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Regio- and stereo-selective biotransformation of 2α,5α,10β,14β-tetra-acetoxy-4(20), 11-taxadiene by *Ginkgo* cell suspension cultures

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Abstract—*Ginkgo biloba* cell suspension cultures were used to bioconvert sinenxan A, 2α,5α,10β,14β-tetra-acetoxy-4(20), 11-taxadiene, a taxoid isolated from callus tissue cultures of *Taxus* spp. Besides two major products, 9α-hydroxy-2α,5α,10β,14β-tetra-acetoxy-4(20), 11-taxadiene **1** and 9α,10β-dihydroxy-2α,5α,14β- triacetoxy-4(20), 11-taxadiene **2**, additional six minor products were obtained and five of them identified as new compounds. On the basis of chemical and spectral data, their structures were identified as 9α,14β-dihydroxy-2α,5α,10β-triacetoxy-4(20), 11-taxadiene **3**, 6α,10β-dihydroxy-2α,5α,14β-triacetoxy-4(20), 11-taxadiene **4**, 6α,9α,10β-trihydroxy-2α,5α,14β-triacetoxy-4(20), 11-taxadiene **5**, 9α,10β-*O*-(propane-2,2-diyl)-2α,5α,14β-triacetoxy-4(20), 11-taxadiene **6**, 9α-hydroxy-2α,5α,10β,14β-tetra-acetoxy-4(20), 11-taxadiene formate **7**, 10β-hydroxy-2α,5α,9α,14β-tetra-acetoxy-4(20), 11-taxadiene formate **8**, respectively. Investigation of the properties of the enzymes responsible for the biocatalysis process of sinenxan A to **1** and **2** revealed that the enzymes were extracellular and constitutive. Using sinenxan A and the two major products (**1** and **2**) as indicators, the stage and concentration of sinenxan A added and the kinetics of the biotransformation reaction were investigated. The results showed that: (1) the optimal stage for sinenxan A addition was the logarithmic phase of the cell growth period, in which sinenxan A was almost completely bioconverted, and the biotransformation rates were up to 60 and 20% for **1** and **2**, respectively; (2) the optimal concentration of sinenxan A added was 60 mg/L; (3) the substrate was mainly converted into **1** and **2** in the first 48 h after addition and then into the minor products.

1. Introduction

The diterpenoid paclitaxel (Taxol®, Scheme 1), originally isolated from the Pacific yew (*Taxus brevifolia* Nutt.) in 1971,[1] exhibited remarkably high cytotoxicity and strong antitumor activity against different tumors resistant to existing anticancer drugs.[2] It has been approved for the treatment of advanced ovarian and breast cancer,[3,4] and it is currently in clinical trials for treatment of lung, skin, head and neck cancers with encouraging results.[5] Since the discovery of paclitaxel in the late 1960s, its unique chemical structure, significant biological activity, as well as its novel mechanism of action[6,7] have led to research by scientists from different fields.[8,9]

Initially the development of paclitaxel was a serious problem since its only approved source was the bark of *T. brevifolia*, which yielded low amounts of paclitaxel (approximately 0.01% of the dry weight). These slow growing, woody gymnosperms were harvested in large amounts to provide paclitaxel for clinical trials: approximately 10,000 kg of dry weight of bark are required to yield 1.0 kg of paclitaxel, in other words, more than 3600 trees must be cut down for the treatment of only 500 patients.[10] This situation illustrates a serious resource crisis that has to be alleviated by using various approaches to produce alternate sources of paclitaxel. Alternative approaches for production of paclitaxel by cell suspension cultures and by semi-synthetic process are being evaluated by various groups.[11] Bristol-Myers Squibb reported alternative methods for the production of paclitaxel, a semisynthetic approach and the application of biocatalysis in enabling the semisynthesis of paclitaxel. Three novel enzymes were discovered that converted a variety of taxanes to a single molecule, 10-deacetyl baccatin III, a precursor for paclitaxel semisynthesis. The concentration of 10-deacetyl baccatin III was increased 5.5–24 fold in the extracts treated with the enzymes, depending upon the type of *Taxus* cultivars used, and the difficult separation of a group of closely related

Keywords: taxane; sinenxan A; biotransformation; enzymes; cell suspension cultures; *Ginkgo biloba*.

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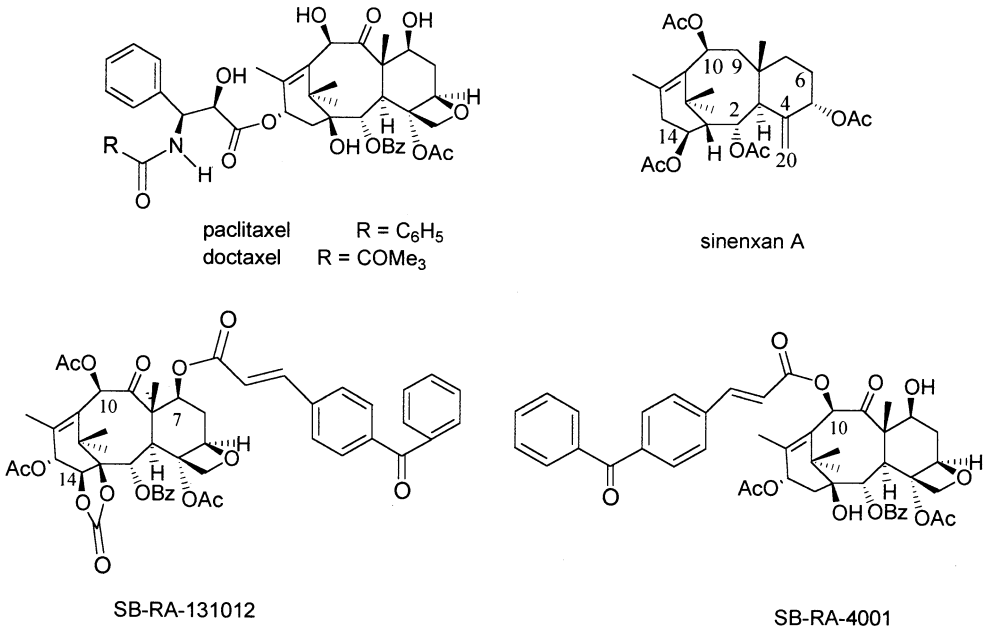
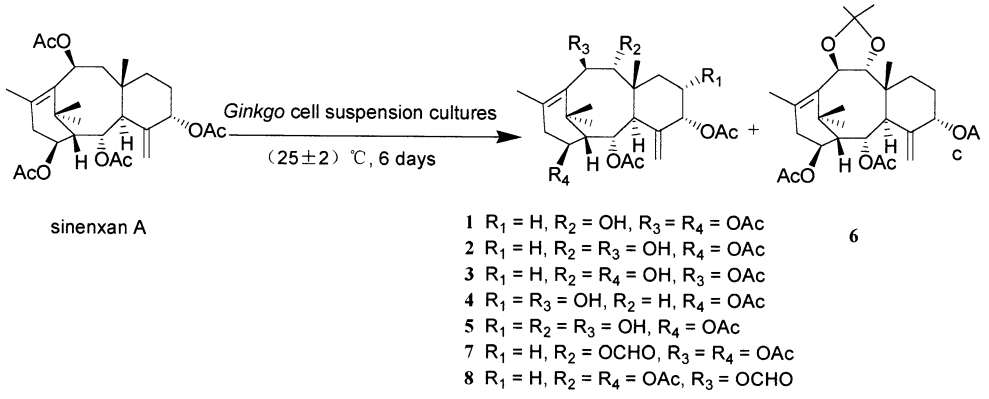


