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Biotransformation of artemisinin by fermentation of *Rhizopus chinensis* and *Cunninghamella elegans*

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Abstract **Object** To study the microbial transformation of antimalarial drug artemisinin (Ⅰ). **Methods** The enzymes secreted by fermentation of *Rhizopus chinensis* Saito CICC 3043 and *Cunninghamella elegans* Lendn. AS 3.1207 in the potato medium were employed to transform artemisinin. **Results** Three products were obtained, among which deoxyartemisinin (Ⅱ) was also found in the controls without microorganisms. The other two were identified as 3α-hydroxydeoxyartemisinin (Ⅲ) and 9β-hydroxyartemisinin (Ⅳ). Compound (Ⅳ) was identified as a new one. **Conclusion** Artemisinin could be transformed by the two title strains of microorganisms. It is easy to release one oxygen atom by breaking of perexide bridge in potato medium and to form deoxyartemisinin, thus making it lose its antimalarial activity. The iron (Fe) in the potato medium may have the above function.

Key words biotransformation; artemisinin; *Rhizopus chinensis saito*; *Cunninghamella elegans* Lendn.

华根霉和雅致小克银汉霉对青蒿素的生物转化研究

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摘 要: 目的 探讨微生物对青蒿素(Ⅰ)的转化作用。方法 通过华根霉和雅致小克银汉霉在土豆培养基中发酵产酶,对青蒿素进行转化。结果 两株菌对底物均有转化作用,分离出去氧青蒿素 deoxyartemisinin (Ⅱ),3α羟基去氧青蒿素 3α-hydroxydeoxyartemisinin (Ⅲ)和 9β羟基青蒿素 9β-hydroxyartemisinin (Ⅳ)共 3个产物,其中Ⅳ为一新化合物,同时振荡条件下底物在无菌培养基中也能发生微量转化得到产物Ⅱ。结论 青蒿素易被实验两株真菌转化,同时过氧桥也易断裂而失去一个氧原子成为去氧青蒿素,丧失抗疟活性,起作用的可能是土豆培养基中的铁元素。

关键词: 生物转化;青蒿素;华根霉;雅致小克银汉霉

中图分类号: R284.1 文献标识码: A 文章编号: 0253-2670(2002)10-0869-04

Artemisinin Ⅰ, also called Qinghaosu, a sesquiterpene lactone endoperoxide isolated from the Chinese herbal plant *Artemisia annua* L., is an important therapeutic agent combating multidrug-resistant *Plasmodium falciparum* strains[1]. Some biotransformations of artemisinin by microorganisms and plant cells were reported[2~6]. Microbial transformation is defined as an enzymatic reaction catalyzed by the enzyme secreted in metabolic activities of microorganisms, by which the structures of specific substrates were modified. Compared with chemical methods, it has highly stereo and chemo-selectivity and other advantages, such as mild reaction conditions, simple operation proce-

* 收稿日期: 2002-02-12

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dures, less cost and lower pollution. Some reactions which can not be fulfilled in chemical approach are facile processes by microbial transformation. Some microbial transformations were established as *in vitro* models for the prediciton of mammalian drug metabolites[8,9]. In this paper the microbial transformation of artemisinin by fermentation of *Rhizopus chinensis* Saito CICC 3043 and *Cunninghamella elegans* Lendn. AS 3.1207 is reported.

1 Results and discussion

Products Ⅱ-Ⅳ were isolated from the fermented broth of *C. elegans* AS 3.1207 and two products Ⅱ, Ⅲ from that of *R. chinensis* CICC 3043. But compound Ⅱ was also obtained from the controls in which the substrate was added without microorganisms.

Compound Ⅱ was obtained as colorless needles. TOFMS of Ⅱ showed a molecular weight of 266 and the characteristic signals of a peroxide bridge were absent in IR spectrum, which suggested an oxygen atom lost. The ^{1}HNMR and ^{13}CNMR were in good agreement with those of deoxyartemisinin, therefore Ⅱ was confirmed to be deoxyartemisinin[4].

