral data for substrates (**1–3**) and metabolites (**1a–1e**, **2a–2b** and **3a–3c**) (DMSO, 125 MHz)

No.	1	1a	1b	1c	1d	1e	2	2a	2b	3	3a	3b	3c
1	29.3	35.9	36.0	29.3	30.3	30.2	29.5	363.2	30.5	29.4	30.4	36.0	36.4
2	27.4	36.6	36.7	27.4	34.6	34.7	27.5	37.0	36.2	27.5	34.8	37.1	37.0
3	64.5	211.5	211.8	64.5	69.8	69.7	64.6	211.6	69.9	64.5	69.8	211.6	212.1
4	32.9	41.5	41.7	32.9	36.0	35.9	33.1	42.1	35.7	33.0	36.0	41.6	41.9
5	35.5	42.8	43.0	35.5	41.1	41.0	35.6	43.0	41.2	35.5	41.1	43.0	43.4
6	25.5	25.1	25.3	25.5	6.0	25.9	26.5	26.3	27.0	25.6	26.1	25.4	25.7
7	20.0	19.3	19.6	20.1	20.3	20.2	21.1	20.7	21.3	20.2	20.4	20.6	20.5
8	32.6	32.4	32.6	32.9	32.9	32.7	41.2	43.1	41.3	33.1	33.3	33.1	32.7
9	38.0	38.6	38.6	38.9	38.9	38.8	34.8	34.6	34.5	38.5	39.3	38.6	35.8
10	34.9	34.6	34.7	34.9	34.8	34.5	34.9	35.1	34.9	35.0	34.5	34.7	35.0
11	20.5	20.6	20.6	20.5	20.4	20.2	21.1	21.0	20.9	20.6	20.4	20.7	30.1
12	38.7	38.3	38.7	38.8	39.0	38.7	40.0	40.0	40.0	38.2	38.3	38.0	74.1
13	44.6	44.5	44.4	44.3	44.4	44.5	48.0	48.0	48.0	44.6	44.6	44.6	51.0
14	71.9	71.7	71.5	70.5	70.6	71.9	83.4	83.4	83.4	74.0	74.0	73.9	73.5
15	59.3	59.4	62.1	61.9	62.0	59.3	32.0	21.9	32.0	59.3	59.4	59.4	59.7
16	74.5	74.4	71.5	71.6	71.7	74.5	28.4	28.6	28.4	31.6	31.6	31.5	32.2
17	48.9	48.9	50.8	50.8	50.8	49.0	50.1	50.3	50.1	46.1	46.2	46.1	42.7
18	16.9	16.8	17.1	17.0	17.1	16.8	16.7	16.8	16.6	16.5	16.5	16.5	16.1
19	23.5	21.8	22.0	23.5	23.1	22.9	23.7	23.4	23.2	23.7	23.1	22.5	22.5
20	116.0	115.9	117.9	117.7	117.9	115.9	122.7	122.7	122.7	122.0	122.0	121.9	121.8
21	152.1	152.1	151.2	151.0	151.1	152.1	149.1	149.2	149.2	150.5	150.5	150.5	150.3
22	148.4	148.4	150.0	149.9	150.0	148.4	147.4	147.5	147.4	147.4	147.4	147.3	146.8
23	112.8	112.8	112.0	111.9	112.0	112.7	114.1	114.2	114.2	114.1	114.2	114.1	115.2
24	160.7	160.6	161.3	161.1	161.3	160.6	161.3	161.3	161.3	161.0	161.0	161.0	162.1
25	169.2	169.2	–	–	–	169.2	–	–	–	–	–	–	–
26	21.1	21.1	–	–	–	20.0	–	–	–	–	–	–	–

