Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Molecular Catalysis B: Enzymatic 36 (2005) 43–46
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Biotransformation of 14-deacetoxyl sinenxan A by *Ginkgo* cell suspension cultures and the cytotoxic activity evaluation

Yulian Zhan [a,b,c], Jian-Hua Zou [a,b], Xiaojun Ma [a,c], Jungui Dai [a,b,*]

[a] *Key Laboratory of Bioactive Substances and Resources Utilization of Chinese Herbal Medicine (Peking Union Medical College), Ministry of Education, Beijing, PR China*
[b] *Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, 1 Xian Nong Tan Street, Beijing 100050, PR China*
[c] *Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, 151 Malianwa North Road, Beijing 100094, PR China*

Received 9 June 2005; received in revised form 5 August 2005; accepted 12 August 2005
Available online 19 September 2005

Abstract

14-Deacetoxyl sinenxan A [2α,5α,10β-triacetoxy-4(20),11-taxadiene, **1**] was converted to two new products, 10β-hydroxy-2α,5α-diacetoxy-4(20),11-taxadiene (**2**) and 10β-butyloxy-2α,5α-diacetoxy-4(20),11-taxadiene (**3**) both about in 20% yields by *Ginkgo* cell suspension cultures. Their structures were identified on the basis of their chemical and spectroscopic data. The three compounds (**1–3**) were preliminarily evaluated for their in vitro cytotoxic activities against two solid tumor cell lines and their drug-resistant counterparts (KB and KB/V, MCF-7 and MCF-7/ADR), and the decreased activities were observed in the case of the two products. The results suggested that biotransformation might be a valuable approach to diversifying natural products and provide some useful information on the study on the structure–activity relationships of the type of compounds.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Biotransformation; Taxane; Cell suspension cultures; *Ginkgo biloba* L.; Cytotoxic activity

1. Introduction

Sinenxan A, 2α,5α,10β,14β-tetra-acetoxy-4(20),11-taxadiene (Fig. 1), is a taxoid isolated from the callus cultures of *Taxus* sp. in high yield (ca. 1–2% of dry weight) [1,2]. The abundant resources and its taxane-skeleton endow it valuable potential for the semisynthesis of paclitaxel or other structurally related bioactive compounds. A number of studies on its structural modification by chemical and biocatalytic approaches were reported and have achieved a lot of intriguing results [3–12]. In our previous reports, highly regio- and stereo-selective hydroxylations at 9α position of sinenxan A and its derivatives were observed in the biotransformations by *Ginkgo* cell suspension cultures [5,6,8,9]. As a part of our ongoing studies, in order to further investigate the effects of different substituents in substrate molecules on the 9α hydroxylation ability of *Ginkgo* cells, i.e., the "substrate-specificity" of the "9α-hydroxylase" of this type of taxanes in *Ginkgo* cells, we prepared many derivatives of sinenxan A for biotransformation. Compound **1** [14-deacetoxyl sinenxan A, 2α,5α,10β-triacetoxy-4(20),11-taxadiene] (Fig. 1), a taxadiene with neither C-14 functional group nor C-13 functional group, was prepared from sinenxan A by chemical method [12,13], and bioconverted as a substrate by *Ginkgo* cell suspension cultures. Moreover, **1** exhibited potent inhibitory effects towards both the sensitive and drug-resistant tumor cell proliferation in our previous bioassay. In an effort to obtain more derivatives with potential for lead compound screening, we have tried biocatalytic modification of **1**. A number of reports [14,15] revealed that the biotransformation approach is a very powerful tool to obtain more useful compounds. The type of biotransformation reactions involve hydroxy-

* Corresponding author. Tel.: +86 10 63165195; fax: +86 10 63017757.
E-mail address: jgdai@imm.ac.cn (J. Dai).

1381-1177/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2005.08.003

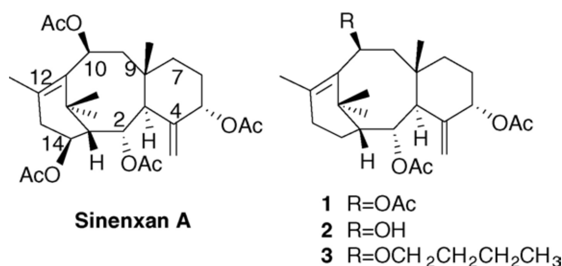

Fig. 1. Chemical structures of sinenxan A and compounds **1–3**.

lation, glycosylation, methylation, acylation, prenylation, sulfation and many others, and this approach possesses more advantages over chemical reactions by regio- and stereo-selectivity, mild conditions and so on. Herein, we report the biotransformation of **1** by *Ginkgo* cell suspension cultures and the cytotoxic activitives of **1** and its two products as well.