Scheme 1.

molecules can be avoided. Biocatalytic processes have also been reported for the preparation of C-13 paclitaxel side chain synthons.[11]

Recently, attention was also paid to the rapid emergence of multidrug resistant (MDR) cancer cells in the chemotherapy of various types of advanced solid tumor, which is mainly caused by the overexpression of P-glycoprotein (pgp), a cell-membrane transporter for a variety of hydrophobic substrates during the course of the clinical use of vincristine, paclitaxel and etoposide which are very effective hydrophobic antitumor drugs for cancer chemotherapy.[12] The overexpression results in decreased accumulation of the drug within the cancer cells, since the cell can efficiently pump out the drug molecules.[13,14] Ojima and co-workers have discovered and developed the 'second-generation' taxoid anticancer agents-paclitaxel congeners that would not be recognized by pgp and would still display potent anticancer activity. Also they discovered taxane-based MDR reversal agents (TRAs, such as SB-RA-131012, SB-RA-4001, etc. Scheme 1). Paclitaxel recovers 95–99.8% of its efficacy against the resistant human breast cancer cells when TRAs are co-administered at 1.0 μM.[15]

Sinenxan A, 2α,5α,10β,14β-tetra-acetoxy-4(20), 11-taxadiene, is a taxoid isolated from the callus cultures of *Taxus* spp. in high yields (ca. 1–2% of dry weight).[16,17] The rich resources and its taxane-skeleton give it valuable potential for the semisynthesis of paclitaxel or other structurally related bioactive compounds, such as 'second-generation' taxoid anticancer agents and TRAs mentioned above. A number of studies on its structural modification by chemical and biocatalytic approaches were reported.[18–21] Recently, we also reported its highly regio- and stereoselective hydroxylation at C-9 and deacetylation at C-10 by *Ginkgo* cell suspension cultures to form two new major products.[22] This paper describes the formation of six additional products. Detailed results concerning the timing and amount of substrate addition, substrate concentration, kinetics of the biotransformation reaction and the basic properties of the enzymes responsible for the biotransformation of the two major products are also provided.



Scheme 2. The biotransformation of sinenxan A by *Ginkgo* cell suspension cultures.

Table 1. ^{1}H NMR spectral data of compounds **3**, **5**–**8** (CD_3Cl, 500 MHz; δ_H mult., *J*, Hz)

Proton	3	5	6	7	8
1	2.00 (brs)	1.90 (d, 2.0)	1.91 (d, 1.5)	1.94 (d, 2.5)	1.95 (d, 2.5)
2	5.41 (dd, 6.0, 2.0)	5.37 (dd, 2.5, 6.0)	5.44 (dd, 6.0, 2.0)	5.42 (dd, 2.5, 6.5)	5.42 (dd, 2.5, 6.5)
3	2.88 (d, 6.0)	2.96 (d, 6.0)	2.82 (d, 5.5)	3.00 (d, 6.5)	2.97 (d, 6.5)
4					
5	5.28 (brs)	5.08 (brs)	5.32 (brs)	5.31 (brs)	5.31 (brs)
6	1.81 (m)	3.93 (m)	1.83 (m), 1.75 (m)	1.86 (m)	1.86 (m)
7	1.54 (m), 1.77 (m)	1.62 (m), 2.00 (m)	1.73 (m), 1.61 (m)	1.76 (m)	1.71 (m)
8					
9	4.22 (d, 10.0)	4.01 (d, 9.5)	4.19 (d, 9.0)	5.88 (d, 10.5)	5.85 (d, 10.5)
10	5.82 (d, 10.0)	4.75 (d, 9.5)	4.83 (d, 9.5)	6.03 (d, 10.5)	6.14 (d, 10.5)
11					
12					
13	2.67 (dd, 19.0, 9.0), 2.53 (dd, 5.0, 19.0)	2.82 (dd, 18.6, 8.6), 2.44 (dd, 5.1, 18.6)	2.82 (dd, 9.5, 19.0), 2.50 (dd, 4.5, 19.0)	2.85 (dd, 9.0, 19.5), 2.45 (dd, 4.0, 19.5)	2.86 (dd, 9.5, 19.0), 2.45 (dd, 4.0, 19.5)
14	4.09 (dd, 9.0, 5.0)	4.98 (dd, 9.3, 5.1)	4.96 (dd, 4.5, 9.0)	4.98 (dd, 4.5, 9.0)	4.98 (dd, 4.5, 9.0)
15					
16	1.59 (s)	1.64 (s)	1.70 (s)	1.73 (s)	1.72 (s)
17	1.17 (s)	1.24 (s)	1.21 (s)	1.13 (s)	1.13 (s)
18	2.11 (s)	2.20 (s)	2.17 (s)	2.15 (s)	2.15 (s)
19	1.04 (s)	1.20 (s)	0.98 (s)	0.89 (s)	0.86 (s)
20	5.31 (brs), 4.95 (brs)	5.01 (s), 5.46 (s)	5.31 (brs), 4.89 (brs)	5.34 (brs), 4.90 (brs)	5.34 (brs), 4.89 (brs)
$OCOCH_3$	2.11 (s), 2.08 (s), 2.15 (s)	2.02 (s), 1.96 (s), 2.06 (s)	2.05 (s), 2.02 (s), 2.01 (s)	2.18, 2.05, 2.01, 2.01	2.18, 2.05, 2.01, 2.01
OCHO				8.12 (s)	8.05 (s)
$C(CH_3)_2$			1.47 (s), 1.41 (s)		