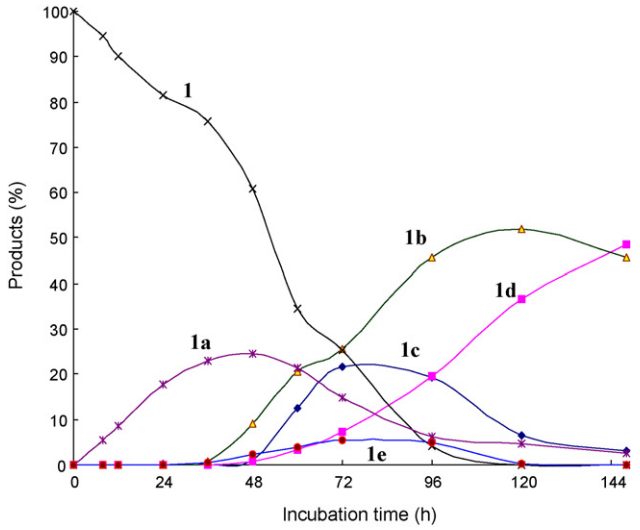

Fig. 2. Time course of biotransformation of cinobufagin (**1**) by *P. aurantigriseum* AS 3.4512 ($n = 3$).

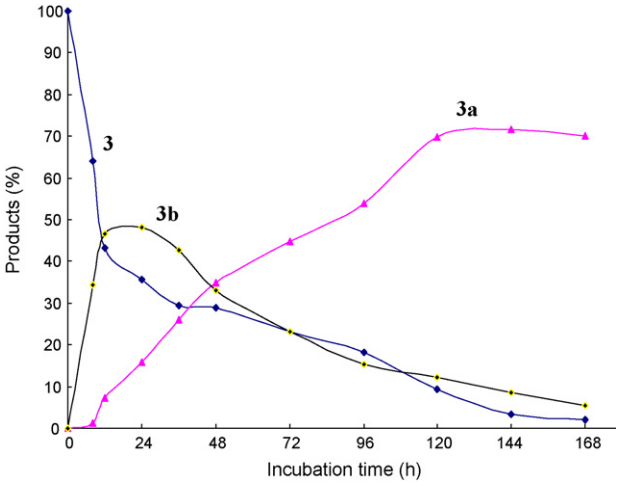

Fig. 3. Time course of biotransformation of resibufogenin (**3**) by *P. aurantigriseum* AS 3.4512 ($n = 3$).

the biotransformation rates were determined to be 52% and 49% by HPLC analysis. The maximum concentrations of compound **1a** reached on the 48 h, and the biotransformation rate was 25%. On the 72 h, the peak rate of biotransformation for compound **1c** was 22%. The minor compound **1e** reached the peak concentration at the 72 h with 5.5%, from which a possible biotransformation pathway was proposed as illustrated in Fig. 1.

The compound **2** was also actively metabolized by *P. aurantigriseum*. After 4 days of incubation, two metabolites (**2a** and **2b**) were isolated from the culture supernatant. They were identified as 3-ketobufalin (**2a**) and 3-epibufalin (**2b**), respectively. The yields for two products **2a** and **2b** were 23% and 28%, respectively. Similarly, *P. aurantigriseum* could also transform **3** into the metabolite **3a** with 72% yield at 6th day (Fig. 3).

3.5. Identification of in vivo metabolites in rat serum and liver

Five metabolites (**1a**–**1e**) of cinobufagin by *P. aurantigriseum* were isolated from the culture liquids. It was very interesting that these metabolites were exactly same with the metabolites

Table 2
HPLC–DAD–APCI–MS characterization of biotransformation products of bufadienolides **1**–**3** by *Penicillium aurantigriseum* AS 3.4512

