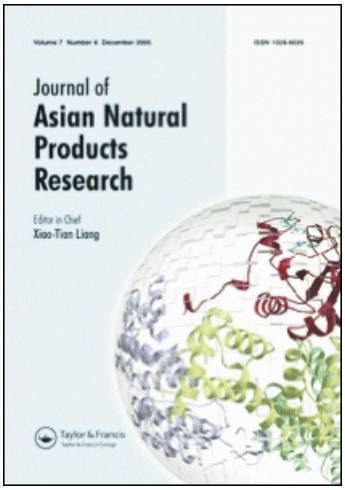

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

Microbial biotransformation of cryptotanshinone by *Cunninghamella elegans* and its application for metabolite identification in rat bile

Jiang-Hao Sun[a]; Min Yang[b]; Xiao-Chi Ma[a]; Jie Kang[a]; Jian Han[a]; De-An Guo[ab]
[a] The State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing, China [b] Shanghai Institute of Materia Medica, Shanghai Research Center for Modernization of Traditional Chinese Medicine, Chinese Academy of Sciences, Shanghai, China

To cite this Article Sun, Jiang-Hao , Yang, Min , Ma, Xiao-Chi , Kang, Jie , Han, Jian and Guo, De-An(2009) 'Microbial biotransformation of cryptotanshinone by *Cunninghamella elegans* and its application for metabolite identification in rat bile', Journal of Asian Natural Products Research, 11: 6, 482 — 489
To link to this Article: DOI: 10.1080/10286020902877754
URL: http://dx.doi.org/10.1080/10286020902877754

Journal of Asian Natural Products Research
Vol. 11, No. 6, June 2009, 482–489

Taylor & Francis
Taylor & Francis Group

Microbial biotransformation of cryptotanshinone by *Cunninghamella elegans* and its application for metabolite identification in rat bile

Jiang-Hao Sun[a], Min Yang[b], Xiao-Chi Ma[a], Jie Kang[a], Jian Han[a] and De-An Guo[ab]*

[a]*The State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing 100083, China;* [b]*Shanghai Institute of Materia Medica, Shanghai Research Center for Modernization of Traditional Chinese Medicine, Chinese Academy of Sciences, Shanghai 201203, China*

(*Received 8 December 2008; final version received 4 January 2009*)

Cryptotanshinone (**1**) is one of the major bioactive constituents in *Salvia miltiorrhiza* Bunge. Preparative-scale biotransformation of cryptotanshinone by *Cunninghamella elegans* (AS 3.2082) produced three new products, which were identified as (3*R*,15*R*)-3-hydroxycryptotanshinone (**2**), (3*S*,15*R*)-3-hydroxycryptotanshinone (**3**), and (4*S*,15*R*)-18-hydroxycryptotanshinone (**4**), respectively. The structural elucidation was based primarily on 1D and 2D NMR and HR-ESI-MS analyses. The absolute configuration of these three products was confirmed by comparison of their circular dichroism spectra with those of the known compounds. These biotransformed metabolites were used as for the comparison of *in vivo* metabolites in rat bile sample after intravenous administration and they are identical to three of the minor hydroxylated metabolites *in vivo*, which suggested that microbial biotransformation model was a useful and feasible approach for the preparation of mammalian metabolites in trace.

Keywords: biotransformation; cryptotanshinone; *Cunninghamella elegans*; diterpenes; *Salvia miltiorrhiza*

1. Introduction

Tanshinones are abietane diterpenes with a common *ortho*- or *para*-naphthoquinone chromophore, which is one class of the major bioactive constituents in Dan-Shen (*Salvia miltiorrhiza* Bunge), a well-known traditional Chinese medicinal herb [1]. Modern pharmacological studies have demonstrated a variety of activities of tanshinones such as neuroprotective effects [2], antioxidant effect on DNA damage by lipid peroxidation [3], *in vitro* cytotoxicity [4], and inducing the apoptosis of cancer cells [5,6]. Among all the tanshinones, cryptotanshinone (**1**) is used as one of the marker compounds to control the quality of crude materials and traditional Chinese preparations containing Dan-Shen for its diverse pharmacological activities [7–9].