2. Results

Sinenxan A was efficiently bioconverted by *Ginkgo* cell suspension cultures. The substrate was administered to 15-day-old cell cultures and eight products were isolated by chromatographic methods after an additional 6 days of incubation. On the basis of the spectral and chemical data, their structures were identified as 9α-hydroxy-2α,5α,10β,14β-tetra-acetoxy-4(20), 11-taxadiene **1**, 9α,10β-dihydroxy-2α,5α,14β-triacetoxy-4(20), 11-taxadiene **2**, 9α,14β-dihydroxy-2α,5α,10β-triacetoxy-4(20), 11-taxadiene **3**, 6α,10β-dihydroxy-2α,5α,14β-triacetoxy-4(20), 11-taxadiene **4**, 6α,9α,10β-trihydroxy-2α,5α,14β-triacetoxy-4(20), 11-taxadiene **5**, 9α,10β-*O*-(propane-2,2-diyl)-2α,5α,14β-triacetoxy-4(20), 11-taxadiene **6**, 9α-hydroxy-2α,5α,10β,14β-tetra-acetoxy-4(20), 11-taxadiene formate **7** and 10β-hydroxy-2α,5α,9α,14β-tetra-acetoxy-4(20), 11-taxadiene formate **8**, respectively. Among them, the two major products (**1** and **2**) were previously reported. Their yields (measured by HPLC) were determined to be 70 and 12%, respectively. The other six minor products except **4**[21] were new taxanes obtained by this approach in low yields. The biotransformation reaction is illustrated in Scheme 2.

The FAB mass spectrum of **3** showed a quasi molecular ion peak $[M+Na]^+$ at *m/z* 501, consistent with the molecular formula of $C_{26}H_{38}O_8$. The ^{1}H NMR spectrum of **3** was similar to that of **1** except that the signal of H-14α (δ 4.97, dd, *J*=4.5, 9.5 Hz) had disappeared, and one oxygen-bearing methine signal appeared at δ 4.09 (dd, *J*=5.0, 9.0 Hz), suggesting the presence of a hydroxyl group rather than an acetoxy group at C-14. This conclusion was supported by the appearance of a carbon resonance at δ 67.5 instead of δ 70.0 in its ^{13}C NMR spectrum. Therefore, compound **3** was elucidated to be the 14-deacetyl derivative of compound **1**. The ^{1}H and ^{13}C NMR spectral data of **3** were summarized in Tables 1 and 2.

Compound **4** was identified as a known structure. Its NMR spectral data, IR spectral data and optical rotation were in good agreement with those of 6α,10β-dihydroxy-2α,5α,14β-triacetoxy-4(20), 11-taxadiene reported by Hu et al.[21]

The FAB mass spectrum of **5** showed a quasi molecular ion peak $[M+Na]^+$ at *m/z* 517, consistent with the molecular formula of $C_{26}H_{38}O_9$. The ^{1}H NMR spectrum of **5** was similar to that of **4**, except that signals corresponding to H-9α (δ 1.67, dd, *J*=5.5, 15.0 Hz) or H-9β (δ 2.39 m) in **4** had disappeared, while an oxygen-bearing methine signal was observed at δ 4.01 (d, *J*=9.5 Hz), suggesting the existence of a hydroxyl group at the C-9 position. As a result, the signal of H-10α (δ 5.06, dd, *J*=6.0, 12.0 Hz) shifted upfield (δ 4.75, d, *J*=9.5 Hz). This was confirmed by the signal of C-9 significantly shifting to lower field at δ 79.3 in the ^{13}C NMR spectrum. Therefore the structure of **5** was identified as 6α,9α,10β-trihydroxy-2α,5α,14β-triacetoxy-4(20), 11-taxadiene, and all the ^{1}H and ^{13}C NMR signals of **5** were assigned according to its ^{1}H–^{1}H COSY and HMQC spectra (see Tables 1 and 2).

The FAB mass spectrum of **6** showed a quasi molecular ion peak $[M+Na]^+$ at *m/z* 541, consistent with the molecular formula of $C_{29}H_{42}O_8$. The ^{1}H NMR spectrum of **6** was similar to that of **1**, but one additional methyl group signal occurred. The ^{13}C NMR spectrum of **6** was also similar to that of **1**, except that an additional quaternary carbon signal appeared at δ 107.0. According to the literature,[23–25] an acetonide bridge might be introduced at the C-9 and C-10 position. This deduction was supported by ^{13}C NMR, ^{1}H–^{1}H COSY, HMQC and HMBC spectra of **6**. All the ^{1}H and ^{13}C NMR signals of **6** (see Tables 1 and 2) were assigned according to its DEPT, ^{1}H–^{1}H COSY, HMQC and HMBC spectra. Interestingly, taxane with an acetonide bridge has not been previously found in *Taxus* spp. This type of reaction has never been reported in the field of

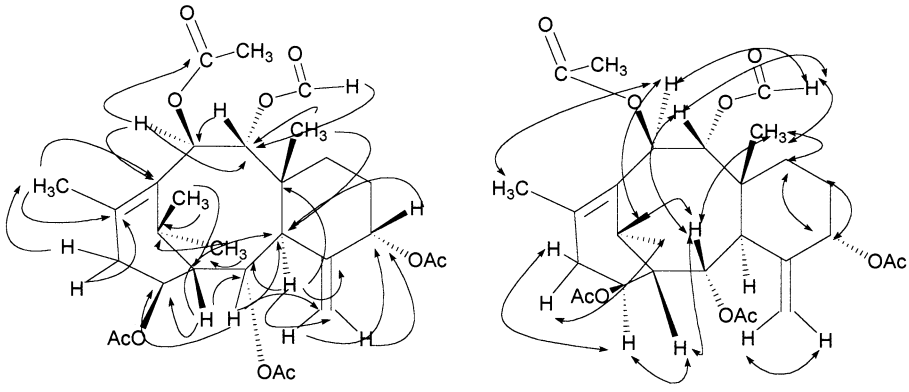
Table 2. ^{13}C NMR spectral data of compounds **3**, **5**–**8** (CD_3Cl, 125 MHz; δ_c)

