Specifically enzymatic conversion of 4(20),11-taxadienes from *Taxus* cell cultures

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Taxuylannanine C, 2α,5α,10β,14β-tetraacetoxytaxa-4(20),11-diene 1, and its analogues, 2, 3 and 4, are the major C-14 oxygenated taxanes produced by the tissue and cell cultures of *Taxus* sp. in high yields (ca. 5-6% of the dry weight)\(^1\)\(^-\)\(^3\). Their high content in the cultures and their taxane-skeleton endow them with valuable potential for the semi-synthesis of paclitaxel (Taxol\(^\circledR\)), one of the most effective anticancer agents, and other structurally related bioactive agents, such as anti-MDR (multi-drug resistance) cancer agents or anti-MDR cancer reversal agents (Scheme 1)\(^4\)-\(^6\). Unfortunately, these taxanes have fewer functional groups on the skeleton in comparison with paclitaxel and other bioactive taxoids. The regio- and stereo-selective introduction of oxygen functional groups at their C-1, C-7, C-9 and C-13 positions seems very difficult by traditional chemical methods. In this context, enzymatic conversion by using microorganisms or plant cell suspension cultures is a potential alternative, and some interesting progress has been achieved\(^2\)-\(^13\). Furthermore, the enzymatic systems of microorganisms or plant cell cultures may be useful tools to mimic some steps of taxoid biosynthesis and can provide some useful help for the study of taxoid biosynthesis, especially for extensive oxidation of the taxane skeleton. In this communication, we mainly describe the specific oxidation of these taxanes by a plant, *Ginkgo biloba*, and a fungus, *Absidia coerulea* IFO 4011 cell cultures.

![Scheme 1 Paclitaxel and taxanes from tissue and cell cultures of *Taxus*](image)

1. Bioconversion by *Ginkgo* cell suspension cultures

The substrate, taxuylannanine C, was added into 15-day-old cell cultures of *Ginkgo*, and after additional 3 days of incubation, the two major products hydroxylated specifically at the 9α position were obtained in ca. 70% and 20% yields, respectively (Scheme 2). The specific hydroxylation at the unactivated C-9 position of 1 by *Ginkgo* cells constitutes an important step in the semisynthesis from 1 to taxol and other bioactive taxoids. In an endeavor to optimize the biotransformation condition to increase the yields of the two major products (5 and 6), the effects of the timing and concentration of 1 added and the kinetics of the biotransformation reaction were investigated.

First, the kinetics of *Ginkgo* cell growth and pH value, as well as the amount of residual 1 and the yields of 5 and 6 corresponding to different stages of 1 addition were investigated. The *Ginkgo* cells grew very fast under the culture conditions, the overall growth period lasted 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 around 4.0-6.0. The results also disclosed that the optimal timing for 1 addition was at the logarithmic-phase (9-18th day)
of the cell growth period. The substrate added at this phase, especially on the 18th day - the late logarithmic phase or the early stationary phase was converted into the two major products efficiently. The substrate was almost completely converted, and the yields reached their highest levels, approximately 70% and 12% for 5 and 6, respectively (HPLC). These may result from different activities of the responsible enzymes in the respective cultural stages and the difference in sensitivity of the cultured cells to exogenous substrates.

![Diagram](image)

Scheme 2 9α Hydroxylation of taxanes by *Ginkgo* cell cultures

In order to determine the optimal concentration of 1 in this experiment, the effects of concentration of 1 (15 mg/L, 30 mg/L, 45 mg/L, 60 mg/L and 75 mg/L, final concentration) on the bioconversion were investigated, and the results showed that the optimal concentration of 1 was 60 mg/L. At this concentration, 1 was efficiently converted and residual 1 could hardly detected; meanwhile, the two major products reached their highest yields, about 40 mg/L and 13 mg/L for 5 and 6, respectively. The results of this experiment also suggested that the concentration of exogenous substrate addition affects the substrate in the bioprocess to products.