In our previous metabolic studies of **1**, we found several minor oxidative metabolites of **1** in rat bile under either oral or intravenous administration using liquid chromatography tandem multi-stage mass spectrometry (LC–MS^n; [10,11]). The chemical structures of these minor metabolites were not confirmed due to the unavailability of the reference compounds. As the concentration of the oxidative metabolites is extremely low in biofluids, it is difficult to

*Corresponding author. Email: gda5958@163.com; gda@bjmu.edu.cn

ISSN 1028-6020 print/ISSN 1477-2213 online

DOI: 10.1080/10286020902877754
http://www.informaworld.com

obtain them by accumulation and separation of the biosamples using chromatographic approaches, which is obviously a tedious and time-consuming course.

Microbial biotransformation technique is by far one of the most feasible approaches for the achievement of the mammal *in vivo* metabolites. It was firstly reported as *in vitro* model for mammalian metabolism of xenobiotics in the early 1970s [12]. In recent years, our laboratory has carried out a series of comparative studies between the microbial transformation and the mammalian metabolism on L-tetrahydropalmatine, evodiamine, bufadienolide, ginsenoside Rb, etc. [13–16], which could be used as models for partly predicting the mammalian metabolism. Though the use of microbial biotransformation cannot predict the real drug metabolism procedure in mammals totally, it is a feasible alternative method for the preparation of the reference compounds of drug metabolites. By using this approach, the use of animals in research can be reduced and this procedure appears to be more widely accepted for such purposes.

In this study, the three microbial biotransformation metabolites of **1** by *Cunninghamella elegans* were characterized as (3*R*,15*R*)-3-hydroxycryptotanshinone (**2**), (3*S*,15*R*)-3-hydroxycryptotanshinone (**3**), and (4*S*,15*R*)-18-hydroxycryptotanshinone (**4**), which were all new compounds. These three biotransformed products were also used as chromatographic standards for the identification of the metabolites in rats and they are consistent with three of the minor hydroxylated metabolites in rat bile after intravenous administration. Furthermore, the similarities and differences of microbial and mammalian metabolisms of **1** were also compared. The results demonstrated that *C. elegans* could be used as an *in vitro* model for the achievement of the *in vivo* minor metabolites of cryptotanshinone.

2. Results and discussion

2.1 Structure identification of biotransformation products

The structures of transformed products were elucidated on the basis of their ^{1}H, ^{13}C NMR, HMQC, and HMBC spectral data. Incubation of cryptotanshinone **(1)** with *C. elegans* for 5 days yielded three products (Figure 1). Their structures were confirmed as (3*R*,15*R*)-3-hydroxycryptotanshinone **(2)**, (3*S*,15*R*)-3-hydroxycryptotanshinone

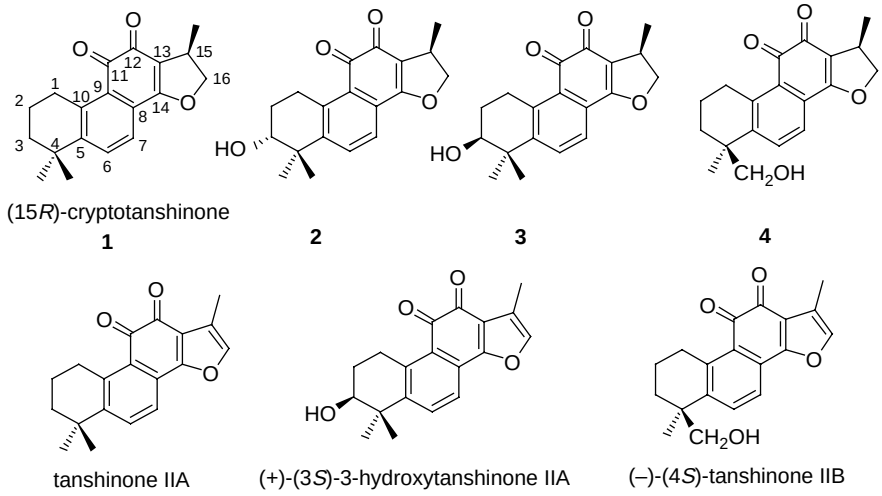

Figure 1. The chemical structures of tanshinones.

(3), and (4*S*,15*R*)-18-hydroxycryptotanshinone **(4)**. Products **2** and **3** were a pair of stereo-isomers with identical ^{1}H and ^{13}C NMR spectral data. The hydroxylated products at C-3 and C-4 side chains were firstly reported as new compounds and they are consistent with three of the *in vivo* metabolites in rat bile sample after administration of cryptotanshinone.

