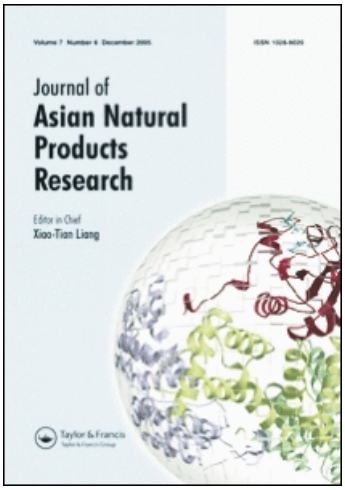

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BIOTRANSFORMATION OF 4(20),11-TAXADIENES BY CELL SUSPENSION CULTURES OF *PLATYCODON GRANDIFLORUM*

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Platycodon grandiflorum cell suspension cultures were employed to biotransform the taxane diterpenoids 2α,5α, 10β,14β-tetraacetoxy-4(20),11-taxadiene (**1**) and 9α-hydroxy-2α, 5α,10β,14β-tetraacetoxy-4(20),11-taxadiene (**2**). One product, 10β-hydroxy-2α,5α,14β-triacetoxy-4(20),11-taxadiene (**3**) was obtained from **1** and two products, 9α,10β-dihydroxy-2α,5α,14β-triacetoxy-4(20),11-taxadiene (**4**) and 10β-hydroxy-2α,5α,9α,14β-tetraacetoxy-4(20),11-taxadiene (**5**) were obtained from **2** incubated with *Platycodon* cultured cells respectively, among which **5** is characterized as a new taxoid compound. The effects of the addition stage for **1** and **2** on the biotransformation were investigated and the results revealed that: (1) the optimal addition stage for **1** was in the early logarithmic phase (6th day) of the cell growth period, in which 78% of **1** was converted and the yield for **3** reached 75%; (2) the optimal addition stage for **2** was on the mid-logarithmic phase (12th day) of the cell growth period, in which 25.3% of **2** was converted and the yields for **4** and **5** reached 18.9 and 14.5%, respectively.

Keywords: 4(20),11-Taxadiene; Biotransformation; Cell suspension cultures; *Platycodon grandiflorum*

INTRODUCTION

Since the discovery of paclitaxel (Taxol®) and its clinical anticancer activity [1], paclitaxel and related taxane compounds have attracted great attention around the world. More than 350 taxane diterpenoids have been isolated from *Taxus* spp. [2], and the diterpenoid family is still expanding. Taxanes can be used as starting materials for semi-synthesis and further modifications for useful compounds and new taxane drugs are under active exploration [3]. The 2α,5α,10β,14β-tetraacetoxy-4(20),11-taxadiene (**1**) is one of the major taxanes isolated from the callus cultures of *Taxus* spp. in high yield (ca. 1–2% of dry weight) [4,5], and a number of remarkable studies on its structural modification by chemical and biocatalytic approaches have been reported [6–9]. Recently, we also reported its highly regio- and stereo-selective hydroxylation at the 9α position using *Ginkgo* cell suspension cultures [10]. In the present paper, we report the biotransformation of **1** and **2** by employing *Platycocdon* cell suspension cultures, the latter compound is one of the conversion products obtained from

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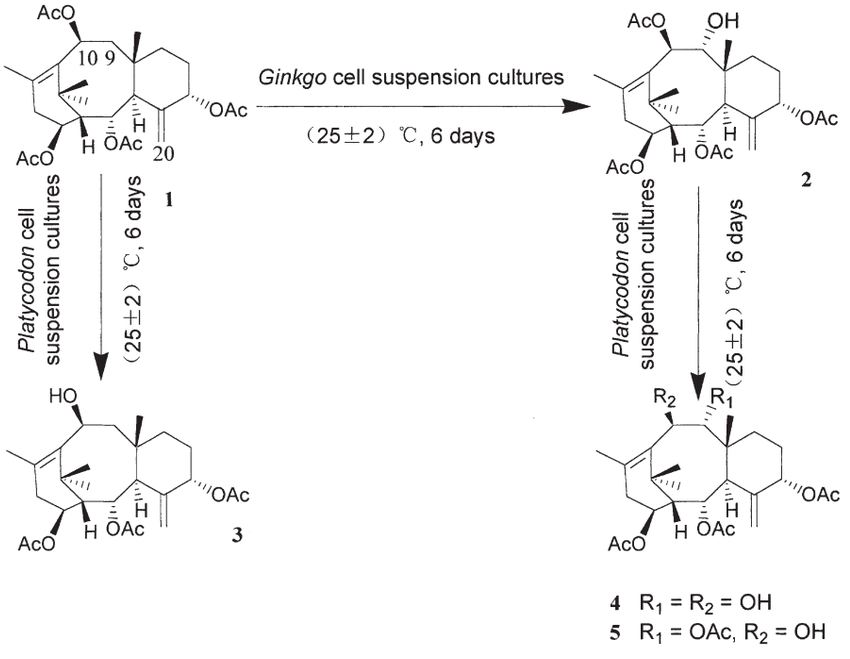
the biotransformation of **1** by *Ginkgo* cells [10], and the effects of the addition stage for substrates on the bioprocess were also investigated.