Based on the above results, the kinetics of the substrate in the bioconversion were also investigated. 60 mg/L of 1 was added into the 18-day-old cultures and incubated for 24 hours, 48 hours, 72 hours, respectively. The HPLC analysis results (Figure 1) revealed that: 1) the biotransformation rates of 1 were about 40%, 80% and 100%, 2) the yields of 5 were about 26%, 60% and 59.5%, and 3) the yields of 6 were about 13%, 18% and 21.6%, at the above mentioned three incubation periods. Therefore, the optimal incubation time should be 48 hours as far as the production of 5 and 6 is concerned.

![Graph](image)

Figure 1 Time course of 1 conversion to 5 and 6 by *Ginkgo* cell suspension cultures

To investigate the specific hydroxylation ability of *Ginkgo* cells and to gain insight into the effects of different substituents on the hydroxylation, seven other taxadienes (2, 3, 4, 7, 8, 9 and 10, Scheme 2) were used as substrates and converted by *Ginkgo* cells. The results showed that these
taxadienes were all specifically hydroxylated at the 9α position, and different yields also resulted from substrates with different substituents (Table 1).

Table 1  The effects of different substituent groups of taxanes on the biotransformation by Ginkgo cell suspension cultures*

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Relative Biotransformation Rates of Substrates</th>
<th>The yields of 9α-Hydroxylated Products (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>70±6.5</td>
</tr>
<tr>
<td>2</td>
<td>80±5.8</td>
<td>60±3.8</td>
</tr>
<tr>
<td>3</td>
<td>50±3.5</td>
<td>20±1.2</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>75±3.3</td>
</tr>
<tr>
<td>8</td>
<td>60±6.0</td>
<td>40±2.5</td>
</tr>
<tr>
<td>9</td>
<td>50±4.6</td>
<td>30±1.8</td>
</tr>
<tr>
<td>10</td>
<td>90</td>
<td>50</td>
</tr>
</tbody>
</table>

*Each value was the mean of three replicates ± SE, the inoculum was 5 g/L cell cultures (dry weight), the concentration of the added substrates was 35 mg/L, and the substrates added on the 15th culture day, the reaction was quenched on day 21.

Table 2 The basic properties of enzymes responsible for substrate biotransformation to compound 5 and 6 a

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Amount of residual substrate (mg/L)</th>
<th>Yield of compound 5 (mg/L)</th>
<th>Yield of compound 6 (mg/L)</th>
<th>Harvested cell cultures (g/L, dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1b</td>
<td>31.6±1.40</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2c</td>
<td>25.4±2.10</td>
<td>4.32±0.36</td>
<td>0.88±0.11</td>
<td>16.4±0.75</td>
</tr>
<tr>
<td>3d</td>
<td>0.78±0.12</td>
<td>17.8±2.50</td>
<td>7.5±0.56</td>
<td>21.6±1.12</td>
</tr>
<tr>
<td>4e</td>
<td>2.65±0.32</td>
<td>21.5±2.11</td>
<td>9.6±1.12</td>
<td>18.5±0.86</td>
</tr>
<tr>
<td>5f</td>
<td>1.32±0.15</td>
<td>40.6±3.22</td>
<td>16.8±1.64</td>
<td>21.1±0.75</td>
</tr>
</tbody>
</table>

*a 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. b 35 mg/L of substrate solution was added to the flask without cell cultures inoculated. c 35 mg/L of substrate solution was added on the 15th day to the flask from which the cell cultures were filtered out. d 35 mg/L of substrate solution was added on the 15th day to the flask with cell cultures. e 35 mg/L 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 35 mg/L of substrate solution was added to the same flask. f 35 mg/L of substrate solution was added on the 15th day to the flask, and on the 18th day, an additional 35 mg/L of substrate solution was added to the same flask.