The HR-FAB-MS of **2** gave the $[M+H]^+$ ion peak at *m/z* 313.1412, together with the ^{1}H and ^{13}C NMR spectral data, suggesting the molecular formula of $C_{19}H_{21}O_4$. The ^{1}H NMR spectrum of **2** further showed three methyl signals at δ 1.37 (3H, d, $J = 8.0$ Hz), 1.34 (3H, s), and 1.35 (3H, s), almost identical to those corresponding signals in compound **1**. However, the CH_2 signals of **1** at δ 1.65 were replaced by the oxygenated methane of **2** at δ 3.78, indicating the hydroxylation of C-3 in **2**, which was supported by the carbon signal at δ 74.2. Moreover, H-1, H-18, and H-19 had HMBC correlations with C-3, respectively, which also confirmed the above results.

The HR-FAB-MS of **3** gave the $[M+H]^+$ ion peak at *m/z* 313.1417, indicating the molecular formula of $C_{19}H_{21}O_4$. The ^{1}H and ^{13}C NMR spectral data were almost identical to those of **2**. The introduction of hydroxyl group will produce one more chiral carbon in the structure. Hence, **2** and **3** were a pair of stereo-isomers. The absolute configurations of **2**–**4** will be discussed in Section 2.2.

The HR-FAB-MS of **4** gave the $[M+H]^+$ ion peak at *m/z* 313.1453, indicating the molecular formula of $C_{19}H_{21}O_4$. In comparison with that of the substrate, the C-18 methyl signals were replaced by an oxygen-bearing methylene. Judging from the ^{13}C NMR and HSQC spectra of **4**, an additional oxygen-bearing methylene at δ 74.2 in **4** was observed, and one methyl carbon at δ 31.9 in **1** disappeared, suggesting **4** to be a hydroxylate of the substrate. In accordance, the two oxygen-bearing methylene proton signals (δ 3.81, 1H, d and δ 3.66, 1H, d) had long-range correlations with the signals of C-5, C-3, and C-19 in the HMBC spectrum, which suggested that a hydroxyl group substituted at the position of C-18 (Figure 2). Therefore, **4** was identified as 18-hydroxycryptotanshinone.

The ^{1}H and ^{13}C NMR data of **2**–**4**, and the substrate were shown in Tables 1 and 2. The signals were assigned according to ^{1}H, ^{13}C NMR, HSQC, and HMBC spectra. The chemical structures of **2**–**4** are shown in Figure 1.

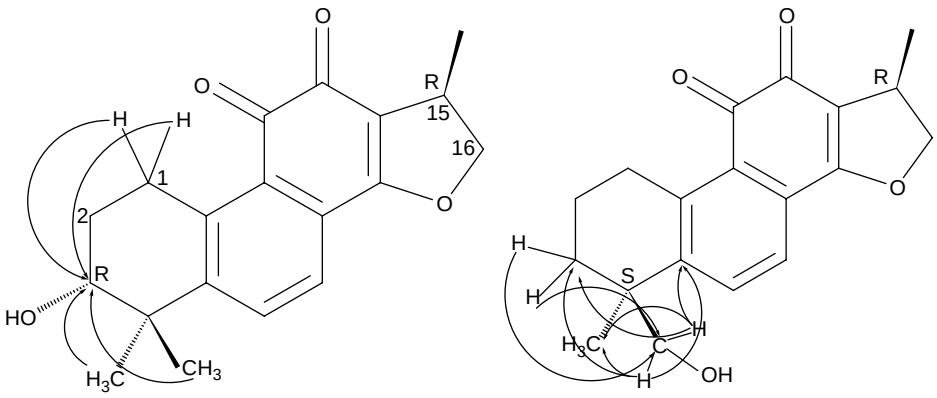

Figure 2. The selected HMBC correlation of **2** and **4**.

Table 1. ^{1}H NMR spectral data of compounds **2**–**4** and cryptotanshinone (**1**) (500 MHz, $CDCl_3$, δ: ppm, J: Hz).