Substrate	Products	Retention time (min)	UV λ_{max} (nm)	Prominent ions	Identification
1	**1a**	42.3	296	441.4 $[M+H]^+$ (30%), 458.4 $[M+NH_4]^+$ (9%), 482.4 $[M+H+MeCN]^+$ (100%)	3-Ketocinobufagin
	1b	27.8	296	399.4 $[M+H]^+$ (41%), 416.4 $[M+NH_4]^+$ (18%), 422.4 $[M+H+MeCN–H_2O]^+$ (20%), 440.3 $[M+H+MeCN]^+$ (100%)	3-Ketodeacetycinobufagin
	1c	20.2	294	401.2 $[M+H]^+$ (50%), 418.4 $[M+NH_4]^+$ (22%), 442.8 $[M+H+MeCN]^+$ (100%)	Deacetycinobufagin
	1d	26.1	300	401.4 $[M+H]^+$ (41%), 418.4 $[M+NH_4]^+$ (20%), 442.4 $[M+H+MeCN]^+$ (100%)	3-Epideacetycinobufagin
	1e	38.4	300	443.2 $[M+H]^+$ (30%), 460.4 $[M+NH_4]^+$ (12%), 468.5 $[M+H+MeCN–H_2O]^+$ (6%), 484.4 $[M+H+MeCN]^+$ (100%)	3-Epicinobufagin
2	**2a**	31.2	300	385.4 $[M+H]^+$ (35%), 408.5 $[M+H+MeCN–H_2O]^+$ (100%), 426.5 $[M+H+MeCN]^+$ (25%)	3-Ketobufalin
	2b	27.0	296	387.5 $[M+H]^+$ (35%), 404.3 $[M+NH_4]^+$ (8%), 410.5 $[M+H+MeCN–H_2O]^+$ (35%), 428.5 $[M+H+MeCN]^+$ (100%)	3-Epibufalin
3	**3a**	36.2	296	385.4 $[M+H]^+$ (25%), 408.5 $[M+H+MeCN–H_2O]^+$ (10%), 426.5 $[M+H+MeCN]^+$ (100%)	3-Epiresibufogenin
	3b	40.7	296	383.1 $[M+H]^+$ (17%), 406.5 $[M+H+MeCN–H_2O]^+$ (8%), 424.4 $[M+H+MeCN]^+$ (100%)	3-Ketoresibufogenin
	3c	15.3	294	401.4 $[M+H]^+$ (40%), 406.4 $[M+H+MeCN–2H_2O]^+$ (26%), 424.4 $[M+H+MeCN–H_2O]^+$ (18%), 442.5 $[M+H+MeCN]^+$ (100%)	12β-Hydroxyl-3-ketoresibufogenin

of **1** by liver microsomes *in vitro* [17], which suggesting that the enzymes systems of *P. aurantigriseum* were similar with the P-450 enzymes of the liver. However, utilizing *P. aurantigriseum* as a bioreactor to obtain **1a**–**1e** was a facile process with the advantages of low cost and simple operation procedures than liver microsomes culture. In order to investigate similarity between biotransformation of **1** by *P. aurantigriseum* and the *in vivo* metabolism of **1**, we investigated the *in vivo* metabolism after oral administration of **1**, and using compounds (**1a**–**1e**) as the reference standards with LC–MS^n analysis (Table 2). APCI and ESI in both negative and positive models were attempted. The results indicated that APCI in positive model was more sensitive to substrate and metabolites. According to our result, a major *in vivo* metabolite of **1** was detected in the serum and liver of 10, 30 and 50 min after oral administration of **1**. The retention time of *in vivo* metabolite of **1** was same with that of **1d** (Fig. 4). Its on-line UV spectra had UV absorption maxima at 300 nm, due to its characteristic α-pyrone ring at the C-17. The APCI–MS spectra also provided same prominent ion such as $[M+H]^+$ (*m/z* 401.4) and $[M+NH_4]^+$ (*m/z* 418.4) and $[M+H+MeCN]^+$ (*m/z* 442.4) with that of **1d** (Fig. 5A and B), all of which suggesting that the major metabolite of **1** in serum and liver of rat should be 3-epideacetycinobufagin (**1d**).

Our result showed that compound **1** was absorbed rapidly after oral administration, and transformed into **1d** by P-450 enzymes of liver *in vivo*, and then transferring into blood. This suggested that the bioconversion of **1** by microorganism was similar with the metabolism of **1** by liver *in vivo*. In addition, **1d** might be an active or toxic molecule *in vivo*, therefore the pharmacological and toxicological effects of **1d** should be lucubrated widely in the future. And our result provided a simple and efficient way to obtain it with high yield, which was very helpful to study the metabolism of toxic drug (**1**) in human.