RESULTS AND DISCUSSION

Compound **1** was administered to the 15-day-old cell cultures of *P. grandiflorum*, and incubated for six further days. The media were harvested and extracted separately as described in the Experimental section. One product was isolated from the medium by chromatographic methods, and its structure was identified as 10β-hydroxy-2α,5α, 14β-triacetoxy-4(20),11-taxadiene (**3**) [9], the 10-deacetyl derivative of **1** on the basis of its chemical and physical spectral data. Compound **2** was obtained from the biotransformation process of **1** by *Ginkgo* cell suspension cultures [10], and was subsequently biotransformed by *Platycodon* cell suspension cultures. After six additional days of incubation with *Platycodon* cells, two more polar products, 9α,10β-dihydroxy-2α, 5α,14β-triacetoxy-4(20),11-taxadiene (**4**) [10] and 10β-hydroxy-2α,5α,9α,14β-tetra-acetoxy-4(20),11-taxadiene (**5**) were obtained from the medium, among which **5** was a new taxane. The biotransformation reactions of **1** and **2** by *Platycodon* cell suspension cultures are shown in Scheme 1.

The FAB mass spectrum of **5** showed a quasi molecular ion peak $[M + Na]^+$ at *m/z* 543, consistent with the molecular formula of $C_{28}H_{40}O_9$. The ^{1}H-NMR spectrum of **5** was similar to that of **2**, except that the signal of H-9 at δ 4.21 (d, $J = 9.5$ Hz) in the spectrum of **2** shifted to a lower field at δ 5.67 (d, $J = 10.0$ Hz), while the signal of H-10 at δ 5.83 (d, $J = 9.5$ Hz) in the spectrum of **2** shifted to higher field at δ 4.92 (d, $J = 10.0$ Hz). These suggested that an acetoxyl group rather than a hydroxyl group was present at the C-9 position, and a hydroxyl



SCHEME 1 The proposed biotransformation pathway of **1** by *Platycodon* cultured cells.

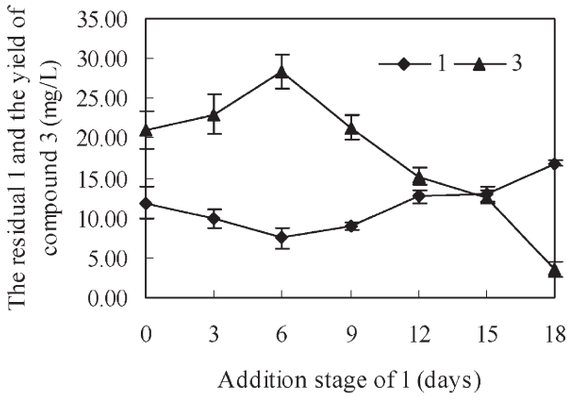


FIGURE 1 The biotransformation kinetics of **1** during the culture period.

group rather than an acetoxyl group was present at the C-10 position. The above deduction was confirmed by the fact that the carbon resonance of C-9 at δ 76.2 in the ^{13}C-NMR spectrum of **2** shifted to higher field at δ 80.2, which is also supported by the data in references [11,12]. Therefore, the structure of **5** was characterized as 10β-hydroxy-2α,5α, 9α,14β-tetraacetoxy-4(20),11-taxadiene. The ^{1}H-NMR and ^{13}C-NMR spectral data of **5** are summarized in the Experimental section.

In a different experiment, we found that **3** obtained from the biotransformation of **1** by *Platycodon* cell suspension cultures could be subsequently and effectively biotransformed by *Ginkgo* cell suspension cultures. Both bioprocesses yielded **4**, in other words, the hydroxylation by *Ginkgo* cells and the deacetylation by *Platycodon* cells could be combined to yield **4** effectively. This suggested that one substrate might be subsequently biotransformed to some desirable products by various plant cells (or microorganisms) and an efficient biotransformation route could be designed.