H	**2**	**3**	**4**	Cryptotanshinone (**1**)
1	3.30 (1H, m), 3.47 (1H, m)	3.30 (1H, m), 3.45 (1H, m)	3.20 (2H, m)	3.19 (2H, dd, $J = 12.8, 12.8$)
2	1.95 (1H, m), 2.07 (1H, m)	1.95 (1H, m), 2.06 (1H, m)	1.97 (1H, m), 1.78 (1H, m)	1.79 (2H, m)
3	3.78 (1H, m)	3.78 (1H, m)	1.87 (1H, m), 1.58 (1H, m)	1.65 (2H, m)
6	7.67 (1H, d, $J = 8.0$)	7.67 (1H, d, $J = 8.0$)	7.67 (1H, d, $J = 8.0$)	7.63 (1H, d, $J = 8.1$)
7	7.54 (1H, d, $J = 8.0$)	7.54 (1H, d, $J = 8.0$)	7.51 (1H, d, $J = 8.0$)	7.47 (1H, d, $J = 8.1$)
15	3.59 (1H, m)	3.59 (1H, m)	3.59 (1H, m)	3.59 (1H, m)
16	4.90 (1H, t, $J = 9.5$), 4.37 (1H, dd, $J = 9.0, 6.0$)	4.90 (1H, t, $J = 9.5$), 4.37 (1H, dd, $J = 9.0, 6.0$)	4.89 (1H, t, $J = 9.0$) 4.37 (1H, dd, $J = 9.0, 6.0$)	4.90 (1H, dd, $J = 9.3, 9.5$), 4.37 (1H, dd, $J = 9.3, 6.1$)
17	1.37 (3H, d, $J = 8$)	1.37 (3H, d, $J = 8.0$)	1.35 (3H, d, $J = 7.0$)	1.37 (3H, d, $J = 7.1$)
18	1.34 (3H, s)	1.34 (3H, s)	3.81 (1H, d, $J = 11.0$), 3.66 (1H, d, $J = 11.0$)	1.31 (3H, s)
19	1.35 (3H, s)	1.35 (3H, s)	1.29 (3H, s)	1.31 (3H, s)

Table 2. ^{13}C NMR spectral data of compounds **2**–**4** and cryptotanshinone (**1**) (125 MHz, $CDCl_3$, δ: ppm, J: Hz).

C	**2**	**3**	**4**	Cryptotanshinone (**1**)
1	26.2	25.9	29.5	29.7
2	26.5	26.3	18.8	19.0
3	74.2	74.0	32.2	37.8
4	40.2	40.0	40.1	34.8
5	151.2	151.0	148.2	152.3
6	133.1	132.8	132.9	132.5
7	123.2	123.0	122.4	122.5
8	126.9	126.7	126.7	126.2
9	128.2	127.9	128.4	128.4
10	142.3	142.0	145.3	143.7
11	183.9	184.1	183.9	184.6
12	175.9	175.5	175.4	175.7
13	118.8	118.5	118.5	118.3
14	170.8	170.5	170.5	170.7
15	34.9	34.6	34.6	34.6
16	81.7	81.5	81.5	81.4
17	19.1	18.8	18.7	18.8
18	25.5	25.2	71.5	31.9
19	29.5	29.3	26.7	31.9

2.2 Confirmation of the absolute configuration of compounds 2–4

The absolute configuration of compounds **2**–**4** was determined by circular dichroism (CD) spectra. Compounds **2** and **3** showed totally different CD behaviors under the same conditions. Compound **2** showed a negative Cotton effect at 365 nm, while compound **3** showed a positive Cotton effect. In comparison with that of substrate 15*R*-cryptotanshinone, which owns one chiral carbon (C-15) in the structure,

we found that the Cotton effect at 365 nm was produced by the additional chiral carbon at C-3. By comparing the CD spectra of tanshinone IIA (no chiral carbon in structure) and a known compound (+)-(3*S*)-3-hydroxytanshinone IIA (one chiral carbon in structure), we found that the S-configuration at C-3 position is responsible for the positive Cotton effect at 365 nm, which was consistent with that of **3**. Hence, **3** was identified as (3*S*,15*R*)-3-hydroxycryptotanshinone and **2** was identified as (3*R*,15*R*)-3-hydroxycryptotanshinone, accordingly. The substitution of hydroxyl group at C-18 position of **4** produced one more chiral carbon at C-4. Similarly, the absolute configuration of **4** was confirmed by comparing the CD spectra of the known compound (−)-(4*S*)-tanshinone IIB and it was characterized as (4*S*,15*R*)-18-hydroxycryptotanshinone.