Carbon	3	5	6	7	8
1	63.2 d	58.7 d	59.4 d	58.1 d	58.1 d
2	70.6 d	69.8 d	69.9 d	69.3 d	69.3 d
3	44.0 d	43.6 d	42.9 d	43.7 d	43.7 d
4	142.3s	138.2s	141.2s	140.9s	140.9s
5	79.0 d	82.0 d	78.7 d	77.7 d	77.7 d
6	28.4 t	69.8 t	28.4 t	27.7 t	27.7 t
7	26.4 t	34.2 t	26.3 t	26.7 t	26.7 t
8	44.7 s	44.1 s	40.7 s	43.8 s	43.8 s
9	76.0 d	79.3 d	82.0 d	77.0 d	77.0 d
10	76.2 d	72.1 d	74.4 d	71.6 d	71.6 d
11	137.5 s	136.7 s	138.1 s	137.2 s	137.2 s
12	133.3 s	134.8 s	133.6 s	132.0 s	132.0 s
13	42.3 t	37.4 t	40.0 t	39.0 t	39.0 t
14	67.5 d	70.2 d	70.5 d	69.6 d	69.6 d
15	37.5 s	39.5 s	37.7 s	36.5 s	36.5 s
16	25.6 q	31.8 q	24.9 q	25.4 q	25.2 q
17	31.2 q	26.0 q	31.9 q	31.0 q	30.9 q
18	21.9 q	21.6 q	21.7 q	20.4 q	20.4 q
19	17.4 q	21.6 q	17.3 q	16.9 q	16.8 q
20	117.4 t	121.1 t	117.3 t	17.7 t	17.6 t
$OCOCH_3$	170.5 s, 169.7 s, 169.6 s, 21.1 q, 21.3 q, 21.5 q	169.8 s, 169.4 s, 170.0 s, 20.8 q, 21.3 q, 21.3 q	169.9 s, 169.9 s, 169.7 s, 20.6 q, 21.3 q, 21.5 q	169.3 s, 169.2 s, 169.1 s, 169.0 s, 20.4 q, 20.7 q, 20.7 q, 21.2 q	169.3 s, 169.2 s, 169.1 s, 169.0 s, 20.4 q, 20.7 q, 20.7 q, 21.2 q
OCHO				159.4 d	159.4 d
$C(CH_3)_2$			107.0 s, 27.2 q, 26.8 q		

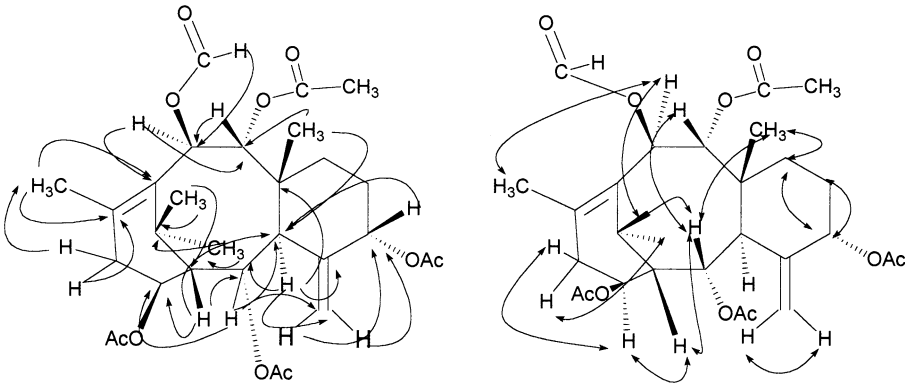
biotransformations. However, it often occurs in chemical synthesis to protect *o*-dihydroxy with acetone under acidic conditions. It was therefore suggested that **6** might not be an enzymatic product, since acetone was introduced in the course of isolation.

The FAB mass spectrum of **7** showed a quasi molecular ion peak $[M+Na]^+$ at *m/z* 571, consistent with the molecular formula of $C_{29}H_{40}O_{10}$. The ^1H NMR spectrum of **7** was similar to that of **1**, except that one proton signal appeared at lower field (δ 8.12) and the signals corresponding to H-9 and H-10 both shifted to lower field at δ 5.88 (d, J=10.5 Hz) and δ 6.03 (d, J=10.5 Hz), respectively. The ^{13}C NMR spectrum showed the appearance of a formate group signal, which was determined to be a quaternary carbon by a DEPT experiment at δ 159.4. All of the above analysis suggested the presence of a formate group at C-9 or C-10 position. The HMBC (Scheme 3) spectrum showed that the proton signal at δ 8.12 was correlated to C-9, and NOESY (Scheme 3) showed the NOE relation between the proton signal at δ 8.12 and the C-19 methyl group, so the formate group should be attached to C-9 rather than C-10. Therefore, **7** was identified as the C-9 formate derivative of **1**, and all the ^1H and ^{13}C NMR signals of **7** were assigned according to its DEPT, ^1H–^1H COSY, HMQC and HMBC spectral data. (see Tables 1 and 2).

The FAB mass spectrum of **8** showed a quasi molecular ion peak $[M+Na]^+$ at *m/z* 571, consistent with the molecular formula of $C_{29}H_{40}O_{10}$. The ^1H NMR spectrum of **8** was similar to that of **7**, except that the signal corresponding to the formate group shifted upfield to δ 8.05, and the signal corresponding to formate methine proton shifted to lower field at δ 6.14, suggesting that the formate group was present at C-10 position rather than at C-9 position. This conclusion was supported by the HMBC and NOESY experiments, since the proton signal at δ 8.05 corresponding to the formate group correlated to C-10 in the HMBC