2. Materials and methods

2.1. General

Optical rotations were measured with a Perkin-Elmer 343 polarimeter. IR spectra were obtained on a Nicolet IMPACT-400 spectrophotometer (KBr). NMR spectra (^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, DEPT, HMQC and HMBC) were recorded with Varian INOVA-501 instrument (^{1}H NMR, 500 MHz; ^{13}C NMR, 125 MHz) using $CDCl_3$ as solvent and internal standard. HRFABMS spectra were measured on a Macromass Autospec-Ultima ETOF mass spectrometer in the positive mode. Semi-preparative HPLC was performed on a Waters-600 HPLC instrument with an YMC-Pack ODS-A stainless steel column (250 mm × 10 mm i.d.) and a Waters-486 UV detector. The *Ginkgo* suspension cells were cultured in 500 ml flask with 150 ml of medium (MS medium supplemented with 0.5 mg/l of 6-BA, 0.5 mg/l of NAA, 0.2 mg/l of 2,4-D and 3% sucrose) in the dark at 25 °C, shaking at 110 rpm [16]. The pH value of medium was adjusted to 5.8 before autoclaving at 121 °C for 20 min.

2.2. Substrate

14-Deacetoxyl sinenxan A (**1**) was prepared as described in the Refs. [12,13], and the structure was determined by chemical and spectral methods.

*2.3. Biotransformation of **1** by Ginkgo cell suspension cultures*

2.3.1. Biotransformation and separation

Seventy milligrams of **1** was dissolved in 7 ml acetone and distributed into 14 flasks with 14-day-old cell cultures. After additional 7 days of incubation, the cell cultures were filtered out under reduced pressure. The filtrate was collected and extracted with ethyl acetate (5 × 1000 ml). The dried cell cultures were extracted by sonication with ethyl acetate (3 × 200 ml, 30 min each time). The two parts of extract solutions were combined and concentrated under reduced pressure at 40 °C. The obtained residue (1.2 g) was chromatographed on a silica gel column eluting gradiently with acetone–petroleum ether (60–90 °C) (1:19–1:2) to give three fractions: Fr1 (22 mg), Fr2 (9 mg, ca. 13%; substrate, determined by TLC and ^{1}H NMR), Fr3 (23 mg). Fr1 and Fr3 were successively purified with Sephadex LH-20 to afford Fr1-1 (18 mg) and Fr3-1 (19 mg), respectively. Fr3-1 was further purified by semi-preparative HPLC (mobile phase: methanol–acetonitrile–water, 50:15:35, v/v/v; flow rate: 2 ml/min) to afford **2** (13 mg, ca. 20%). Fr1-1 was further purified by semi-preparative HPLC (mobile phase: methanol–acetonitrile–water, 75:15:10, v/v/v; flow rate: 2 ml/min) to afford **3** (13 mg, ca. 20%).

2.3.2. Structural identification of the new products

The structures of the products (**2** and **3**) were identified on the basis of their chemical and spectroscopic analyses, the data were shown as follows.

*2.3.2.1. 10β-Hydroxy-2α,5α-diacetoxy-4(20),11-taxadiene (**2**).* White powder; $[\alpha]_D^{20}$ +52.9° (*c* 0.93, CH_3Cl); IR ν_{max} (KBr): 3624, 3000, 2948, 1726, 1642, 1440, 1374, 1316, 1250, 1222, 1206, 1106, 1086, 1020 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.38 (1H, dd, $J = 1.8$, 6.0 Hz, H-2), 5.25 (1H, t, $J = 2.8$ Hz, H-5), 5.24 (1H, s, H-20a), 5.09 (1H, dd, $J = 5.6$, 11.7 Hz, H-10), 4.90 (1H, s, H-20b), 3.08 (1H, d, $J = 6.1$ Hz, H-3), 2.44 (1H, m, H-13a), 2.34 (1H, dd, $J = 11.8$, 14.8 Hz, H-9a), 2.11 (3H, s, 5-OAc), 1.95 (1H, m, H-14a), 2.03 (3H, s, 2-OAc), 1.97 (1H, m, H-13b), 1.94 (3H, s, H-18), 1.91 (1H, m, H-7a), 1.80 (2H, m, H-6), 1.76 (1H, m, H-1), 1.69 (1H, m, H-14b), 1.65 (3H, s, H-16), 1.63 (1H, m, H-9b), 1.20 (1H, m, H-7b), 1.11 (3H, s, H-17), 0.85 (3H, s, H-19); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 169.8 (s, 5-OAc), 169.7 (s, 2-OAc), 143.5 (s, C-4), 137.2 (s, C-11), 134.8 (s, C-12), 116.4 (t, C-20), 78.9 (d, C-5), 72.4 (d, C-2), 67.7 (d, C-10), 52.2 (d, C-1), 47.0 (t, C-9), 41.0 (d, C-3), 39.6 (s, C-8), 37.2 (s, C-15), 33.7 (t, C-7), 32.0 (q, C-17), 30.1 (t, C-13), 29.0 (t, C-6), 25.3 (q, C-16), 22.5 (q, C-19), 21.9 (q, 5-OAc), 21.6 (q, 2-OAc), 21.3 (q, C-18), 18.3 (t, C-14); HRFABMS (positive) m/z 405.2656 $[M+H]^+$(calc 405.2642 for $C_{24}H_{37}O_5$).