2.3 *Comparison of biotransformed products and the* in vivo *metabolites in rat bile sample*

The *in vivo* metabolites in rat bile were confirmed by the retention behavior and the MS/MS data with the reference compounds obtained by microbial biotransformation. The 24-h bile and microbial biotransformation samples were analyzed by the same HPLC–MS/MS method. The retention behaviors of **2**–**4** and three of the hydroxylated metabolites in rat bile sample exhibited identical under the same HPLC eluting system. The quasi-molecular ion at *m/z* 313 ($[M+H]^+$) was detected in the MS analysis of these two samples. The main product ions at *m/z* 295 ($[M+H-H_2O]^+$), *m/z* 277 ($[M+H-2H_2O]^+$), *m/z* 267 ($[M+H-H_2O-CO]^+$), *m/z* 249 ($[M+H-2H_2O-CO]^+$), etc., with the almost identical abundance were observed in the multistage mass spectra. Therefore, these three rat metabolites were characterized as (3*R*,15*R*)-3-hydroxycryptotanshinone, (3*S*,15*R*)-3-hydroxycryptotanshinone, and (4*S*,15*R*)-18-hydroxycryptotanshinone, respectively.

The microorganism and rats exhibited different metabolic routes for cryptotanshinone. Hydroxylated metabolites are the major biotransformed products in microorganism, while the dehydrogenated metabolite, tanshinone IIA, is dominated in rat. Tanshinone IIA was also observed as the main metabolite in rat plasma and bile sample in this study and in the other literatures [10,11,17–20]. The hydroxylated metabolites produced by oxidation *in vivo* were occupied no more than 1% of the total metabolites judging by the peak area ratio. However, it could be seen that for the full identification of minor metabolites *in vivo*, microbial biotransformation model is undoubtedly a good approach for the achievement of the reference standards.

3. Experimental

3.1 *General experimental procedures*

UV spectra were measured on a YV-1091 UV–VIS spectrophotometer. IR spectra were recorded on a Thermo Nicolet 5700 spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a Varian INOVA-500 spectrometer (Varian, Palo Alto, CA, USA) in $CDCl_3$ at ambient temperature with TMS as the internal standard. Standard pulse sequences were used for HMQC and HMBC experiments. HR-MS were performed on Autospec Ultima-TOF spectrometer, which equipped with EI/FAB ion source (Micromass, Altrincham, UK). CD spectra were recorded on a J-500C CD chiroptical spectrometer (Jasco, Tokyo, Japan).

HPLC–MS were performed with an Agilent 1100 Series HPLC systems and a Finnigan LCQ Advantage Mass Spectrometer System (San Jose, CA, USA). The HPLC conditions were the same as above. ESI-MS^n experiments were conducted using an LCQ Advantage ion trap mass spectrometer under positive ionization with the conditions as follows: sheath gas (N_2) flow rate, 30 U; capillary temperature, 340°C; spray voltage, 4.5 kV; capillary

voltage, 30 V; tube lens offset, 15 V; scan range, *m/z* 100–500. The $[M+H]^+$ ion was selected as precursor ion and fragmented up to MS^3 stage. The isolation width was 1.0 Th.

3.2 Chemicals and materials

HPLC-grade acetonitrile and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was prepared using a Milli-Q purification system (Millipore, Bedford, MA, USA). Ethyl acetate and all the other chemicals and solvents were all of analytical grade. Substrate cryptotanshinone together with tanshinone IIA, (+)-(3*S*)-3-hydroxytanshinone IIA, and (−)-(4*S*)-tanshinone IIB were isolated by the authors from the standardized extract of total tanshinones (Masson Pharma, Guangzhou, China). The structure was fully characterized by NMR spectroscopy and MS. The purity was over 95% determined by HPLC-DAD analysis.

C. elegans AS 3.2082, *C. elegans* AS 3.1207, *C. blakesleana* AS 3.970, *Aspergillus niger* AS 3.795, *A. niger* AS 3.1858, *A. niger* AS 3.739, *Mucor alternata* AS 3.2450, *M. subtilissimus* AS 3.2454, *M. subtilissimus* AS 3.2456, and *Fusarium avenaceum* AS 3.4594 were purchased from China General Microbiological Culture Collection Center in Beijing, China.