In order to shed light on the effects of the stage of substrate addition on the biotransformation process, the growth curve for *Platycodon* cell culture and the curve for pH values over the cell culture growth period as well as the biotransformation curves for substrates **1** and **2**, products **3**–**5** were recorded (Figs. 1 and 2). The *Platycodon* cell cultures

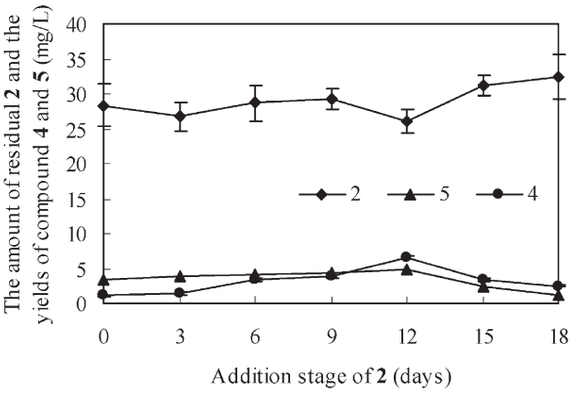


FIGURE 2 The biotransformation kinetics of **2** during the culture period.

grew rapidly under the culture conditions as described in the Experimental section, and the biomass of cell cultures was 25 g/l of dry weight after a complete period of cultivation. The growth period involved three phases (data not shown): (1) lag phase (0–8th day), (2) logarithmic phase (8–12th day), (3) stationary phase (12–16th day) and (4) death phase (16–21st day). The pH values remained relatively stable in the course of the cell growth period ranging from 5.0 to 6.0. The results also revealed that: (1) the optimal addition stage for **1** was at the lag phase (especially on the 6th cultural day), and correspondingly the biotransformation rate of **1** reached 78.6%, and the yield for **3** reached 75%; (2) the optimal addition stage for **2** was at the late logarithmic phase (especially on the 12th culture day), and the biotransformation rate of **2** reached 25.3%, and the yields for **4** and **5** reached 18.9 and 14%, respectively. Additional tests showed that the substrates **1** and **2** under the above pH condition without cell cultures yielded no products, which showed both **3** and **4** to be enzymatic products.

In conclusion, we report here a powerful method for preparation of derivatives by *Platycodon* cell cultures alone or combined with *Ginkgo* cell cultures from **1** which is a readily available product from tissue and cell cultures of *Taxus* spp. Furthermore, the genes encoding the enzymes responsible for the bioprocess could be cloned and transferred to a particular microorganism, then the substrates could be biotransformed to the desired products by employing the genetic microorganism effectively. The results may not only provide a useful tool for preparing bioactive taxoids or precursors for the semisynthesis of other bioactive taxoids, but also give some hints as to the biosynthesis of paclitaxel.

EXPERIMENTAL SECTION

General Experimental Procedures

IR spectra were obtained on a Perkin–Elmer 983B spectrophotometer (KBr). NMR spectra (^{1}H-NMR, ^{13}C-NMR) were recorded in $CDCl_3$ on Bruker DRX-500 or INOVA-500 spectrometers, and chemical shifts were recorded in ppm using TMS as internal standard. FAB mass spectra were measured on a KYKY-ZHP-5$^{\#}$ mass spectrometer in the positive mode. All chemicals were obtained from the Beijing Chemical Factory.

Tissue and Cell Culture of *P. grandiflorum*

The seedlings of *P. grandiflorum* (identified by Professor De-An Guo, Peking University) were obtained from the Green House of Peking University Health Science Center in November, 1999. Young leaves and stems were used to initiate calli. The explants were disinfected by immersing them in 70% ethanol for 30 s, followed by 0.1% $HgCl_2$ for 10 min, washed five times with sterilized water, then cut into small pieces (about $0.5 \times 0.5\,cm^2$ for leaves, 1 cm for stems) and aseptically transferred to Murashige and Skoog's Medium (MS) supplemented with 5.0 mg/l of naphthaleneacetic acid (NAA). The pH of the medium was adjusted to 5.8 before it was autoclaved at 121°C for 20 min. The calli initiated from all of the explants within four weeks of cultivation in a dark culture room at (25 ± 2)°C. The callus cultures were maintained under the same medium and culture conditions by sub-culture every four weeks. Three-week-old friable calli were used for initiation of suspension cultures. MS medium supplemented with 0.5 mg/l of 6-benzylaminopurine (6-BA), 0.5 mg/l of NAA and 0.2 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D) proved to be best for good growth of the cultures in liquid conditions. Cell cultures were sub-cultured every three weeks

at the inoculum of 5 g/l dry weight in 500 ml Erlenmeyer flasks with 150 ml of fresh medium and incubated on a rotary shaker at 110 rpm at (25 ± 2)°C in darkness. Suspension cultures were harvested from the flask every three days to determine the pH values and dry weights for kinetic studies. The pH value of the sampled flask was directly measured by acidimeter before cell cultures were filtered out. The cell cultures from a sampled flask were filtered under vacuum and washed with a large amount of distilled water, then dried at 50°C to a constant weight.