Similarly, the *in vivo* metabolites of bufalin (**2**) and resibufogenin (**3**) were also investigated. The *in vivo* metabolite of **2** was detected in the serum and liver of 10, 30 and 50 min after oral administration of bufalin. By comparing the retention time, UV spectra and MS spectrum (Figs. 5C, D and 6) with those of the standard compounds (**2**, **2a** and **2b**), the metabolite of **2** was identified as 3-epibufalin (**2b**).

In addition, one *in vivo* metabolite of **3** was also detected in the serum and liver of 20, 40 and 60 min after oral administration of resibufogenin (**3**). Comparing with the reference standards (**3**, **3a**, **3b** and **3c**), its retention time and maxima UV absorption were same with that of **3a** (Fig. 7). Meanwhile, its MS spectrum was also same with **3a** at *m/z* 385.4 $[M+H]^+$, 408.5 $[M+H+MeCN–H_2O]^+$ and 426.5 $[M+H+MeCN]^+$ (Fig. 5E and F). Hence this major metabolite of **3** *in vivo* was identified as 3-epiresibufogenin (**3a**), and it was the firstly reported metabolite of **3** *in vivo*. All of these indicated that the bioconversion of **2** and **3** by *P. aurantigriseum* were also similar with their *in vivo* metabolism in rat.

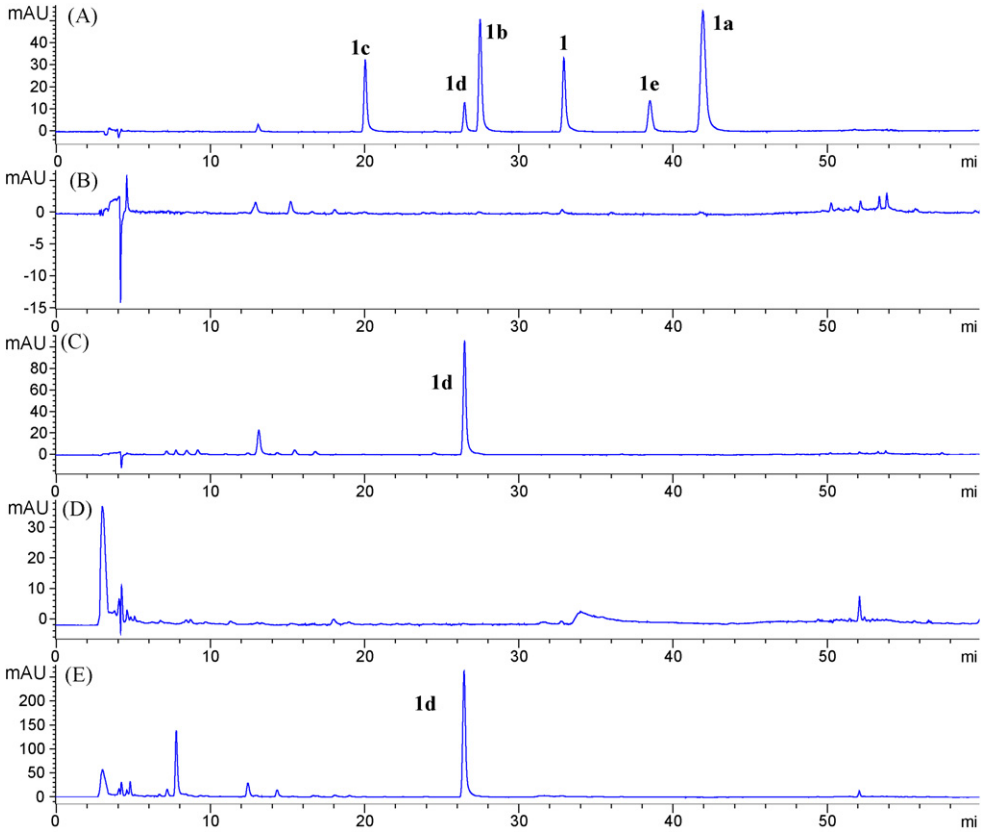

Fig. 4. HPLC chromatograms of standard mixture (A), blank rat plasma sample (B), rat serum sample at 30 min after an oral dose of 30 mg/kg cinobufagin (**1**) (C), blank rat liver sample (D), rat liver sample at 30 min after an oral dose of 30 mg/kg **1** (E).