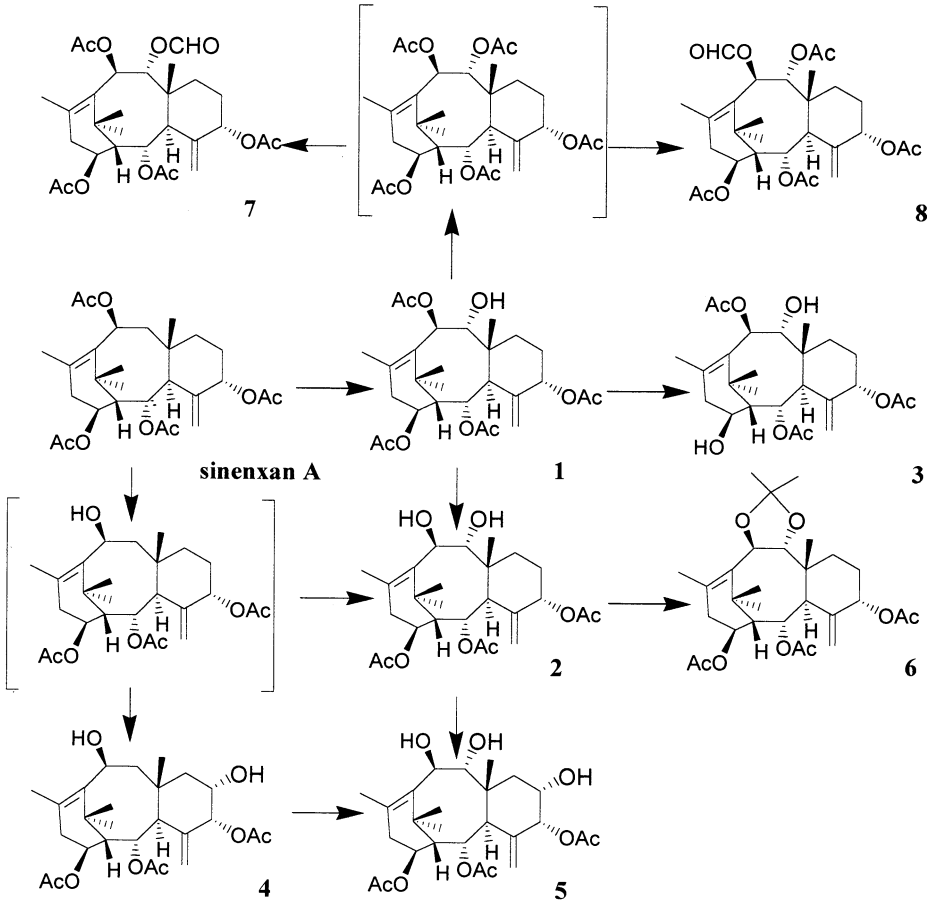
Scheme 3. HMBC and NOESY correlations of compound **7**.



Scheme 4. HMBC and NOESY correlations of compound **8**.

spectrum, and no NOE relation between the formate and C-19 methyl group was observed by NOESY. Therefore, **8** was identified as the 10-formate derivative of **1**, and all the ^{1}H and ^{13}C NMR signals of **8** were assigned according to its DEPT, ^{1}H–^{1}H COSY, HMQC and HMBC spectral data. (see Tables 1 and 2, Scheme 4).

From the above results, the biotransformation of sinenxan A by *Ginkgo* cell suspension cultures involved various reactions, such as: (1) selective hydroxylation at C-9 and C-6 positions; (2) selective deacetylation at C-10 and C-14 positions, which were proven to be enzymatic reactions since sinenxan A is very stable in the pH range (4.0–6.0), which was displayed in the course of the growth period of *Ginkgo* cells; (3) selective formylation at C-9 and C-10 positions; and (4) selective acetylation of C-9 hydroxyl group. In other words, there are several types of enzymes involved in the bioprocess. Product **1** was subsequently used for further biotransformation by *Ginkgo* cell cultures and **2** was identified as the major product. Therefore **2** might be biosynthesized from **1** by a deacetylation process. Though no more experimental data were available on the order in which these reactions took place, a comparison of the structures of the substrate and biotransformed products leads to the proposed biotransformation pathway as shown in Scheme 5.



Scheme 5. The proposed biotransformation pathway of sinenxan A by Ginkgo cell suspension cultures.

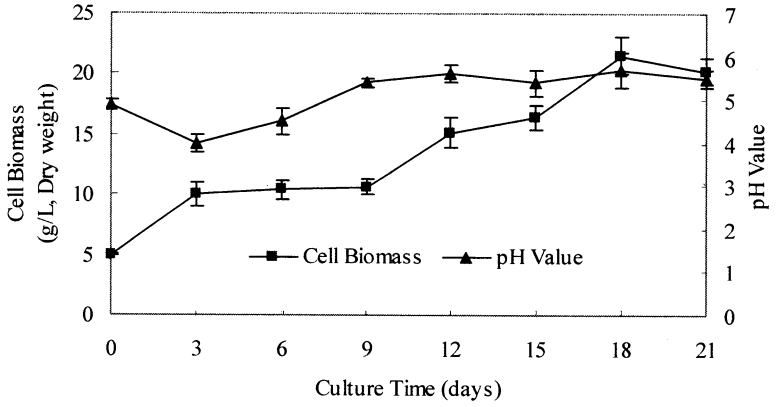


Figure 1. The kinetics of the growth and pH value of *Ginkgo* cell suspension cultures.

The specific hydroxylation at the unactivated C-9 position of sinenxan A by *Ginkgo* cells constitutes an important step in the semisynthesis from sinenxan A to paclitaxel and other bioactive taxoids. In an endeavor to optimize the biotransformation conditions to increase the yields of the two major products (**1** and **2**), the effects of the addition stage and concentration of sinenxan A, also the kinetics of the biotransformation reaction were investigated.

