Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 17 (2007) 3195–3197
Bioorganic & Medicinal Chemistry Letters

Biotransformation of gentiopicroside by asexual mycelia of *Cordyceps sinensis*

Dong Wang, Min Xu, Hong-Tao Zhu, Ke-Ke Chen, Ying-Jun Zhang* and Chong-Ren Yang*

State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, China

Received 17 November 2006; revised 5 March 2007; accepted 8 March 2007
Available online 12 March 2007

Abstract—The biotransformation of gentiopicroside by asexual mycelia of *Cordyceps sinensis* yielded two products, one of which was proved to be a new pyridine monoterpene alkaloid. The possible mechanisms were discussed.

The fungi of genus *Cordyceps* belong to the family Clavicipitaceae of the order Hypocreales. It is generally regarded that there are more than 300 species in this genus.[1] *Cordyceps sinensis* (Berk.) Sacc. (CS) is a fungal parasite on the larvae of *Lepidoptera*. In late autumn, the fungus infects the caterpillar and devours its host. In early summer of the following year, the grass-like fruiting body protrudes from the ''head'' of the dead host. Because of this particular life cycle, it is called ''winter-worm, summer-grass'' or ''worm grass'' in China. CS is a valuable medicinal fungus of traditional Chinese medicine and has long been used as a general tonic and an aphrodisiac medicine.[2–4] Recent studies revealed a wide range of its biological functions.[5] Nevertheless, because it is a very rare herb growing slowly only in high altitude areas, the supply of CS is inadequate for the demand. Moreover, as a result of the immoderate exploitation, the resource of CS is in severe danger. Though the key mechanisms of fungal infection are not clear, and the cultivation of whole parasitic complex is still far from success, the mycelia of this fungus have been cultured and are used as substitute for CS in China.

Gentiopicroside (**1**), a secoiridoid glycoside, commonly exists in the gentianaceous plants, especially in the genera *Gentiana* and *Swertia*, of which many species are used as folk drugs for the treatments of indigestion, hepatitis, rheumatism, wounds, and sores. As one of the main principles of these medicinal plants, **1** was proved to be active in antibacterial, liver protecting, and free radical scavenging tests.[6,7]

Gentiopicroside (**1**) is chemically unstable, it can be transformed into gentianine (**2**) and gentianal (**3**) with the presence of NH_4^+.[8] Van der Sluis and Ishiguro reported the enzymatic hydrolysis of **1** with β-glucosidase and the product gentiopicral (**4**) was obtained.[9–11] A.I. El-Sedway et al. studied the metabolism of **1** by human intestinal bacteria and five metabolites (**5**–**9**) were isolated.[12]

In this paper, we report the study on the biotransformation of gentiopicroside (**1**) by asexual mycelia of CS.

Asexual mycelia of CS were kindly donated by Assoc. Prof. Dai-Fang Li, Kunming Institute of Botany, Chinese Academy of Sciences. The mycelia (1 cm^2) were inoculated into a 100 mL flask containing 30 mL PDA medium[13] and agitated at 80 rpm with a rotary shaker in darkness at 28 °C. After 10 days' incubation, gentiopicroside (**1**)[14] (30 mg) (dissolved in 1 mL H_2O, filter-sterilized with 0.22 μm filter) was added. The culture liquid was sampled at the intervals of three days and analyzed by TLC and HPLC.[15] Two products can be detected after three days' incubation and increased slowly with the time. The transformation quickened after 15 days, and the products reached the highest level

Keywords: Biotransformation; Gentiopicroside; *Cordyceps sinensis*; New pyridine monoterpene alkaloid.
* Corresponding authors. Tel.: +86 871 5223235; fax: +86 871 5150124 (Y.-J.Z.); e-mail addresses: zhangyj@mail.kib.ac.cn; cryang@mail.kib.ac.cn

0960-894X/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2007.03.022

at the 21st day, while the substrate (**1**) reduced to the lowest level (Fig. 1).

In parallel control experiments, the medium containing only substrate but without fungus and the culture without substrate were incubated, respectively, in the same condition and analyzed by HPLC. Both tests showed negative results. Therefore, it could be concluded that the detected products were derived from 1 by the fungal biotransformation with CS.

