Agric. Chem. Biotechnol. **43**(3), 176-179(2000)

Isolation and Identification of Nonpolar Taxane Derivatives from the Plant Cell Culture of *Taxus chinensis*

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Received July 11, 2000

Nonpolar taxoides extracted from a large-scale cell culture of *Taxus chinensis* were isolated through the normal and reverse phase column chromatographies, and their compounds were identified via NMR spectroscopy. The complete separation method was systematically established and described. In dichloromethane, dissolved paclitaxel and other taxoids with hexane were precipitated during the purification of paclitaxel from the plant cell culture of *T. chinensis* through a large-scale process while the relatively nonpolar taxane derivatives remained dissolved in the hexane phase. 13-Deoxy baccatin III (I), baccatin VI (II), taxchinin I (III), 2α, 5α, 10β, 14β-tetraacetoxy-4(20),11-taxadiene (IV), 1-deoxy baccatinVI (V), and taxayuntin C (VI) were isolated through column chromatography and identified via NMR spectroscopy. Compounds I and IV were found to be the major components, aside from paclitaxel, in the plant cell culture of *T. chinensis*. The concentrations of I and IV were compared with the that concentration of the paclitaxel in each batch of plant cell culture. The possible applications of compounds I, II, IV, and V were discussed.

Key words : *Plant cell culture, nonpolar taxane derivatives, paclitaxel, isolation, identification.*

Paclitaxel (Taxol®), recognized as a promising plant-derived anticancer drug, was intensively investigated in chemical as well as biological aspects. Its related terpenoides have also attracted considerable interests for their use in chemotherapy.

Through the plant cell culture of *Taxus chinensis* producing up to 173 ppm paclitaxel in a large-scale process, the formation of other types of taxoides and how their concentrations related to the concentration of the paclitaxel in each batch were investigated. For this purpose, the nonpolar compounds **I**, **II**, **III**, **IV**, **V**, and **VI** were isolated from the hexane phase during the purification of paclitaxel in order to identify their structures and also to search further for novel and potent antitumor agents or other NGF-like substances. Their structural identifications via NMR spectroscopy, mass spectrometry, and/or x-ray crystallographic methods were all described previously,[1-6] and the results were compared with those of the present study. These six compounds were mainly formed, just as paclitaxel, during the cell culture of *T. chinensis*. Among these taxoides, the biosynthetic pathway of 2α,5α,10β,14β-tetraacetoxy-4(20),11-taxadiene(**IV**) was, in a previous study, postulated through the isotope-labeled NMR analysis in which the taxane ring system is not synthesized via mevalonate and its four isoprenoid moieties have identical labeling pattern.[7] This compound was also described to be a novel physiologically active substance which has an NGF-like activity.[8] Compound **III**, known as abeotaxoide could be classified into three different types through the structural analyses via NMR and x-ray crystallographic techniques.[4] Compound **VI** was first isolated and identified by Chen *et al.* from the bark of *T. yunnanensis* and was found to be composed of four different compounds, taxayuntin A, B, C, and D.[9]

In this paper, we established the systematic separation method of nonpolar compounds from large-scale plant cell cultures. After the isolation of these compounds, their structures, in particular, the structures of the compounds **I** and **IV** which were two of the main compounds formed in the plant cell cultures of *T. chinensis*, were identified. To our knowledge, this is the first report for the quantitative analysis, isolation, and identification of taxane derivatives from the commercialized large-scale plant cell culture of *T. chinensis*.

Materials and Methods

Plant materials and cell culture conditions. The cells of *T. chinensis* were grown in a large scale (32 M^3) bioreactor at 24°C with an impeller speed of 7.5 rpm. A liquid medium consisting of inorganic salt formulation of Gamborg,[10] 30 g/L sucrose, 10 μL naphthalene acetic acid, 0.2 μM 6-benzamino purine, 1 g/L casein hydrolate, and 1 g/L 2-[N-Morpholino]ethanesulfonic acid were used. For a prolonged cultivation of the cell suspension, 1 and 2%

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Abbreviation: NGF, neural growth factor.

maltose were added to the culture broth on the 7th and the 21th days, respectively. $AgNO_3$ (4 μM) was added on day 0 as an elicitor for the production of paclitaxel.