Compound Ⅲ was obtained as colorless needles. The TOFMS of Ⅲ suggested its molecular weight of 282. IR spectrum of Ⅲ showed a strong absorption at 3 495 cm^{-1} and no characteristic signals of the peroxide bridge observed. After a comparison of the ^{1}HNMR and ^{13}CNMR spectra with those of 3α-hydroxydeoxyatemisinin, Ⅲ was identified as 3α-hydroxydeoxyatemisinin. It was previously reported to be a microbial transformed product of artemisinin by *Penicillium chrysogenum* (ATCC 9480)[4].

Product Ⅳ was obtained as colorless needles. TOFMS of Ⅳ showed a molecular weight of 298. A strong absorption at 3 491 cm^{-1} and the signals of the peroxide bridge were observed, suggesting a hydroxy group introduced into the substrate molecule. DEPT analysis showed that the number of secondary carbon changed from four to three and that of tertiary carbon increased from five to six. A new peak at δ 73.5 in the ^{13}CNMR spectrum was found, which indicated Ⅳ was a hydroxylated product of substrate and the hydroxy group must be added at a secondary carbon. The hydroxy group was determined from COSY, HSQC and HMBC spectra to be at C-9 position. Comparing ^{13}CNMR spectrum of Ⅳ with that of artemisinin, it was found that C-10, C-8 shifted downfield and C-1, C-7, C-14 shifted upfiled, which confirmed Ⅳ to be a 9-hydroxy product. In the 2D ^{1}H-^{1}H NOESY spectrum, the correlation of 9α-H to 1α-H and 7α-H strongly suggested that the orientation of 9-OH as β configuration. Therefore Ⅳ was identified as 9β-hydroxyartemisinin, which is a new compound (See Fig. 1).

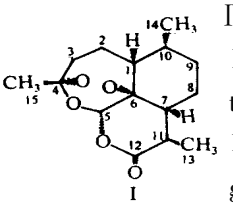

Fig 1. The structure of artemisinin

Product Ⅱ was also isolated from the substrate controls, which showed that it was not a biotransformed product but a product of chemical reaction catalyzed by the iron ion exited in potato medium possibly. It could be inferred that Ⅲ was a hydroxylated product of deoxyartemisinin Ⅱ. A two-step reaction including a chemical and a biological reaction resulted in product Ⅲ.

Product Ⅳ was a hydroxyl product of artemisinin Ⅰ owing to the enzyme secreted by *C. elegans* AS 3.1207. The biological activities of Ⅳ are currently under investigation (See Fig. 2).

In conclusion, microbial transformation is a useful tool in new drug research. By using various kinds of enzymes of microorganisms and some chemical methods, it is possible to transform different natural products, modify their structures or active sites and obtain new bioactive compounds for new drug development.

2 Experimental

2.1 General experimental procedures: Melting points were determined on a micromelting point apparatus and uncorrected. 1D and 2D NMR spectra were recorded in CD_3Cl on an INOVA-500 instrument at 500 MHz by using TMS as internal standard. IR spectrum was run on a Perkin-Elmer 983