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 properties of enzymes are well investigated, the biotransformation condition could be greatly optimized, and the yields of the 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 the enzymes could be cloned and transferred into a microorganism to yield products industrially. With this view, a series of experiments (Table 2) were designed to characterize the enzymes responsible for the biocatalytic conversion of 1 to products 5 and 6. The results of treatment of 2 in which the added 1 was metabolized and compounds 5 and 6 were produced, solidly suggest that the enzymes
are constitutive and extracellular, and the results from the other treatments also confirmed it.

2. Bioconversion by the fungus *A. coerulea* IFO 4011

To 2-day-old cell cultures of fungus *A. coerulea* IFO 4011 (obtained from Institute for Fermentation, Osaka, Japan), 1 was added, and after another one week of incubation, 7β hydroxyl product 11 was obtained in 5% yield (Scheme 3). To confirm the specific hydroxylation capacity of the fungus and to gain insight into the influence of the different substrates on the biotransformation process, two other related compounds, 3 and 4, were also used as exogenous substrates and incubated with the cell cultures. As expected, the 7β hydroxylated products (12, 13) were obtained under the same incubation conditions in 10% and 15% yield (Scheme 3), respectively. It is interesting that the longer the alkyl chain of the acyloxy groups at C-14 became, the higher the yield of 7β-hydroxylated products became.

![Scheme 3](image_url)

**Scheme 3** 7β Hydroxylation of taxanes by *Absidia coerulea* IFO 4011

![Scheme 4](image_url)

**Scheme 4** β-Cyclodextrin effects on the biotransformation of taxanes by *Absidia coerulea* IFO 4011

In an attempt to enhance the yield of 11, β-cyclodextrin, which has been used commonly and successfully in the biotransformation for the increase of the yield, was co-administered to the cell cultures of the fungus with 1. The yield of the desired product 11 was ca. 5% and was not increased as expected; however, very intriguingly, three other products were produced in addition to 11. Their structures were determined as 5α,9α,10β,13α-tetraacetoxytaxa-4(20), 11-dien-1β-ol (14), 5α,9α,10β,13α-tetraacetoxytaxa-4(20),11-dien-1β-ol (15) and 5α,9α,10β,13α-tetraacetoxy-11(15→1)abotaxa-4(20),11-dien-15-ol (16) (Scheme 4). 14 and 15 were produced in about 5% yields individually, and 16 in a trace yield. The results suggested that the substrate go, in the presence of β-cyclodextrin, into the organelles of the cells where there are many different enzymes and these completely different reactions took place by their actions. We observed very substantial difference in the reaction modes of these biotransformations in the presence and in the absence of β-cyclodextrin. Obviously, the difference of reaction mode means that each step of taxoid biotransformation takes place in different compartments in the cells. This biotransformation by fungus gave hypothetical biosynthetic intermediates of paclitaxel and its analogues 11, 14 and 15 from C-14 oxygenated taxoids, in fair yield. Since the abundance of taxanes bearing functionalized group both at C-13 and C-14 such as 14 is very limited in yew trees, this kind of taxanes may be the
intermediates between C-14 functionalized taxanes and C-13 functionalized taxanes, even the intermediates of paclitaxel biosynthesis. The fact that the functional group at C-2 in 1 was removed after incubation with the fungus cell cultures suggested that the same reaction probably takes place in the Taxus plant.

In conclusion, we have obtained a powerful method for preparation of 9α hydroxylated derivatives by Ginkgo cells from readily available products from Taxus tissue and cell cultures. In addition, here we have reported a useful 7β hydroxylation, 9α and 13α acetoxylation of C-14 oxygenated taxanes by employing fungus A. coerulea IFO 4011 cell cultures. To the best of our knowledge, these are the first examples of hydroxylation or acetoxylation of the C-9, C-7 or C-13 methylene of taxane derivatives by biocatalytic reactions. These biotransformations would provide not only valuable intermediates for the synthesis of paclitaxel or other bioactive taxoids, but also some helpful hints for determination of or establishment of the taxoid biosynthetic pathway in the Taxus plant.

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References