3.3 Preliminary screening experiments

Ten strains of fungi were screened for the biotransformation of cryptotanshinone. The fungal mycelia from the agar slant were transferred to liquid medium. Cultures were incubated in 250-ml flasks containing 60 ml of medium at 25°C with rotary shaking at 150 rpm in the dark. The substrate was dissolved in acetone and diluted to the concentration of 10 mg/ml. After the culture had been incubated for 48 h, 0.2 ml of substrate was added to each flask. The incubation was allowed to continue for 5 days. The culture was filtered and extracted with an equal volume of ethyl acetate for three times. The organic layer was affiliated and then evaporated to dryness by rotary evaporation. The residue was dissolved in 1 ml of methanol for analysis. Culture controls consisted of fermentation blanks in which microorganisms were incubated under identical conditions without substrate. Substrate controls were composed of sterile medium to which substrate was added and were incubated without the microorganisms.

3.4 Preparative-scale experiments

C. elegans AS 3.2082 from agar slant was transferred to two 250-ml flask containing 60 ml of liquid potato medium. The fungus was incubated for 24 h to make a stock inoculum. Then 5 ml of stock inoculum was added to the 1-l flask containing 400 ml liquid potato medium and then incubated for 24 h. Ten micrograms per milliliter of substrate cryptotanshinone were added to the medium and then incubated for 5 days in 25°C with rotary shaking at 150 rpm. The incubation conditions and procedures were identical to those for the preliminary screening experiments described above.

3.5 Separation, purification, and identification of biotransformation products

Cryptotanshinone could be transformed by AS 3.1207 and AS 3.2082 according to the HPLC analyses and the substrate was stable in the substrate controls. *C. elegans* AS 3.2082 was the most potent strain for transforming cryptotanshinone; hence it was selected for the preparative-scale biotransformation. The EtOAc extract was purified by silica gel column and preparative HPLC to obtain

compounds **2** (2.6 mg), **3** (4.2 mg), and **4** (2.2 mg).

3.6 Determination of CD spectra

(−)-(15*R*)-Cryptotanshinone, tanshinone IIA, (+)-(3*S*)-3-hydroxytanshinone IIA, (−)-(4*S*)-tanshinone IIB, and compounds **2**–**4** were prepared at the concentration of 0.02 mg/ml with methanol. The spectra were monitored from 210 to 400 nm.

3.7 Animals, drug administration, and bile sampling

Two male Sprague–Dawley rats weighing 250 ± 20 g were obtained from the Laboratory Animal Center of Peking University Health Science Center (Beijing, China). The animals were kept in fully acclimatized room at constant temperature and humidity on a 12 h light/dark cycle. The common bile duct was cannulated with PE-10 tubing (i.d. = 0.08 cm; Becton Dickinson, Franklin Lakes, NJ, USA) for collection of the bile samples as described before [10,11]. The 4 mg/kg of cryptotanshinone in propylene glycol was administrated to the animals by intravenous administration when the animals recovered conscious. Bile samples were collected for 24 h and stored at − 80°C until additional extraction and analysis. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 85-23, revised).

3.8 Bile samples preparation

The bile samples were extracted with EtOAc at the ratio of 1:3 (v/v) for three times. Extraction was performed by vortex mixing for 5 min, followed by centrifugation for 10 min at 4024 *g*. The organic layer was transferred and evaporated to dryness at 30°C by rotary evaporation. The residue was reconstituted in 1 ml methanol. After filtration by 0.22 μm filter, 5 μl of the aliquot was injected for HPLC–MS^n analysis.

3.9 Separation and identification of biotransformation products

3.9.1 (3R,15R)-3-Hydroxycryptotanshinone (2)

Red powder; $C_{19}H_{21}O_4$; UV λ_{max} MeOH (nm): 263.0; CD MeOH (nm) $[\theta]_{290.0}$ -9.05×10^3, $[\theta]_{365.0}$ -9.94×10^3; IR ν (cm^{-1}): 3400, 2922, 1715, 1458; ^1H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$) spectral data were shown in Tables 1 and 2, respectively. HR-FAB-MS: *m/z* 313.1412 $[M+H]^+$ (calcd for $C_{19}H_{21}O_4$, 313.1440).

3.9.2 (3S,15R)-3-Hydroxycryptotanshinone (3)

Red powder; UV λ_{max} MeOH (nm): 263.0; CD MeOH (nm) $[\theta]_{290.0}$ -3.08×10^3, $[\theta]_{365.0}$ $+14.90 \times 10^3$; IR ν (cm^{-1}): 3400, 2924, 1720, 1460; ^1H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$) spectral data were shown in Tables 1 and 2. HR-FAB-MS: *m/z* 313.1417 $[M+H]^+$ (calcd for $C_{19}H_{21}O_4$, 313.1440).