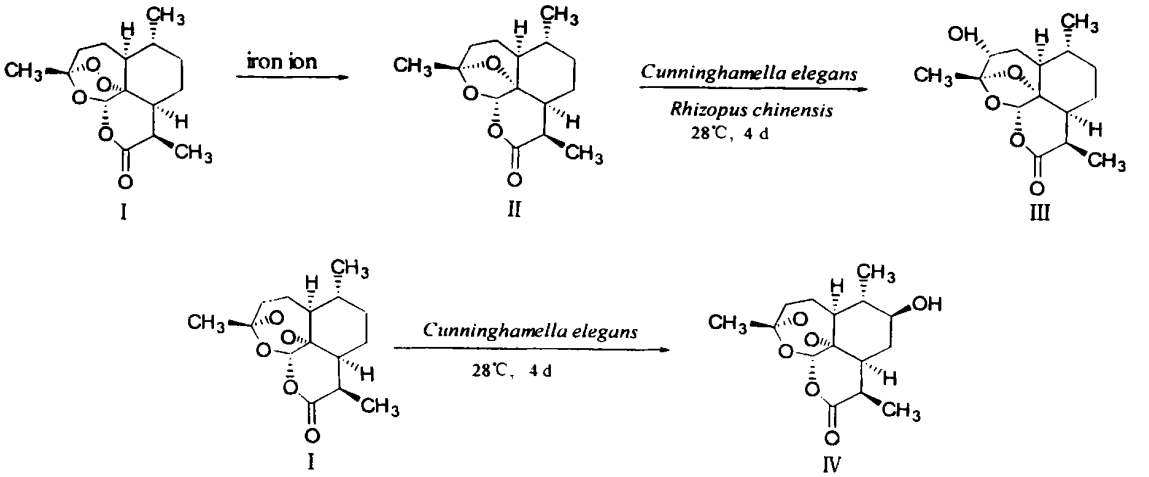

Fig 2. The possible biotransformation pathways of artemisinin by *Rhizopus chinensis* and *Cunninghamella elegans*

FT-IR and recorded in KBr pellets. Optical rotations were measured on Perkin-Elmer 243 spectrometer using MeOH as solvent. TOFMS was obtained with a Perkin-Elmer QSTAR mass spectrometer. TLC analyses were performed on silica gel G. Separation and purification were carried out by column chromatography on silica gel. The silica gel was obtained from Qingdao Haiyang Chemical Group Co., P. R. China.

2.2 Microorganisms: *R. chinensis* CICC 3043 was obtained from China Center of Industrial Culture Collection and *C. elegans* AS 3.1207 from China General Microbiological Culture Collection Center.

2.3 Medium: All the experiments of culture and biotransformation were carried out in potato medium.

2.4 Cultural and general biotransformation procedure: Microorganisms used in this study were inoculated in to 250 mL Erlenmeyer flasks containing 50 mL of potato medium from PDA (potato dextrose agar) slants. The fermentation flasks were placed on rotary shakers, operating at 180 r/min at 28℃. Artemisinin was prepared as a 20 mg/mL solution in acetone. After 48 h of incubation, 0.3 mL of substrate solution was added into the fermentation broth. The cultures were maintained under the identical conditions for further four days. Cultural controls consisted of fermentation blanks in which microorganisms were grown without substrate. Substrate controls consisted of sterile medium containing the same amount of substrate and incubated under the same conditions.

After additional four days, the transformed broth and the controls were filtered and the filtrates were extracted with the same volume of EtOAc for three times. The solvent was evaporated to dryness under reduced pressure and the residue was dissloved in acetone. TLC analyses were carried out on silica gel plates using petroleum ether (60℃~ 90℃)-acetone (5∶ 2) as the developing system, and visualization of plates was performed by using 10% H_2SO_4-EtOH as spray reagent. The results showed both strains could biotransform the substrate.

Preparative scale biotransformation experiments by *R. chinensis* CICC 3043 and *C. elegans* AS 3.1207 were carried out in twenty 1 L Erlenmeyer flasks containing 250 mL of potato medium respectively. Artemisinin, 200 mg, was used in each biotransformation system. All operations were performed under the conditions described above.

2.5 Isolation and characterization of biotransformed products: yellow residue, 600 mg, was obtained from the fermented broth of *R. chinensis* CICC 3043 and 650 mg from that of *C. elegans* AS 3.1207. The residues were choromatographed on silica gel columns. Both columns were eluted with pertroleum ether (60℃~ 90℃)-acetone (10∶ 1).

Product Ⅱ, 3 mg, and Ⅲ, 66 mg, were obtained from *R. chinensis* CICC 3043, while Ⅱ, 2 mg, Ⅲ, 47 mg, and Ⅳ, 70 mg from *C. elegans* AS 3.1207.

9β-hydroxyartemisinin Ⅳ, $C_{15}H_{22}O_6$, colorless needles, mp 194 ℃~ 196 ℃, $[\alpha]_D^{20}$ + 50.0 (c 0.20, EtOH) Positive TOFMS m/z 299 [M+ 1]$^+$, 316 [M+ NH_4]$^+$, 321 [M+ Na]$^+$, 337 [M+ K]$^+$, 614 [M+ NH_4]$^+$, 619 [2M+ Na]$^+$, 635 [2M+ K]$^+$; IR $\nu_{max}^{(KBr)}$ (cm^{-1}): 3 491 (OH), 1 741 (C= O), 835, 883 and 1 113 (the peroxide bridge) ^{1}HNMR and ^{13}CNMR data see Table 1.