*2.3.2.2. 10β-Butyloxy-2α,5α-diacetoxy-4(20),11-taxadiene (**3**).* White powder; $[\alpha]_D^{20}$ +26.4° (*c* 0.36, $CHCl_3$); IR ν_{max} (KBr): 2935, 1734, 1633, 1464, 1367, 1238, 1095, 1005 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.36 (1H, dd, $J = 1.8$, 6.3 Hz, H-2), 5.24 (1H, t, $J = 2.8$ Hz, H-5), 5.21 (1H, s, H-20a), 4.86 (1H, s, H-20b), 4.65 (1H, dd, $J = 5.3$, 11.8 Hz, H-10), 3.43 (1H, m, 10-$OCH_2CH_2CH_2CH_3$, H-a), 3.26 (1H, m, 10-$OCH_2CH_2CH_2CH_3$, H-b), 3.08 (1H, d, $J = 6.0$ Hz, H-3), 2.42 (1H, m, H-13a), 2.28 (1H, dd, $J = 12.0$,

14.8 Hz, H-9a), 2.10 (3H, s, 5-OAc), 2.04 (1H, m, H-13b), 2.00 (3H, s, 2-OAc), 1.96 (1H, m, H-7a), 1.93 (3H, s, H-18), 1.90 (1H, m, H-14a), 1.77 (2H, m, H-6), 1.74 (1H, m, H-1), 1.66 (1H, m, H-14b), 1.62 (1H, m, H-9b), 1.56 (3H, s, H-16), 1.54 (2H, m, 10-$OCH_2CH_2CH_2CH_3$), 1.37 (2H, m, 10-$OCH_2CH_2CH_2CH_3$), 1.19 (H, m, H-7b), 1.07 (3H, s, H-17), 0.91 (3H, t, $J = 7.3$ Hz, 10-$OCH_2CH_2CH_2CH_3$), 0.82 (3H, s, H-19); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 169.9 (s, 2-OAc), 169.7 (s, 5-OAc), 143.7 (s, C-4), 136.1 (s, C-12), 135.9 (s, C-11), 116.2 (t, C-20), 78.9 (d, C-5), 74.5 (d, C-10), 72.5 (d, C-2), 67.4 (t, 10-$OCH_2CH_2CH_2CH_3$), 52.1 (d, C-1), 45.3 (t, C-9), 41.3 (d, C-3), 39.6 (s, C-8), 37.0 (s, C-15), 33.8 (t, C-7), 32.3 (t, 10-$OCH_2CH_2CH_2CH_3$), 31.5 (q, C-17), 30.3 (t, C-13), 29.0 (t, C-6), 24.8 (q, C-16), 22.5 (q, C-19), 22.0 (q, 5-OAc), 21.6 (q, 2-OAc), 21.2 (q, C-18), 19.7 (t, 10-$OCH_2CH_2CH_2CH_3$), 18.3 (t, C-14), 14.1 (q, 10-$OCH_2CH_2CH_2CH_3$); HRFABMS (positive) *m/z* 461.3297 $[M+H]^+$ (calc 461.3268 for $C_{28}H_{45}O_5$).

2.4. Cytotoxicity bioassays of **1–3**

2.4.1. Cells and culture conditions

Human epidermoid cancer cell line (KB) and human breast cancer cell line (MCF-7) were obtained from ATCC, human epidermoid cancer cell line resistant to vincristine (KB/VCR) and human breast cancer cell line resistant to adriamycin (MCF-7/ADR) were established by continuous exposure to increasing VCR or ADR concentrations [13]. Cells were maintained in RRMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. Cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere.