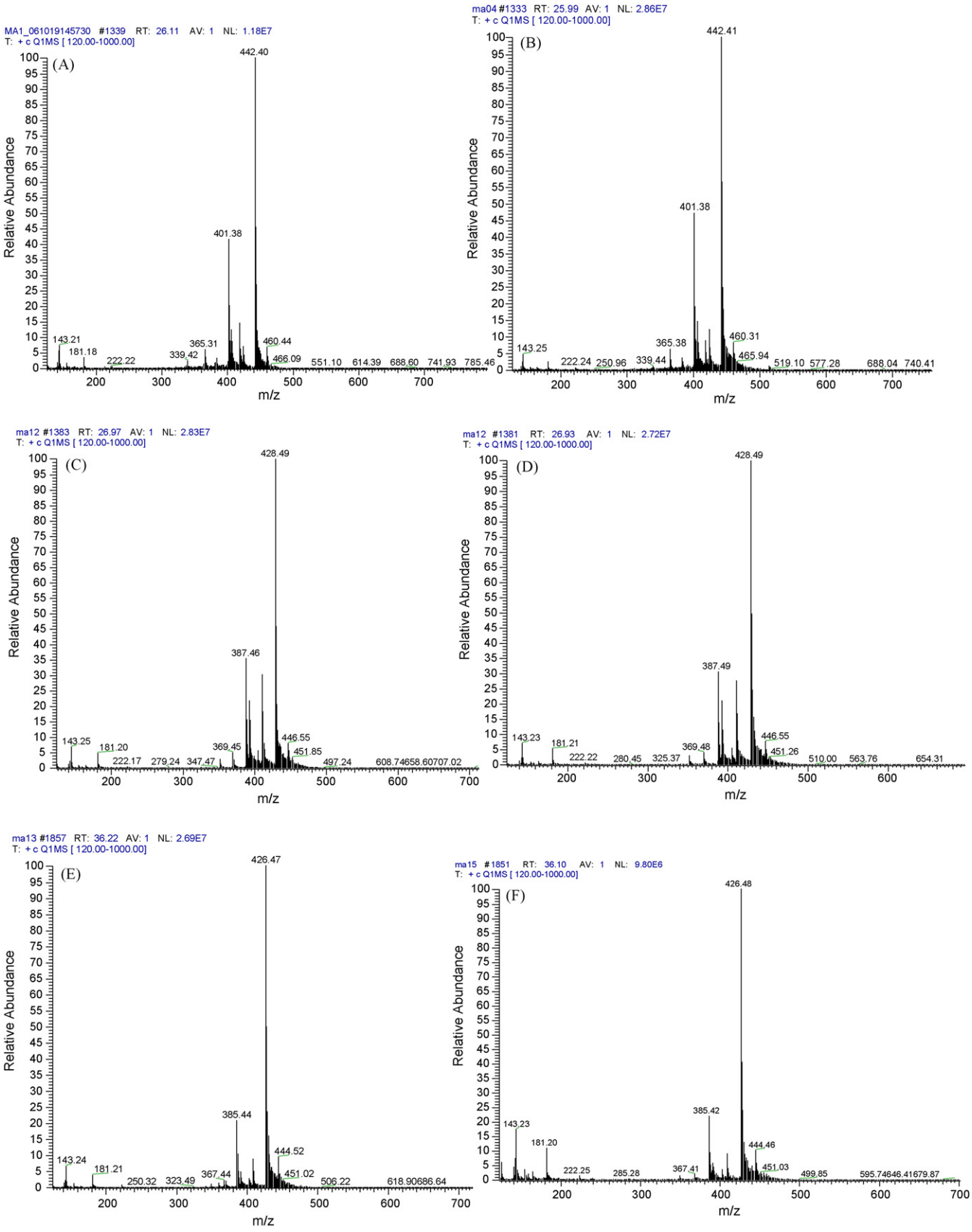

Fig. 5. Mass spectra of **1d** (A), the peak at 26.1 min in HPLC chromatogram of rat serum sample at 30 min after oral administration **1** (B), the standard compound **2b** (C), the peak at 26.9 min in HPLC chromatogram of rat serum sample at 30 min after oral administration **2** (D), the standard compound **3a** (E), the peak at 36.1 min in HPLC chromatogram