Table 1. ^{1}HNMR and ^{13}CNMR spectral data of compound Ⅳ

Position	δ_C	δ_D	DEPT
1	42.9	1.52 m	CH
2	24.7	1.52 m, 2.00 m	CH_2
3	35.7	2.07 m, 2.43 m	CH_2
4	105.5		C
5	93.4	5.93 s	CH
6	78.8		C
7	42.2	1.91 dq (14.0, 5.0)	CH
8	32.1	1.18 m, 2.12 m	CH_2
9	73.5	3.29 m	CH
10	44.4	1.39 m	CH
11	32.5	3.38 m	CH
12	171.6		C
13	12.6	1.23 d (7.0)	CH_3
14	15.5	1.12 d (6.0	CH_3
15	25.2	1.46 s	CH_3

In addition, substrate, 80 mg, was added into four 1 L flasks containing 250 mL of blank medium. They were maintained on the rotary shakers under identical conditions. Finally product Ⅱ, 1 mg, was isolated from the medium.

Acknowledgement We thank the National Outstanding Youth Foundation by NSF of China and Trans-Century Training Program Foundation for the Talents by the Ministry of Education for financial support.

References

[1] Klayman D L. Qinghaosu (artemisinin): antimalarial drug form China [J]. Science, 1985, 228: 1049-1055.

[2] Zhou L G, Ruan D C, He Z D, *et al*. Biotransformation of artemisinin by hairy roots of *Cyanotis arachnoidea* [J]. Acta Bot Yunnan, 1998, 20(2): 229-232.

[3] Hufford C D, Khalifa S I K, Wiggers F T, *et al*. 1α-hydroxy-artemisinin, a new microbial transformation product [J]. J Nat Prod, 1995, 58: 751-755.

[4] Lee I S, Elsohly H N, Croom E M, *et al*. Microbial metabolism of the antimalarial sesquiterpene artemisinin [J]. J Nat Prod, 1989, 52: 337-341.

[5] Orabi K Y, Galal A M, Ibrahim A R S, *et al*. Microbial metabolism of artemisitene [J]. Phytochemistry, 1999, 51: 257-261.

[6] Bhattacharya A K, Sharma R P. Recent development on the chemistry and biological activity of artemisinin and related antimalarials-an update [J]. Heterocycles, 1999, 51: 1681-1745.

[7] Zhan J X, Zhang Y X, Guo D A. Biotransformation of Gastrodin by *Mucor spinosus* [J]. J Chin Pharm Science, 2001, 10 (4): 187-189.

[8] Khalifa S I, Baker J K, Rogers R D, El-Feraly F S, *et al*. Microbial and mammalian metabolism studies of the semisynthetic antimalarial, anhydrohydroartemisinin [J]. Pharm Res, 1994, 11: 990-994.

[9] Clark A M, Hufford C D. Use of microorganisms for the study of drug metabolism; an update [J]. Med Res Rev, 1991, 11: 473-501.

蝉翼藤茎化学成分研究 (Ⅱ)

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摘 要: 目的 对蝉翼藤 *Securidaca inappendiculata* 的化学成分进行研究。方法 利用硅胶柱色谱和中压液相色谱方法对蝉翼藤茎的 95% 乙醇提取物进行分离; 采用 UV, IR, MS, 1D和 2DNMR等技术对所得化合物进行结构研究。结果 分离鉴定了 6个化合物, 分别为: 4, 4′-二甲基-1, 7-庚二酸 (Ⅰ), 肌醇 (Ⅱ), 豆甾醇 (Ⅲ), 维太菊苷 (Ⅳ), 鼠李糖 (Ⅴ) 和蔗糖 (Ⅵ)。结论 Ⅰ为首次从该植物中分离得到的化合物, 其他化合物均为首次从本属植物中分离得到。

关键词: 蝉翼藤; 远志科; 4, 4′-二甲基-1, 7-庚二酸

中图分类号: R284.1 文献标识码: A 文章编号: 0253-2670(2002)10-0872-03

* 收稿日期: 2001-12-25

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