3.9.3 18-Hydroxycryptotanshinone (4)

Red powder; $C_{19}H_{21}O_4$; UV λ_{max} MeOH (nm): 263.0; CD MeOH (nm) $[\theta]_{290.0}$ -3.29×10^3, $[\theta]_{365.0}$ $+3.17 \times 10^3$; IR ν (cm^{-1}): 3367, 2930, 1721, 1462; ^1H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$) spectral data were shown in Tables 1 and 2. HR-FAB-MS: *m/z* 313.1453 $[M+H]^+$ (calcd for $C_{19}H_{21}O_4$, 313.1440).

Acknowledgements

This work was supported by Program for Changjiang Scholars and Innovative Team in University (985-2-063-112).

References

[1] X. Wang, S.L. Morris-Natschke, and K.H. Lee, *Med. Res. Rev.* **27**, 133 (2007).
[2] B.Y. Lam, A.C. Lo, X. Sun, H.W. Luo, S.K. Chung, and N.J. Sucher, *Phytomedicine* **10**, 286 (2003).

[3] E.H. Cao, X.Q. Liu, J.J. Wang, and N.F. Xu, *Free Rad. Biol. Med.* **20**, 801 (1996).
[4] S.Y. Ryu, C.O. Lee, and S.U. Choi, *Planta Med.* **63**, 339 (1997).
[5] W.L. Wu, W.L. Chang, and C.F. Chen, *Am. J. Chin. Med.* **19**, 207 (1991).
[6] M.A. Mosaddik, *Phytomedicine* **10**, 682 (2003).
[7] A.H. Liu, Y.H. Lin, M. Yang, H. Guo, S.H. Guan, J.H. Sun, and D.A. Guo, *J. Chromatogr. B* **846**, 32 (2007).
[8] A.H. Liu, Y.H. Lin, M. Yang, J.H. Sun, H. Guo, and D.A. Guo, *J. Pharm. Pharm. Sci.* **9**, 1 (2006).
[9] Y.J. Yan, G.D. Yang, and L.C. He, *Chin. Tradit. Herb. Drugs* **33**, 363 (2002).
[10] J.H. Sun, M. Yang, X.M. Wang, M. Xu, A.H. Liu, and D.A. Guo, *J. Pharm. Biomed. Anal.* **44**, 564 (2007).
[11] J.H. Sun, M. Yang, J. Han, B.R. Wang, X.C. Ma, M. Xu, P. Liu, and D.A. Guo, *Rapid Commun. Mass Spectrom.* **21**, 2211 (2007).
[12] R.V. Smith and J.P. Rosazza, *J. Pharm. Sci.* **11**, 173 (1975).
[13] L. Li, R.X. Liu, M. Ye, X.Y. Hua, Q. Wang, K.S. Bi, and D.A. Guo, *Enzyme Microb. Technol.* **39**, 561 (2006).
[14] L. Li, M. Ye, K.S. Bi, and D.A. Guo, *Biomed. Chromatogr.* **20**, 95 (2006).
[15] X.C. Ma, J. Cui, J. Zheng, and D.A. Guo, *J. Mol. Catal. B: Enzym.* **48**, 4842 (2007).
[16] G.T. Chen, M. Yang, Y. Song, Z.Q. Lu, J.Q. Zhang, H.L. Huang, S.H. Guan, L.J. Wu, and D.A. Guo, *Biomed. Chromatogr.* **22**, 779 (2008).
[17] H.P. Hao, G.J. Wang, P. Li, J. Li, and Z.Q. Ding, *J. Pharm. Biomed. Anal.* **13**, 382 (2006).
[18] J. Li, G.J. Wang, P. Li, and H.P. Hao, *J. Chromatogr. B* **826**, 26 (2005).
[19] M. Song, T.J. Hang, Z.X. Zhang, R. Du, and J. Chen, *J. Chromatogr. B* **827**, 205 (2005).
[20] M. Xue, Y. Cui, H.Q. Wang, Z.H. Hu, and B. Zhang, *J. Pharm. Biomed. Anal.* **21**, 207 (1999).