2.4.2. Cell proliferation assay

KB, KB/VCR, MCF-7 and MCF-7/ADR tumor cells were seeded in 96-well microtiter plates at 1200 cells/well. After 24 h, the compounds were added to the cells. After 96 h of drug treatment, cell viability was determined by measuring the metabolic conversion of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into purple formazan crystals by active cells [17,18]. MTT assay results were read using an MK 3 wellscan (Labsystem Drogon) plate reader at 570 nm. All compounds were tested in five concentrations and were dissolved in 100% DMSO with a final DMSO concentration of 0.1% in each well. Each concentration of the compounds was tested in three parallel wells. IC_{50} values were calculated using Microsoft Excel software.

3. Results and discussion

3.1. Biotransformation of **1** by Ginkgo cell suspension cultures

Substrate (**1**) was administered to the 14-day-old cell cultures, and two products (Fig. 1) were obtained by chromatographic methods after additional 7 days of incubation. On the basis of the spectral and chemical analyses, their structures were identified as 10β-hydroxy-2α,5α-diacetoxy-4(20),11-taxadiene (**2**, ca. in 20% yield) and 10β-butyloxy-2α,5α-diacetoxy-4(20),11-taxadiene (**3**, ca. in 20% yield). They are two new taxanes obtained by biocatalytic approach.

The HRFAB mass spectrum of **2** showed a quasi molecular ion peak $[M+H]^+$ at *m/z* 405.2606, which was consistent with the molecular formula of $C_{24}H_{36}O_5$, suggesting the removal of one acetyl group in comparison with the molecular of **1**. The presence of two OAc groups in ^1H NMR and ^{13}C NMR spectra of **2** and the IR absorption at 3624 cm^{-1} confirmed the deduction. The ^1H NMR spectrum of **2** was similar to that of **1** except that the signal of H-10β shifted to an upper field at δ 5.09 (dd, $J = 5.6$, 11.7 Hz) as compared with δ 6.06 (dd, $J = 5.6$, 12.2 Hz) in **1**, suggesting the existence of an OH group rather than an OAc group at C-10. It was supported by its ^{13}C NMR spectrum in which the signal of C-10 shifted to an upper field at δ 67.7 (*d*) compared with δ 70.5 (*d*) in **1**. Therefore, the structure of **2** was identified as 10β-hydroxy-2α,5α-diacetoxy-4(20),11-taxadiene, a derivative of **1** via enzymatic hydrolysis of C-10 acetyl group (the spectral data are shown in Section 2).

The HRFAB mass spectrum of **3** showed a quasi molecular ion peak $[M+H]^+$ at *m/z* 461.3297, consistent with the molecular formula of $C_{28}H_{44}O_5$. The ^1H NMR, ^{13}C NMR spectra of **3** were similar to those of **1** except that two OAc groups were observed, and an additional butyloxyl group appeared according to ^1H-^1H COSY, DEPT, HMQC and HRFAB mass data together. The signal of C-10 (δ 70.5 in **1**) obviously shifted to a lower field at δ 74.5 in **3**, moreover, the signal of H-10 (δ 6.06, dd, $J = 5.6$, 12.2 Hz in **1**) shifted remarkably to an upper field at δ 4.65 (dd, $J = 5.3$, 11.8 Hz in **3**), suggesting the presence of the butyloxyl group at C-10 position. Thus, the structure of **3** was identified as 10β-butyloxy-2α,5α-diacetoxy-4(20),11-taxadiene (the spectral data are shown in Section 2). Butylation reaction is very popular in biosynthesis field in nature, but it is not very popular in biotransformation. It might be formed by acyl-transferase with butyl CoA.

In our previous works (Refs. [5–9,11]), many taxanes bearing C-14 or C-13 functional groups could be hydroxylated at 9α position by *Ginkgo* cells. However, the substrate (14-deacetoxyl sinenxan A) used in this work did possess neither C-14 nor C-13 functional group and could not be hydroxylated at C-9 position. These results suggested the substantial effects of C-14 functional group on the biotransformation and the rigorous substrate-specificity of the "9α-hydroxylase" of taxadienes in *Ginkgo* cells.