Analysis of paclitaxel and taxane derivatives (compounds I and IV). Suspension cultures were homogeneously taken and extracted overnight in methyl-t-buthyl ether (MtBE) with 1% cetylpyridiniumchloride (CPC) solution. After extraction, the supernatant was loaded on an LC-NH_2 SPE column (Supelco) and eluted with MtBE and methanol mixture (85/15, v/v). The eluant was mixed well and dried in a speed vacuum evaporator. Dried residue was resolved in methanol and used for the quantitative analysis of paclitaxel. HPLC system (Hewlett-Packard Series1100 with Chem station) with Cuosil-PFP-column (Phenomenex, 4.6×250 mm, 5 μm) was used for the quantitative analysis. The elution was performed in a gradient condition with the mixture of acetonitrile and water from 65/35 (v/v) to 35/65 (v/v) within 30 min (flow rate 1.0 ml/min) and held for 3 min. Absorption was measured at 227 nm with a photo diode array detector. For the quantitative analyses of paclitaxel and compounds **I** and **IV** from the cell biomass, the regression curve was used with an external standard in each case.

Isolation of nonpolar taxane derivatives. Paclitaxel was purified from the cell biomass of *T. chinensis* via the successive extraction and evaporation of methanol, liquid/liquid extraction with dichlorimethane, adding of the adsorbent of active clay (Mizukalife Chemical Co., Japan), filtrating, and precipitating with hexane. Paclitaxel and the relatively polar taxoides were precipitated with hexane while the relatively nonpolar compounds **I~VI** remained dissolved in the hexane phase which were evaporated to dryness.

Approxmately 20 g of the dark yellowish powder dissolved in hexane was subjected to the open silica gel column chromatography. The sample was first fractionated with a gradient mixture of hexane (H) and ethylacetate (E) from H/E 50/1 (v/v) to 20/1 (v/v), 10/1 (v/v), 5/1 (v/v), 2/1 (v/v), and 1/1 (v/v). The detection of each compound was followed via TLC (silica gel $60F_{254}$, Merck, H/E 1/1, v/v). After developing, the plate was viewed at 254 nm, sprayed with 3% H_2SO_4, and heated.

Compound **IV** was detected in the second (H/E 20/1, v/v) and third fractions (H/E, 10/1, v/v). After the evaporation of the solvents of these fractions, washing with H/E (20/1, v/v), and filtration, IV was dissolved in ethylacetate and crystallized out with hexane. Its purity showed >99% through HPLC analysis. Compounds **I, V,** and **VI** were collected from the fifth fraction (H/E 2/1, v/v), and **II** and **III** from the sixth fraction (H/E 1/1, v/v) as separated through the first silica column chromatography.

Approximately 2 g sample obtained from the first through the sixth fractions was dissolved in 5~10 mL methanol and loaded into the reverse phase column (Lichroprep RP-18, 40~63 μm, Merck, column size: 18×3 cm) for the separation of each compound. The sample was elutriated with a gradient mixture of methanol (M)/water (W) at 50/50 (v/v), 60/40 (v/v), and 70/30(v/v), successively. The compounds were detected via TLC (RP-18 F_{254}s, Merck, M/W 80/20, v/v) during the column chromatography. The compound **I** was collected by the gradient mixture of M/W 60/40 (v/v). The fractionated compound **I** was dried, resolved in methanol, and crystallized out with water. The obtained crystal showed a purity of 97.8% via HPLC analysis.

Approximately 120 mg mixture of the compounds **V** and **VI** was collected from the gradient of M/W 70/30 (v/v) through the reverse phase chromatography. This mixture was separated again through silica column (size: 25×2.5 cm) at the elution of dichloromethane/methanol 200/1 (v/v). Three fractions were collected with approximately 400 mL of the gradient mixture was used for each fraction. The detection was done via TLC (silica gel $60F_{254}$, Merck, dichloromethane/methanol 20/1). Compound **V** received from the third fraction, compound **VI** from the first fraction, and the mixture of both compounds from the second fraction. This mixture was separated via crystallization in hexane with ethylacetate. Showing purities of 97.2% for compound **V** and 97.3% for compound **VI** as determined through HPLC analysis.

