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Biotransformation of 2α,5α,10β,14β-tetra-acetoxy-4(20), 11-taxadiene by *Ginkgo* cell suspension cultures

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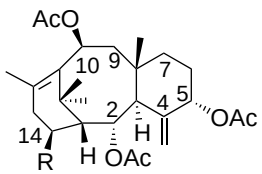
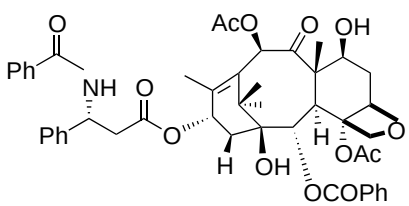
Abstract—*Ginkgo biloba* cell suspension cultures were used to biotransform 2α,5α,10β,14β-tetra-acetoxy-4(20),11-taxadiene. Two novel compounds were obtained and their structures were identified as 9α-hydroxyl-2α,5α,10β,14β-tetra-acetoxy-4(20), 11-taxadiene **1** and 9α, 10β-dihydroxyl-2α,5α,14β-tri-acetoxy-4(20), 11-taxadiene **2**, respectively, on the basis of their physical and chemical data. Compound **1** was subsequently used as a substrate for the bioconversion by *Ginkgo* cell cultures, and the product obtained was confirmed to be the same as **2**, which suggested that **2** is biosynthesized from **1**. Investigation on properties of the related enzymes responsible for the biotransformation reaction through the experimental techniques of cell-free culture and substrate/product concentration analysis revealed that the enzymes were extracellular and constitutive.

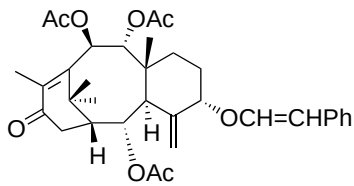
Sinenxan A, 2α,5α,10β,14β-tetra-acetoxy-4(20),11-taxadiene and its analogs (sinenxan B and C) (Scheme 1) are three main taxane compounds isolated from the callus cultures of *Taxus yunnanensis*, *T. cuspidata*, *T. chinesis* and *T. chinensis* var. *mairei*.[1] These taxane compounds are abundant in the callus cultures of the above plants, in which the total concentration of sinenxans could reach 5–6% of the callus dry weight.



sinenxan A R=OCOCH$_3$
sinenxan B R=OCOCH$_2$CH$_3$
sinenxan C R=OCOCH(CH$_3$)CH$_2$CH$_3$



taxol



taxinine

Scheme 1.

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

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Their high concentration and their taxol skeleton vest sinenxans have valuable potential for the semisynthesis of taxol, an anti-cancer drug,[2] or other structurally related bioactive compounds, such as taxinine, a multi-drug-resistance (MDR) reversal agent[3] (Scheme 1). Recently, attention has been paid to the rapid emergence of MDR cancer cells in the chemotherapeutic treatment of various types of advanced solid tumors. This is mainly caused by the over-expression of P-glycoprotein (P-gp),[4] a cell-membrane transporter for a variety of hydrophobic substrates, during the course of the clinical use of vincristine, taxol and etoposide which are very useful hydrophobic antitumor drugs for cancer chemotherapy. Kobayashi and his colleagues reported that a number of new and known taxoids, such as taxinine and its derivatives, isolated from the Japanese yew, *T. cuspidata*,[3,5,6] having neither an oxetane ring at C-4 and C-5 nor an *n*-acylphenyl–lisoserine group at C-13 could increase cellular accumulation of vincristine (VCR) in MDR tumor cells, which is as potent as verapamil, and efficiently inhibited [^{3}H]-azidopine photolabeling of P-gp. Sinenxan A is structurally analogous to taxinine, and therefore it was chosen for structural modification by a biotransformation approach to synthesize taxinine-type compounds or other potential bioactive compounds.