of rat serum sample at 30 min after oral administration **3** (F).

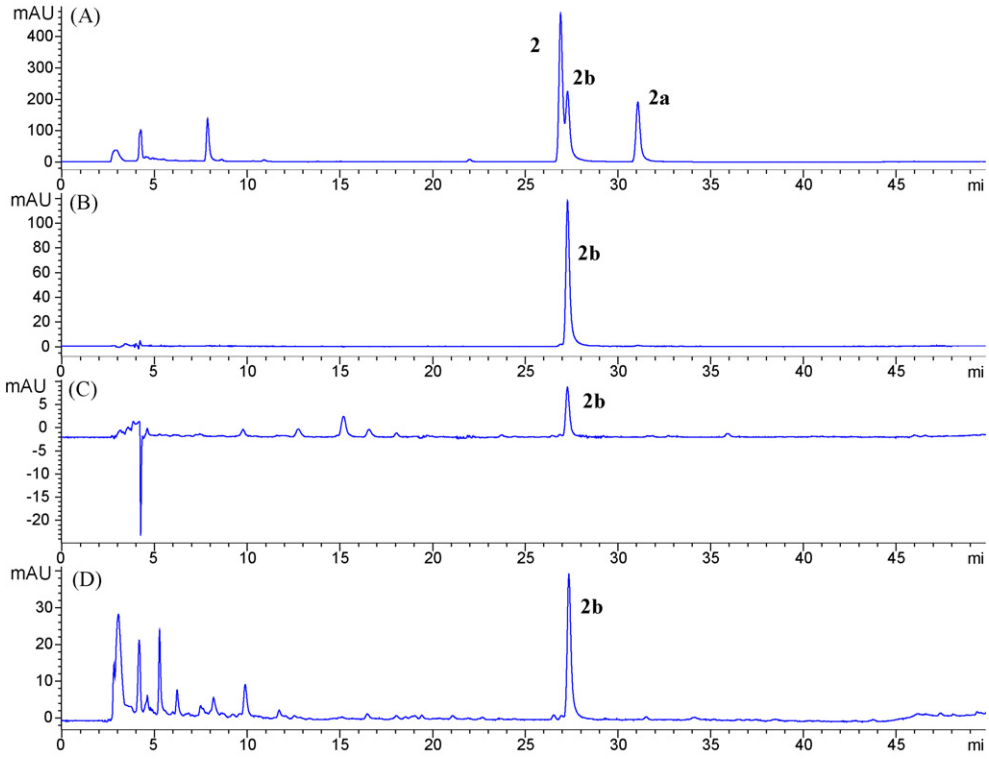

Fig. 6. HPLC chromatograms of sample of administration **2** after 96 h by *P. aurantigriseum* AS 3.4512 (A), standard compound **2b** (B), rat serum sample at 40 min after an oral dose of 20 mg/kg bufalin (**2**) (C), rat liver sample at 40 min after an oral dose of 20 mg/kg **2** (D).