In order to obtain the products, biotransformation was scaled up by adding 10 g of gentiopicroside (**1**) into five 1000 mL flasks (2 g for each), in which mycelia of CS had been incubated for 10 days in 300 mL PDA medium. Three weeks after the addition of **1**, the culture medium was collected and applied to a separation procedure[16] to afford two purified products (compounds **A** and **B**).

Compound **A** was obtained as a white amorphous powder, $[\alpha_D^{25}] + 6.4^\circ$ (0.0057, H_2O). It gave orangy red color with the presence of dragendorff reagent, suggesting the presence of nitrogen. The molecular formula was established as $C_{10}H_{13}NO_3$ on the basis of HRESI MS (*m/z* 219.0629, $[M+1+Na]^+$, calcd 219.0660) and NMR data (Table 1), which indicated five degrees of unsaturation. The IR spectrum showed the presence of carbonyl (1701 cm^{-1}) and NH/OH group (3345 cm^{-1}). Positive ESI MS gave the quasi-molecular ion peaks at *m/z* 415 $[2(M+H)+Na]^+$, 196 $[M+H]^+$, 219 $[M+H+Na]^+$, and fragment ion peaks at *m/z* 179 $[M-OH+H]^+$, 151 $[M-OH-C_2H_4+H]^+$. The ^{13}C NMR (including DEPT) data revealed ten carbon signals consisting of one methyl group, three methylenes (including one oxygen-bearing and one nitrogen-bearing carbons), one oxygen-bearing and one olefinic methines, three olefinic quaternary carbons, and one lactone. The ^1H NMR spectrum also exhibited an NH proton at δ 3.72. There were no proton and carbon signals arising from glucopyranosyl unit observed in the NMR spectra of **A**, indicating that the sugar moiety in **1** was hydrolyzed during the biotransformation. Comparison of the NMR data with those of **2**[17] indicated that **A** contained an unsaturated lactone ring similar to that of **2**. However, unlike the pyridine ring in **2**, compound **A** has a 1,2,5,6-tetrahydropyridine-2-ol ring in the molecule. Detailed analysis of the 2D NMR spectra of **A**, including HMQC, ^1H–^1H COSY, and HMBC (Fig. 2), led us to conclude that the novel structure of **A** was (Z)-5-ethylidene-8-hydroxy-3,4,5,6,7,8-hexahydropyrano[3,4-*c*]pyridine-1-one (shown in Chart 1 as compound **10**).

Table 1. ^1H and ^{13}C NMR data of compounds **9** and **10** (δ in ppm, *J* in Hz)

Compound	**9**		**10**	
	δ_C	δ_H	δ_C	δ_H
1	164.3 s		164.3 s	
3	69.1 t	4.96 (br d, 2.8) 4.92 (br d, 3.3)	65.4 t	4.42 (m)
4	113.2 d	5.47 (m)	22.6 t	2.61 (m)
4a	127.8 s		146.0 s	
5	43.3 d	2.61 (t, 7.8)	130.3 s	
6	75.2 d	4.34 (q, 3.2, 6.8)	57.1 t	4.67 (d, 14.2) 4.55 (d, 14.2)
NH				3.72 (br d, 3.4)
8	154.0 d	7.54 (s)	87.0 d	5.83 (s)
8a	102.0 s		121.7 s	
9	58.6 t	3.52 (dd, 8.1, 11.0) 3.69 (dd, 5.6, 11.0)	128.8 d	6.05 (q, 7.1)
10	16.5 q	1.31 (d, 6.8)	13.9 q	1.83 (d, 7.2)

Recorded at 500 MHz for ^1H and 125 MHz for ^{13}C NMR in $CDCl_3$ with TMS as internal standard.

Compound **B** was obtained as a white amorphous powder. Positive FAB MS gave the quasi-molecular ion peak at *m/z* 197 $[M+H]^+$, corresponding to the molecular formula of $C_{10}H_{13}NO_3$, together with the ^{13}C NMR data

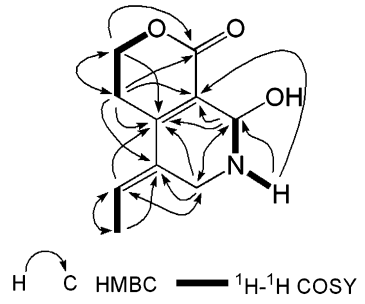


Figure 2. ^1H–^1H COSY and HMBC correlations of **10**.