Firstly, the kinetics of *Ginkgo* cell growth and pH value (Fig. 1), as well as the amount of residual sinenxan A and the yields of **1** and **2** corresponding to different stages of sinenxan A addition (Fig. 2) were investigated. The *Ginkgo* cells grew very fast under the culture conditions and the whole growth period lasted for 21 days and involved three phases: (1) lag phase (0–9th day), (2) logarithmic phase (9–18th day) and (3) stationary phase (18–21st day). The pH values remained relatively stable ranging from 4.0 to 6.0. The results also disclosed that the optimal stage for sinenxan A addition was at the logarithmic-phase (9–18th day) of the cell growth period. Substrate added at this phase, especially on the 18th day—the late logarithmic phase was converted into the two major products efficiently. The substrate was almost completely converted and the yields reached their highest levels, about 16.7 and 5.4 mg/L for **1** and **2**, respectively (HPLC, approximately 70 and 12% of the amount of added substrate). This may result from different activities of the responsible enzymes in different cultural stages and different sensitivities of cultured cells to exogenous substrates.[26,27]

In order to clarify the optimal concentration of sinenxan A addition for further investigation, the effects of concentration of sinenxan A (15, 30, 45, 60 and 75 mg/L, calculated for the resulting solution concentration) on the bioconversion were investigated. The results (Fig. 3) showed that the optimal concentration for addition of sinenxan A was 60 mg/L. At this concentration, sinenxan A was efficiently converted and the residual sinenxan A could hardly be detected, meanwhile, the two major products reached their highest yields, about 40 and 13 mg/L for **1** and **2**, respectively. Generally, exogenous substrates can be toxic for cultured plant cells, however, biotranformation such as hydroxylation and glucosylation are considered to be detoxification reactions. Hydrophobic materials such as terpenoids and sterols may disturb the membranes of cells and organelles when the molecules are incorporated and phenolics may cause generation of active oxygen in the cells. Therefore, if the substrates are not toxic to cells, the cells may not respond to them. For example, *Glycyrrhiza* cultured cells were sensitive to papaverine HCl at concentrations above 250 mg/L and yielded several biotransformation products. No product and no toxic effect were detected in *Saponaria officinalis*, although more than one half the amount of papaverine was taken into the cell when papaverine HCl was added at 1250 mg/L.[28] The results of

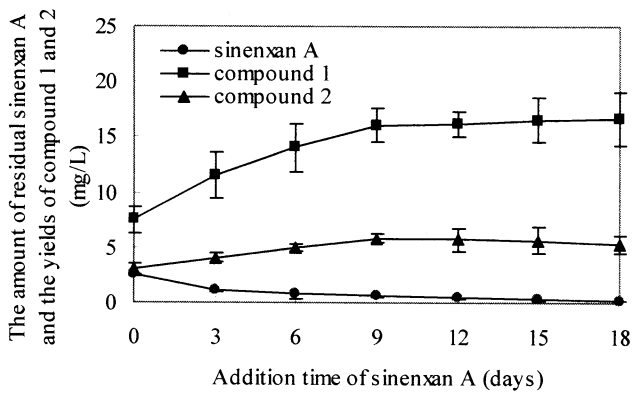


Figure 2. The effects of the timing of sinenxan A addition on its biotransformation by *Ginkgo* cell suspension cultures.

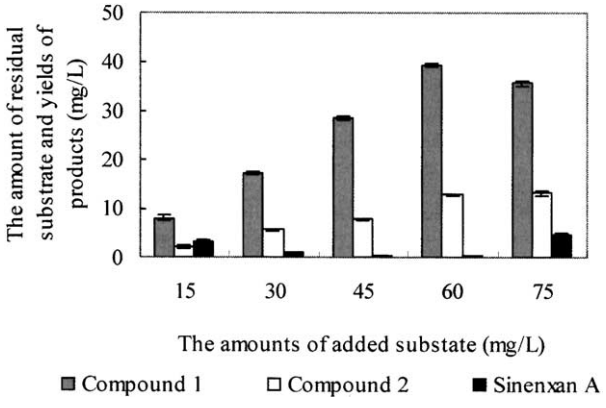


Figure 3. The effects of the concentrations of sinenxan A added on its biotransformation by *Ginkgo* suspension cultured cells.

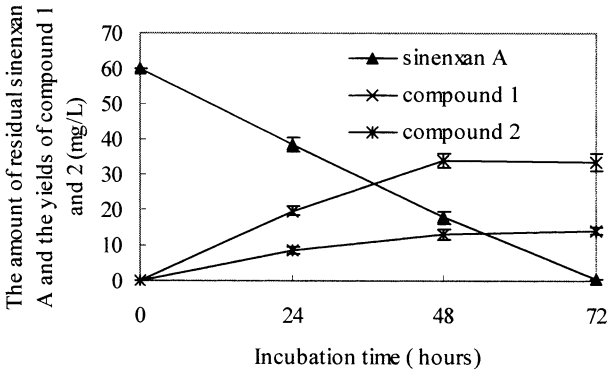


Figure 4. The kinetics of sinenxan A biotransformation and compound **1** and **2** production by *Ginkgo* cell suspension cultures.

this experiment also suggested that the concentration of exogenous substrate addition affects the substrate to product bioprocess.

Based on the above results, the kinetics of the substrate to product bioconversion were also investigated. 60 mg/L of sinenxan A was added to the 18-day-old cultures and incubated for 24, 48 and 72 h, respectively. The HPLC analysis results (Fig. 4) revealed that: (1) the biotransformation rates of sinenxan A were about 40, 80 and 100%; (2) the yield of **1** was about 26, 60 and 59.5%; and (3) the yield of **2** was about 13, 18 and 21.6% for the above mentioned three incubation periods. These results suggested that the metabolism of sinenxan A was consistent with production of two major products within the first 48 h, then production of other minor products. Therefore, the optimal incubation time should be 48 h as far as the production of **1** and **2** are concerned.

Plant cells in vitro could convert the exogenous substrates to new products. In essence, it is the enzymes produced by plant cells that play the role. If the enzymes' properties and additional information were well investigated, the biotransformation condition could be greatly optimized, and the yields of desired products could be improved by modulating the enzymes' activities, or the enzymes could be extracted, purified and immobilized for large-scale production. In addition, the genes encoding to the enzymes could be cloned and transferred into a microorganism to yield products industrially. With this view, a series of experiments (Table 3) were designed to characterize the enzymes responsible for the biocatalytic conversion of sinenxan A to products **1** and **2**. The results of treatment 2 in which the added sinenxan A was metabolized and compound **1** and **2** were produced, suggest that the enzymes are constitutive and extracellular. The results of other treatments confirmed this conclusion. Most probably, the conversion of sinenxan A to **1** is cytochrome P-450-dependent hydroxylation.[29,30]

3. Discussion

Biotransformation refers to the technique that converts various substrates to more useful products using freely suspended, immobilized plant (or microorganisms) cells or enzymes derived from those organisms. Biotransformations employing plant cell cultures cover a wide range of reactions, such as glucosylation, glucosyl esterification, hydroxylation, oxidoreductions, methylation and demethylation, hydrolysis, etc. To a certain extent, plant cells act as a poly-enzyme system that can biocatalyze an exogenous substrate to several products through various types of reactions. In this paper, regio- and stereo-selective hydroxylation (C-9, C-6), deacetylation (C-10, C-14) or demethylation (acetoxyl group at C-9 and C-10) were displayed by employing the same *Ginkgo* cell line, showing the reaction diversity of sinenxan A catalyzed by *Ginkgo* cells. However, the activities or amounts of enzymes responsible for these reactions exhibited great differences, which were suggested by significant different yields of the products. Obviously, the activities or amounts of enzymes responsible for the yields of **1** and **2** were superior to those of others, because the yields of **1** and **2** were much higher. Thus, the major biotransformation of sinenxan A by *Ginkgo* cells might be considered as C-9 hydroxylation. On the other hand, the generation of a product could be controlled depending upon identification of the enzymes reponsible for the process.