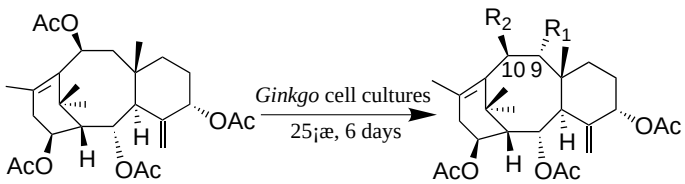
In this paper, we report the successful biotransformation of sinenxan A into two products by the cell suspension cultures of *Ginkgo biloba* L. Those two products were structurally identified as 9α-hydroxyl-2α,5α,10β,14β-tetra-acetoxy-4(20),11(12)-taxadiene **1** and 9α,10β - dihydroxyl - 2α,5α,14β - tri - acetoxy - 4(20), 11(12)taxadiene **2** (Scheme 2) on the basis of their IR, ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H-COSY, ^{13}C–^{1}H-COSY and FABMS[7,8] data. The biotransformation yields for **1** and **2** were determined to be 60 and 20%, respectively. Compound **1** was subsequently used for further biotransformation by *Ginkgo* cell cultures, and it was proved that the converted product was exactly the same as **2**, which suggested that **2** was biosynthesized from **1** through a deacetylation process (Scheme 2).

The fact that hydroxylation reaction took place at the unactivated C-9 position makes it possible to synthesize other taxoids, especially taxinine. In an endeavor to optimize the biotransformation conditions, the growth curve for *G. biloba* cell cultures and the biotransformation curves for the substrate, products **1** and **2** were recorded (data not shown). The results disclosed that the optimal time for substrate addition was at the logarithmic-phase (9th–18th day) during the cell growth period. Substrate addition at this phase, especially on the 18th day, the late logarithmic phase or early stationary phase, resulted in an efficient bioconversion of substrate and yield of products (the yields for **1** and **2** were 2-fold more than those of substrate addition at the beginning of the cell growth period).

In general, the concentration of substrate added affects the substrate to product biotransformation process. Thus, the effects of five different concentrations of substrates (15 mg/L, 30 mg/L, 45 mg/L, 60 mg/L and 75 mg/L) on the substrate bioconversion were investigated and the results showed that the optimal amount of substrate addition was 60 mg/L. At this concentration, the substrate was efficiently converted (100%), and simultaneously the products (**1** and **2**) also reached the highest yields (data not shown).

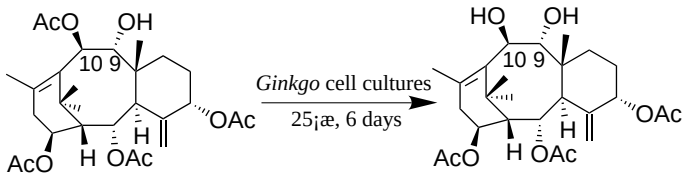
Based on the above results, the kinetics of the substrate/product bioconversion were also investigated. 60 mg/L of substrate was added into the culture on the 18th day, and incubated for 24 h, 48 h and 72 h, respectively. The HPLC analysis results revealed that the substrate was transformed close to completion and the yields of the products **1** and **2** reached the highest levels after 72 h incubation (data not shown).

In order to shed light on the basic properties (constitutive or inducible, extracellular or endocellular) of the



sinenxan A

1 R_1=OH R_2=AcO
2 R_1=OH R_2=OH



1 **2**

Scheme 2.

enzymes responsible for the biotransformation, a series of experiments were designed to characterize the enzymes through the methods of cell-free culture and substrate/product concentration analysis. The results showed that the enzymes are extracellular and constitutive (data not shown). Plant cells in vitro could convert the exogenous substrates to new products, but 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 rate and yield could be optimized through adjusting their activities. Subsequently, the enzymes could be extracted, purified and immobilized for large-scale production of the desired products.

In conclusion, we have obtained a powerful method for preparation of C-9 hydroxylated derivatives by *Ginkgo* cell cultures from sinenxan A, a readily available product from tissue and cell cultures of *Taxus* sp. This might provide a useful tool to prepare bioactive taxoids and to probe some important biosynthetic steps in taxoid biosynthesis in *Taxus* sp. To the best of our knowledge, this is also the first example of 9-hydroxylation of taxoids by cultured plant cells.