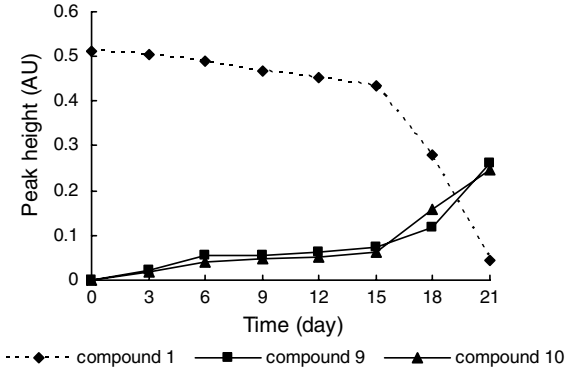


Figure 1. Time course of the biotransformation of **1** with the mycelia of CS (detected by HPLC at 47 nm).

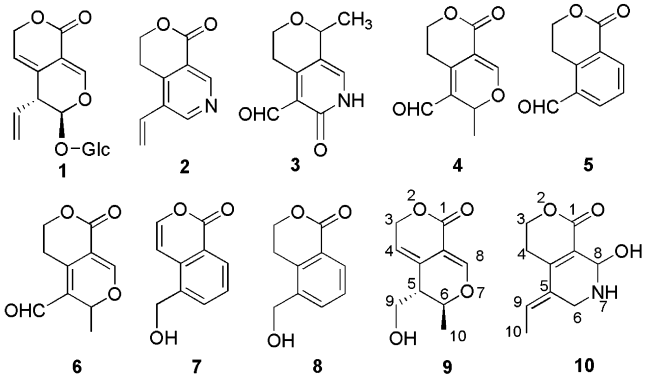
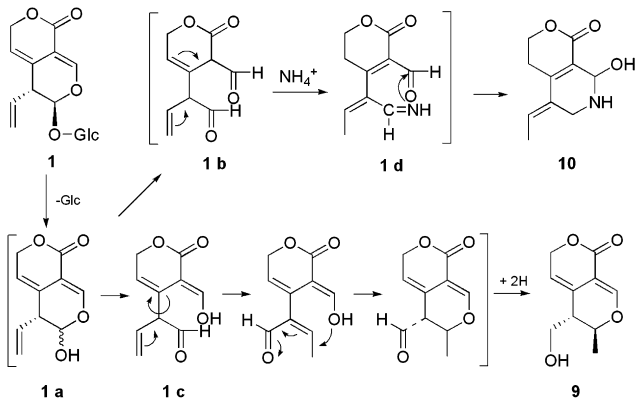


Chart 1.



Scheme 1. Possible biotransformation processes of **1** by CS.

(Table 1). The ^{1}H spectral data of **B** were practically in agreement with those of the reported 5,6-dihydro-5-hydroxymethyl-6-methyl-1*H*,3*H*-pyrano[3,4-*c*]pyran-1-one (**9**), a metabolite of **1** by human intestinal bacteria.[12] Further 2D NMR experiments (HMQC, HMBC, ^{1}H–^{1}H COSY, HMQC–TOCSY, and NOESY spectra) confirmed the structure of **B** to be as the same as that of compound **9**. The coupling constant between H-5 and H-6 was too small to be measured, indicating the ee coupling between these two protons. Moreover, NOESY correlation between H-5 and methyl proton (H-10) was observed. These facts demonstrated a *trans* configuration between the methyl at C-6 and side chain at C-5.