Table 3. The basic properties of enzymes responsible for substrate biotransformation to compound **1** and **2**

Treatments	Amount of residual substrate (mg/L)	Yield of compound **1** (mg/L)	Yield of compound **2** (mg/L)	Havested cell cultures (g/L, dry weight)
1[a]	31.6±1.40	0	0	0
2[b]	25.4±2.10	4.32±0.36	0.88±0.11	16.4±0.75
3[c]	0.78±0.12	17.8±2.50	7.5±0.56	21.6±1.12
4[d]	2.65±0.32	21.5±2.11	9.6±1.12	18.5±0.86
5[e]	1.32±0.15	40.6±3.22	16.8±1.64	21.1±0.75

Each value was the mean of three repeated tests ±SE, the inoculum was 5 g/L of cell cultures (dry weight), and the reaction was quenched on day 21.

[a] 0.5 mL of substrate solution was added to the flask without cell cultures inoculated.

[b] 0.5 mL of substrate solution was added on the 15th day to the flask of which the cell cultures were filtered out.

[c] 0.5 mL of substrate solution was added on the 15th day to the flask with cell cultures.

[d] 0.5 mL of substrate solution was added on the 15th day to the flask, but on the 18th day, the cell cultures were filtered out, then an additional 0.5 mL substrate solution was added to the same flask.

[e] 0.5 mL of substrate solution was added on the 15th day to the flask, and on the 18th day, an additional 0.5 mL of substrate solution was added to the same flask.

In the long run, it would be desirable to produce paclitaxel and analogs thereof by a process that does not depend on the extraction of plant materials, and that can be carried out under controlled conditions. Such a biotechnological process may be based on plant cell culture fermentation or a microbial process with a genetically engineered organism in combination with some chemical/enzymatic steps. The development of such a process would be greatly benefited by an understanding of biosynthesis of paclitaxel in plants, and by characterization of enzymes catalyzing various reactions and identification of genes encoding the key enzymes. Identification of rate-limiting enzymes would be essential to increase paclitaxel synthesis. Some remarkable research results on paclitaxel biosynthesis have been reported, and have suggested that cytochrome P-450 enzymes are responsible for the oxygenation steps.[31–35] However biotransformation of taxanes employing plant or microbial cells may biomimic some steps of taxoid biosynthesis, such as extensive oxidation of the taxane skeleton.

In conclusion, we report a powerful method for preparation of variety of derivatives by *Ginkgo* cells from sinenxan A. These may not only supply new bioactive taxoids or intermediates for synthesis of other new bioactive taxoids, but also give a new alternative approach to study paclitaxel biosynthesis.

4. Experimental

4.1. General methods

Optical rotations were measured with a Perkin–Elmer 243 B polarimeter. IR spectra were obtained on a Perkin–Elmer 983 spectrophotometer (KBr). NMR spectra (^{1}H, ^{13}C NMR, DEPT, ^{1}H–^{1}H-COSY, HMQC, HMBC and NOESY) were recorded in $CDCl_3$ with Bruker DRX-500 or Varian INOVA-500 instrument (^{1}H NMR, 500 MHz; ^{13}C NMR, 125 MHz) and chemical shifts were recorded in δ using TMS as internal standard. FABMS spectra were measured on a KYKY-ZHP-5$^{\#}$ mass spectrometer in positive mode; and HREIMS analyses were performed on a JEOL JMX HX-110 spectrometer (The Instrumental Analysis Center for Chemistry, Tohoku University, Japan). The *Ginkgo* suspension cells were shaked on the rotary shaker at 110 rpm at 25°C in the darkness at the inoculum of 5 g/L of cell cultures (dry weight). The pH values were adjusted to 5.8 before autoclaving for 20 min at 121°C and the amount of sucrose added to the medium was 30 g/L.[36] All chemicals were obtained from Beijing Chemical Factory.

4.2. Substrate

2α,5α,10β,14β-Tetra-acetoxy-4(20), 11-taxadiene was isolated from callus cultures of *T. yunnanensis*, and identified by chemical and spectral methods.[16] The substrate was dissolved in EtOH and diluted to 10.0 mg/mL before use as stock solution.

4.3. Biotransformation of sinenxan A by *Ginkgo* cell suspension cultures

The suspension cells were cultured in 500 mL flask with 150 mL MS liquid medium. 0.5 mL of the prepared substrate solution was added to one flask with suspension cell cultures and 0.5 mL EtOH alone to one additional flask as control. After an additional six days of incubation, the cell cultures were filtered out under vacuum and washed three times with distilled water. The filtrate was collected and extracted three times and the dried cultures extracted once by sonication with an equivalent volume of EtOAc, and all the extracted solutions were concentrated under vacuum at 40°C. The residues were dissolved in acetone and analyzed by TLC developed by the acetone–petroleum ether (60–90°C) (1:2.5) and detected by spraying with 10% H_2SO_4 (in EtOH) followed by heating at 105°C. The TLC results showed that some new spots appeared in the chromatogram of the medium extract of the treatment compared with those of the cultures of treatment and the controls. For preparative biotransformation experiments, the *Ginkgo* suspension cell cultures were cultivated on a large scale using 1000 mL flasks with 330 mL of medium. On the day 15, 1.0 g of substrate was dissolved in 100 mL EtOH and distributed into 50 flasks. After an additional six days of incubation, all the media were collected, extracted and concentrated as described above. The obtained residue (1.5 g) was separated by silica gel chromatography (silica gel H, 5–40 mesh) eluting with acetone–petroleum ether (1:5–1:1) to yield compound **1** (500 mg), **2** (100 mg), **6** (5 mg), a mixture of **7** and **8** (6 mg), a mixture of **3** and **4** (10 mg), and a mixture of **4** and **5** (12 mg). Then the mixture of **3**–**5** was further purified by semi-preparative HPLC (mobile phase: methanol–acetonitrile–water, 40:15:55, v/v/v) to afford **3** (3 mg), **4** (5 mg), and **5** (6 mg).