Substrates

The 2α,5α,10β,14β-tetraacetoxy-4(20),11-taxadiene (**1**) was isolated from callus cultures of *T. yunnanensis*, and identified by chemical and spectral methods. The 9α-hydroxy-2α,5α, 10β,14β-tetraacetoxy-4(20),11-taxadiene (**2**) was obtained by biotransformation of **1** by *Ginkgo* cell suspension cultures [10]. The two substrates were dissolved in EtOH and diluted to 10.0 mg/ml before use.

Biotransformation of 1

A measured quantity of 0.5 ml of the prepared substrate solution was added to one flask with 15-day-old cell cultures, and one additional flask without substrate as control. After six additional days of incubation, the cells were filtered under vacuum and washed three times with distilled water. The filtrate was collected and extracted three times and the dried cultures extracted once on an ultrasonic bath by 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 using acetone–petroleum ether (1:2.5) as the mobile phase and detected by spraying with 10% H_2SO_4 (in EtOH) followed by heating at 105°C. The TLC results showed that a new spot appeared in the chromatogram of the medium extract of the treatment compared with those of the cell extract of the treatment and the control. For preparative biotransformation experiments, the *Platycodon* cell suspension cultures were cultivated on a large scale, 0.5 ml of substrate solution were added to each flask on the 15th day of culture and the total amount of **1** administered was 100 mg. After six additional days of incubation, all of the media were collected, extracted and concentrated as described above. The obtained residue (150 mg) was separated by silica gel chromatography (silica gel H, 5–40 mesh), eluting with acetone–petroleum ether (60–90°C) (1:5–1:1) to yield 50 mg of **3**.

Biotransformation of 2

The procedure was similar to that of **1** as described above, except that the total added amount of **2** was 50 mg, and yielded 4.6 mg of **4** and 3.3 mg of **5**.

Compound **5** 10β-hydroxy-2α,5α,9α,14β-tetraacetoxy-4(20),11-taxadiene, white powder. IR ν_{max} (KBr): 3432, 2940, 1726, 1636, 1373, 1236, 1023 cm^{-1}; ^{1}H-NMR δ 5.67(1H, d, $J = 10.0$), 5.40(1H, dd, $J = 2.5, 6.5$), 5.33(1H, brs), 5.30(1H, brs), 4.96(1H, dd, $J = 5.0, 9.5$), 4.92(1H, d, $J = 10.0$), 4.88(1H, brs), 2.97(1H, d, $J = 6.5$), 2.47(1H, dd, $J = 5.0, 18.5$), 2.18(3H, s), 2.14(3H, s), 2.05(6H, s), 1.92(1H, d, $J = 2.0$), 1.92(1H, m), 1.86(2H, m), 1.65(3H, s), 1.25(1H, m), 1.22(3H, s), 0.85(3H, s); ^{13}C-NMR δ 169.9, 169.9, 169.9, 169.9, 146.0, 136.5, 134.3, 118.1, 80.2, 78.5, 70.8, 70.4, 70.0, 58.9, 44.5, 44.2, 39.5, 37.3, 31.8, 28.4, 26.1, 26.1, 21.8, 21.3, 21.3, 21.3, 21.0, 17.5; FABMS (NBA) *m/z*: 543(M + Na, 28), 419(5), 401(2), 299(6), 281(15), 135(100).

Effect of Addition Time of 1 on the Biotransformation

A measured quantity of 35 mg/l of **1** was administered to the cell cultures every three days from the start of the inoculation by **3** replicates. 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 to give 2.0 ml, then the amounts of residual substrate and **3** were analyzed by HPLC. HPLC analyses were performed by using a Zorbax C_{18} column (25×4.6 mm I.D., 5 μm) with methanol–acetonitrile–water (50:15:35, v/v/v) as mobile phase at flow rate of 1.0 ml/min and detected at 227 nm. The regression equations of **1** and **3** were determined to be $Y = 625889X + 154506$ ($r = 0.9997$) and $Y = 643254X - 18322$ ($r = 0.9998$), respectively. In the equation, Y refers to the peak area, X the injection amount (in μg) and r the correlation coefficients.

Effect of Addition Time of 2 on the Biotransformation

The procedure in this experiment was the same as above, except that the regression equation of **2**, **4** and **5** were determined to be $Y = 839985X + 25292$ ($r = 0.9993$), $Y = 828249X - 42290$ ($r = 0.9993$) and $Y = 625428X + 564529$ ($r = 0.9997$), respectively.

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