The biotransformation of gentiopicroside (**1**) by human intestinal bacteria resulted in pyrano[3,4-*c*]pyran-type and isochroman-type metabolites in different ratios, and no pyridine monoterpene alkaloid was produced.[12] In our experiment, pyrano[3,4-*c*]pyran and pyridine monoterpene alkaloid were formed in nearly equal ratios, but no isochroman-type metabolite was obtained. A.I. El-Sedway presented only ^{1}H NMR data of **9**,[12] we assigned all ^{1}H and ^{13}C NMR signals in this paper. Scheme 1 shows the possible pathway of the biotransformation of **9** and **10** from **1** by CS. Gentiopicroside (**1**) was hydrolyzed by fungal β-glucosidase to give an unstable hemiacetal aglycone (**1a**), which was readily converted to dialdehyde (**1b**) and aldehyde alcohol (**1c**) forms. **1b** reacted with ammonium ion to form a Schiff base (**1d**), which was subjected to intramolecular cyclization to give a pyridine monoterpene alkaloid-type compound (**10**). Simultaneously, **1c** was subjected to another pathway with intramolecular cyclization and hydrogenation to give compound **9**.

Gentiopicroside (**1**) was reported to have different physiological activities, and some of its derivatives such as gentianine (**2**) and gentianal (**3**) are considered to be the bioactive components in practice.[18] In our study, two types of metabolites were obtained. The preparation of these products on a larger scale is in process and the further inquiry about their bioactivities will be carried out in the near future.

References and notes

1. Jiang, Y.; Yao, Y. J. *J. Fungal Res.* **2004**, *2*(1), 58–67.
2. Huang, H. T.; Chou, S. H.; Ho, H. L. *Chin. Pharm. Bull.* **1981**, *16*, 53.
3. Jiang, S. J. *J. Oriental Med.* **1991**, *16*, 128.
4. Chen, Y. C.; Huang, Y. L.; Huang, B. M. *Int. J. Biochem. Cell Biol.* **2005**, *37*, 214.
5. Buenz, E. J.; Bauer, B. A.; Osmundson, T. W.; Motley, T. J. *J. Ethnopharmacol.* **2005**, *96*, 19.
6. Kumarasamy, Y.; Naha, L.; Sarker, S. D. *Fitoterapia* **2003**, *74*, 151.
7. Qian, Y.; Ling, C. Q.; Yu, C. Q. *J. Fourth Mil. Med. Univ.* **1999**, *20*, 916.
8. Guo, Y. J.; Lu, Y. R. *Chin. J. Pharm. Anal.* **1983**, *5*, 268.
9. Van der Sluis, W. G.; Van der Nat, J. M.; Spek, A. L.; Ikeshiro, Y.; Labadie, R. P. *Planta Med.* **1983**, *49*, 211.
10. Van der Sluis, W. G.; Van der Nat, J. M.; Labadie, R. P. *Planta Med.* **1982**, *45*, 161.
11. Ishiguro, K.; Yamaki, M.; Takagi, S. *Planta Med.* **1983**, *49*, 208.
12. El-Sedway, A. I.; Hattori, M.; Kobashi, K.; Namba, T. *Chem. Pharm. Bull.* **1989**, *37*, 2435.
13. Wang, D.; Zhu, H. T.; Zhang, Y. J.; Yang, C. R. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4073.
14. Gentiopicroside (**1**) was isolated from a frequently used medical plant *Gentiana rigescens* Fr. ex Hemsl: Xu, M.; Wang, D.; Zhang, Y. J.; Yang, C. R. *Acta Botanica Yunnanica* **2006**, *28*(6), 669–672.
15. HPLC analysis was conducted on a Waters 2596 system equipped with a 2996 PDA detector. Mobile phase: (CH_3CN/H_2O, 0–30 min: 5–50%), column: symmetry C-18 column (5 μm, 3.9 × 150 mm, Waters), flow rate: 1 mL/min, UV detection at 200–400 nm, column temperature: 35 °C.
16. Isolation of **9** and **10**: the culture medium was filtered and applied to a Diaion HP-20 column chromatography, eluted successively with H_2O and MeOH. The MeOH fraction was concentrated to dryness. The residue (4.5 g) was subjected to repeated column chromatography over silica gel [petroleum ether/EtOAc (2:1)] and RP-18 [MeOH/H_2O (3:7–7:3)] to give **9** (120 mg) and **10** (45 mg).
17. Bailleul, F.; Delaveau, P.; Rabaron, A.; Plat, M.; Koch, M. *Phytochemistry* **1977**, *16*, 723.
18. Yang, X. F.; Song, C. Q. *Chin. J. Chin. Mater. Med.* **2000**, *25*, 673.