3.2. Cytotoxicity bioassays of **1–3**

In our previous pharmacological evaluation, compound **1** exhibited potent cytotoxic activities towards both the sensitive and MDR-resistant tumor cell lines (KB and KB/V, MCF-7 and MCF-7/ADR). The interesting results motivated us to yield more novel and more efficient compounds for

Table 1
The cytotoxicity of compounds **1–3** in vitro

Compound	IC_{50} in μM			
	KB	KB/V	MCF-7	MCF-7/ADR
1	4.3 ± 0.6	0.41 ± 0.05	9.6 ± 1.2	1.4 ± 0.2
2	>10	5.4 ± 0.3	>10	>10
3	3.5 ± 0.4	4.2 ± 0.5	9.8 ± 0.8	>10

KB: human epidermoid cancer cell line; KB/VCR: human epidermoid cancer cell line resistant to vincristine; MCF-7: human breast cancer cell line; MCF-7/ADR: human breast cancer cell line resistant to adriamycin. The IC_{50} values were the means of triplicates ± standard deviations.

further biological evaluation, therefore, *Ginkgo* cell cultures used before were employed as biocatalysts to obtain specifically enzymatic products. Although the "expected" products were not obtained, the primary in vitro cytotoxic activities of the substrate and its two products against the above two pairs of solid tumor cell lines were evaluated. Unfortunately, the decreased activities were observed in the case of the two products (see Table 1). To some extent, the results suggested the C-10 OAc group might be one of the active functional groups in this type taxoids, although the tested number of compounds was very limited.

4. Conclusion

In summary, 14-deacetoxyl sinenxan A was not hydroxylated at C-9 position with *Ginkgo* cells as expected, indicating that the substituents in exogenous compound molecule exert substantial influences, not only on biotransformation yield [5–9,11], but also on the biotransformation mode. The bioassay results suggested that the approach of biotransformation of foreign compounds might provide diverse derivatives for bioassay screening and the valuable information on structure–activity relationship (SAR) studies of the type of compounds.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (No. 30100230).

References

[1] (a) K.D. Cheng, W.M. Chen, W.H. Zhu, Q.C. Fang, PCT Int. Appl. WO9406, 740 (Cl. C07C35/37), March 31, 1994;
(b) K.D. Cheng, W.M. Chen, W.H. Zhu, Q.C. Fang, JP Appl. 92/249, 047, September 18, 1992.
[2] Y. Wu, W. Zhu, J. Lu, Q. Hu, X. Li, Chin. Pharm. J. 33 (1998) 15.
[3] S. Hu, X. Tian, W. Zhu, Q. Fang, J. Nat. Prod. 59 (1996) 1006.
[4] S. Hu, X. Tian, W. Zhu, Q. Fang, Tetrahedron 52 (1996) 8739.
[5] J. Dai, H. Guo, D. Lu, W. Zhu, D. Zhang, J. Zheng, D. Guo, Tetrahedron Lett. 42 (2001) 4677.
[6] J. Dai, M. Ye, H. Guo, W. Zhu, D. Zhang, Q. Hu, J. Zheng, D. Guo, Tetrahedron 58 (2002) 5659.
[7] J. Dai, S. Zhang, J. Sakai, J. Bai, Y. Oku, M. Ando, Tetrahedron Lett. 44 (2003) 1091.
[8] J. Dai, M. Zhang, M. Ye, W. Zhu, J.-Y. Guo, X.-T. Liang, Chin. Chem. Lett. 14 (2003) 804.
[9] J. Dai, M. Ye, H. Guo, W. Zhu, D. Zhang, Q. Hu, J. Zheng, D. Guo, Bioorg. Chem. 31 (2003) 345.
[10] X. Zhao, J. Gu, D. Yin, X. Chen, Bioorg. Med. Chem. Lett. 14 (2004) 4767.
[11] J. Dai, L. Yang, J. Sakai, M. Ando, Tetrahedron 61 (2005) 5507.
[12] M. Zhang, D. Yin, J.-Y. Guo, X.-T. Liang, Tetrahedron 61 (2005) 5519.
[13] M. Zhang, D. Yin, H. Liu, J.-Y. Guo, X.-T. Liang, Acta Pharm. Sin. 38 (2003) 424.
[14] K. Ishihara, H. Hamada, T. Hirata, N. Nakajima, J. Mol. Catal. B Enzym. 23 (2003) 145 (and the references cited therein).
[15] T. Suga, T. Hirata, Phytochemistry 29 (2000) 2393–2460 (and the references cited therein).
[16] J. Dai, W. Zhu, Y. Wu, Q. Hu, D. Zhang, Acta Pharm. Sin. 35 (2000) 151.
[17] T. Mosumann, J. Immunol. Methods 65 (1983) 55.
[18] J. Carmichael, W.G. DeGraff, A.F. Gazdar, J.D. Minna, J.B. Mitchell, Cancer Res. 47 (1987) 936.