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7. 9α-Hydroxyl-2α,5α,10β,14β-tetra-acetoxy-4(20),11(12)-taxadiene **1**: prism-like; $[\alpha]_D^{25}$+44.64 (*c* 0.0083, MeOH); IR λ_{max} (KBr): 3432, 2940, 1726, 1636, 1373, 1236, 1023 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.83 (1H, d, $J=10$, H-10), 5.34 (1H, dd, $J=6.5$, 2.3, H-2), 5.31 (2H, brs, H-5, H-20), 4.98 (1H, dd, $J=5.0$, 9.5, H-14), 4.91 (1H, s, H-20), 4.21 (1H, d, $J=9.5$, H-9), 2.94 (1H, d, $J=5.5$, H-3), 2.85 (1H, dd, $J=10$, 20, H-13), 2.44 (1H, dd, $J=5.0$, 19.5, H-13), 2.18 (3H, d, $J=2.0$, H-18), 2.14 (3H, s), 2.12 (3H, s), 2.06 (3H, s), 2.02 (3H, s), 1.87 (1H, m, H-1), 1.83 (2H, m, H-6, H-7), 1.72 (1H, m, H-6), 1.60 (3H, s, H-16), 1.54 (1H, m, H-7) 1.12 (3H, s, H-17), 1.05 (3H, s, H-19); ^{13}CNMR ($CDCl_3$, 500 MHz) δ 170.51, 169.98, 169.98, 169.76, 141.84 (C-4), 136.41 (C-11), 133.26 (C-12), 117.69 (C-20), 78.67 (C-5), 76.22 (C-9), 76.01 (C-10), 70.34 (C-2), 70.03 (C-14), 58.66 (C-1), 44.78 (C-8), 44.07 (C-3), 39.56 (C-13), 37.12 (C-15), 31.54 (C-17), 28.47 (C-6), 26.19 (C-16), 25.84 (C-7), 21.87 (C-18), 21.40, 21.40, 21.40, 21.02, 17.55 (C-19). FABMS (*m*/*z*): 543 (M+Na, 21), 461 (8), 419 (23), 401 (14), 341 (7), 299 (12), 281 (28), 281 (28), 135 (100).
8. 9α,10β-Dihydroxyl-2α,5α,14β-tri-acetoxy-4(20),11(12)-taxadiene **2**: needle; $[\alpha]_D^{25}$ +40.60 (*c* 0.0061, MeOH); IR λ_{max} (KBr): 3432, 2940, 1726, 1636, 1373, 1236, 1023 cm^{-1}; ^{1}HNMR ($CDCl_3$, 500 MHz) δ 5.37 (1H, dd, $J=6.5$, 2.3, H-2), 5.31 (2H, brs, H-5, H-20), 4.97 (1H, dd, $J=4.5$, 8.5, H-14), 4.90 (1H, brs, H-20), 4.80 (1H, d, $J=9.5$, H-10), 4.09 (1H, d, $J=9.6$, H-9), 2.93 (1H, d, $J=6.5$, H-3), 2.82 (1H, dd, $J=8.5$, 9.5, H-13), 2.46 (1H, dd, $J=5.0$, 19.0, H-13), 2.18 (3H, s, H-18), 2.05 (3H, s), 2.02 (3H, s), 2.00 (3H, s), 1.86 (1H, d, $J=2.1$, H-1), 1.81 (1H, m, H-6), 1.77 (1H, m, H-7), 1.75 (1H, m, H-6), 1.65 (3H, s, H-16), 1.50 (1H, m, H-7), 1.20 (3H, s, H-17), 1.03 (3H, s, H-19). ^{13}C NMR ($CDCl_3$, 500 MHz) δ 169.91, 169.91, 169.91, 142.12 (C-4), 136.70 (C-11), 134.48 (C-12), 117.60 (C-20), 78.84 (C-9), 78.52 (C-5), 72.21 (C-10), 70.62 (C-14), 70.21 (C-2), 59.05 (C-1), 44.46 (C-8), 44.27 (C-3), 39.63 (C-13), 37.48 (C-15), 31.89 (C-17), 28.53 (C-6), 26.57 (C-16), 26.09 (C-7), 21.80 (C-18), 21.33, 21.33, 21.29, 17.60 (C-19). FABMS (*m*/*z*): 501 (M+Na, 6), 401 (2), 341 (1), 299 (2), 154 (100), 136 (94).