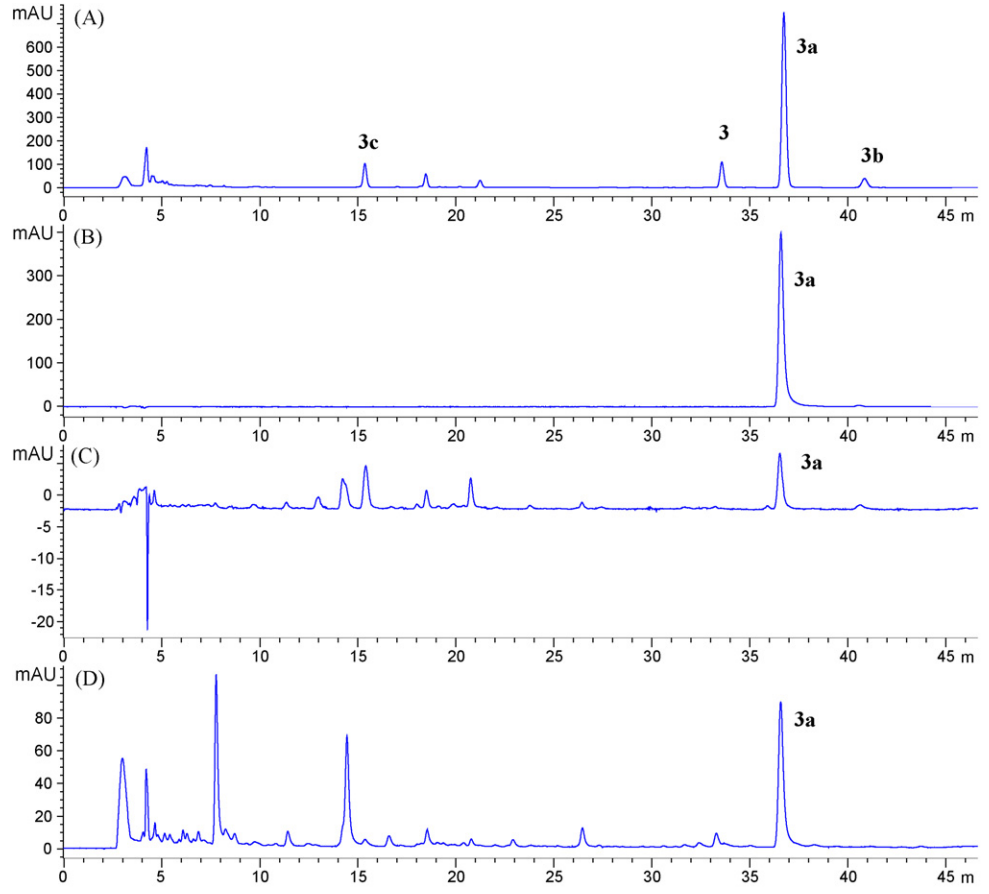

Fig. 7. HPLC chromatograms of sample of administration **3** after 96 h by *P. aurantigriseum* AS 3.4512 (A), standard compound **3a** (B), rat serum sample at 40 min after an oral dose of 30 mg/kg resibufogenin (**3**) (C), rat liver sample at 40 min after an oral dose of 30 mg/kg **3** (D).

4. Conclusions

In this paper, *P. aurantigriseum* was found to be the most potent strain to transform **1**, **2** and **3**. Ten transformed products were obtained and their structures were identified. Among them, six compounds were same with the metabolites of **1** and **2** by liver microsomes *in vitro*. Three compounds were identified as the major metabolites of **1**, **2** and **3** *in vivo*, and 3-epiresibufogenin (**3a**) was the firstly reported metabolite of **3** *in vivo*. Our result strongly suggested that the *P. aurantigriseum* AS 3.4512 and rats had similar metabolism routes to the bufadienolides, that was, isomerization of hydroxyl group at C-3 was major metabolic reaction. At the same time, we could utilize *P. aurantigriseum* AS 3.4512 as a bioreactor to obtain the *in vivo* metabolites of **1**, **2** and **3** with the advantages of low cost, simple operation procedure and obtaining sufficient amounts of metabolites, and then use them as the reference standards to investigate the metabolism of bufadienolides. To some extent, this work also suggested that microbial transformation was an additional means for the investigation of mammalian drug metabolism, especially for toxic drugs.

Acknowledgements

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