4.3.1. 9α,14β-Dihydroxy-2α,5α,10β-triacetoxy-4(20), 11-taxadiene 3. Colorless needles; $[\alpha]_D^{25}=+42.8$ (*c* 0.0049, MeOH); IR ν_{max} (KBr): 3432, 2940, 1726, 1636, 1373, 1236, 1023 cm^{-1}; FABMS (NBA) *m/z*: 501 (M+Na, 15), 441 (1), 419 (2), 401 (3), 341 (4), 299 (6), 154 (77), 136 (100); HREIMS for $C_{26}H_{38}O_8$ requires: 478.2567; found: 478.2566. The ^{1}H and ^{13}C NMR spectral data are summarized in Tables 1 and 2.

4.3.2. 6α,10β-Dihydroxy-2α,5α,14β-triacetoxy-4(20), 11-taxadiene 4. White powder; $[\alpha]_D^{25}=+47.1°$ (*c* 0.0072, MeOH); other data matched Ref. 21.

4.3.3. 6α,9α,10β-Trihydroxy-2α,5α,14β-triacetoxy-4(20), 11-taxadiene 5. Colorless needles; $[\alpha]_D^{25}=+51.2$ (*c* 0.0054, MeOH); IR ν_{max} (KBr): 3432, 2940, 1726, 1636, 1373, 1236, 1023 cm^{-1}; FABMS (NBA) *m/z*: 517 (M+Na, 7), 154 (100), 136 (82); HREIMS for $C_{26}H_{38}O_9$ requires: 494.2516; found: 494.2520. The ^{1}H and ^{13}C NMR spectral data are summarized in Tables 1 and 2.

4.3.4. 9α,10β-*O*-(Propane-2,2-diyl)-2α,5α,14β-triacetoxy-4(20), 11-taxadiene 6. White powder; $[\alpha]_D^{25}=+36.7$ (*c* 0.0062, MeOH); IR ν_{max} (KBr): 3432, 2940, 1726, 1636, 1373, 1236, 1023 cm^{-1}; FABMS (NBA) *m/z*: 541 (M+Na, 18), 401 (3), 341 (6), 299 (10), 281 (16), 154 (40), 135 (66), 91 (100); HREIMS for $C_{29}H_{42}O_8$ requires: 518.2880; found: 518.2884. The ^{1}H and ^{13}C NMR spectral data are summarized in Tables 1 and 2.

4.3.5. 9α-Hydroxy-2α,5α,10β,14β-tetra-acetoxy-4(20), 11-taxadiene formate 7 and 10β-hydroxy-2α,5α,9α,14β-tetra-acetoxy-4(20), 11-taxadiene formate 8. As a

mixture, white powder; FABMS (NAB) *m/z*: 571 (M+Na, 18), 341 (5), 299 (11), 281 (30), 135 (100); HREIMS for $C_{29}H_{40}O_{10}$ requires: 548.2622; found: 548.2629. The ^{1}H and ^{13}C NMR spectral data are summarized in Tables 1 and 2.

4.4. Effect of substrate addition time on the biotransformation

On the 0, 3rd, 6th, 9th, 12th, 15th, 18th day during the cell culture growth period, 35 mg/L of sinenxan A was added to each 500 mL flask with 150 mL of medium (in triplicates). On the 21st day, the medium in each flask was collected, extracted and concentrated as described above. The residues were dissolved in the HPLC mobile phase and diluted with the same solution to give 2.0 mL and the amounts of residual substrate, compound **1** and **2** were determined by HPLC. HPLC analyses were performed by using a Zorbax C_{18} column (25 cm×4.6 mm I.D., 5 μm) eluting with methanol–acetonitrile–water (50:15:35, v/v/v) at the flow rate of 1.0 mL/min and detecting at 227 nm. The regression equations of sinenxan A, **1** and **2** were: $Y=625{,}889X+154{,}506$ ($r=0.9997$), $Y=839{,}985X+25{,}292$ ($r=0.9993$), $Y=683{,}770X-46{,}645$ ($r=0.9999$), respectively, where Y refers to peak area, X the injection amount (in μg), and r the correlation coefficient.

4.5. Effect of substrate concentration on the biotransformation

On the 18th day of culturing, the different concentrations of substrate (15, 30, 45, 60, 75 mg/L) were added (in triplicates). And on the 21st day, the media were collected, extracted and concentrated, and the amounts of residual substrate, compound **1** and **2** were determined by HPLC.

4.6. Biotransformation kinetics

On the day 18, 60 mg/L of sinenxan A was added (in triplicates) and after every 24 h of incubation, the media were collected, extracted and concentrated, and the amounts of residual substrate, compound **1** and **2** were determined by HPLC.

4.7. Basic properties of the enzymes responsible for the production of compound 1 and 2

In this experiment, a 500 mL flask with 150 mL medium was used for cell culturing. Treatment 1: 0.5 mL of substrate stock solution was added to the flask without cell cultures inoculated. Treatment 2: 0.5 mL of substrate stock solution was added to the flask just after the cell cultures were filtered out on the 15th day. Treatment 3: 0.5 mL of substrate stock solution was added to the flask with 15-day-old cell cultures. Treatment 4: 0.5 mL of substrate stock solution was added to the flask with 15-day-old cell cultures, but on the 18th day of culturing, the cell cultures were filtered out under vacuum, then additional 0.5 mL of substrate solution was added to the flask without cell cultures. Treatment 5: 0.5 mL of substrate stock solution was added to the flask with 15-day-old cell cultures, and on the 18th day of culturing, additional 0.5 mL of substrate solution was added to the same flask. On the 21st day, all the media were collected, extracted and concentrated, and the amounts of residual substrate, compound **1** and **2** measured by HPLC. The results were averaged (in triplicate).

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