Approximately 500 mg sample obtained from the sixth fraction (H/E 1/1, v/v) containing compounds **II** and **III**, was separated through the reverse phase column at gradient of M/W 50/50 (v/v), 55/45 (v/v), and finally 60/40 (v/v). Compound **II** was separated as a pure compound by the mixture of 60/40 (v/v), evaporated and, washed with ethylacetate. Its purity was determined to be 97.4% through the HPLC analysis. Compound **III** was eluted in the first (M/W 50/50, v/v) and second fractions (M/W 55/45, v/v) with other impurities. It was evaporated to dryness and purified via preparative TLC (silica gel $60F_{254}$ with 0.5 mm of layer thickness, Merck, dichloromethane/ methanol 20/1, v/v). The obtained layer was dissolved in dichloromethane/methanol (10/1, v/v), filtrated, and crystallized out. Its purity was determined to be 95.9% through HPLC analysis.

All isolated compounds (**I~VI**) were identified via NMR-spectroscopy (Bruker DRX 300, Germany) utilizing 1-D and 2-D NMR techniques. To confirm the suggested molecular structures of the derivatives, FAB-MS spectrometry (VG Masslab, UK) was also used.

Results and Discussion

In the course of purifying of the paclitaxel from the plant cell of *T. chinensis*, the major nonpolar taxane derivatives formed were separated via silica and reverse column chromatography in order to identify their structures and also to search for new compounds. A complete separation procedure via column chromatography was systematically performed. The molecular structures of compounds **I~VI** could be derived from the NMR spectroscopic data (Fig.1). The ^{1}H-NMR data of these compounds were exactly

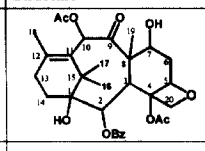
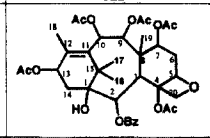
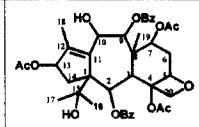
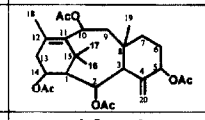
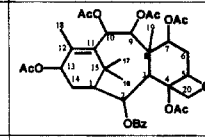
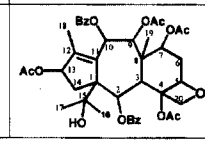
Compounds	Structure	Molecular weight
13-Deoxybaccatin III (I)		570
Baccatin VI (II)		714
Taxchinin I (III)		734
2α,5α,10β,14β-Tetraacetoxy-4(20),11-taxadiene (IV)		504
1-Deoxybaccatin VI(V)		698
Taxayuntin C (VI)		776

Fig. 1. The structures of compounds I~VI and their molecular weights.

identical to those of previous studies.[1-6] During the plant cell culture of *T. chinensis* to produce paclitaxel, it was found that compounds **I** and **IV** were detected as major products aside from paclitaxel. 13-Deoxy bacctin III (**I**) was suggested as an intermediate product to paclitaxel formation by plant cell culture and was also used as an important starting material through the semi-synthesis of paclitaxel. 7-TES baccatin III could be synthesized from compound **I**, and the 7-TES baccatin III again an intermediate material for the synthesis of paclitaxel.[1]

Table 1 shows the concentration of compounds **I** and **IV** in relation to the concentration of paclitaxel through biosynthetic pathway at the end of the plant cell culturing ca. 55 days. Their concentrations were evaluated via HPLC analysis where they were quantitatively determined for each batch, though their exact time course study on the ratio of these compounds was not performed. However, it could generally be recognized that high amount of compound **IV** formed at low concentration of paclitaxel. The lowest amount (38.2 ppm) of compound **I** showed at the paclitaxel concentration of 42.2 ppm. But compound **I** seemed to be formed similar amount in all fermented batches, exceptionally the batch No.3. Compound **I** used for semi-synthesis of paclitaxel and its purification yield conducted up to 66% in large-scale process. Biosynthetic pathway of Baccatin III and paclitaxel are now investigating whether Baccatin III formed from compound **I** via enzymatic

Table 1. Concentration of paclitaxel, 13-deoxy baccatin III (I), and 2α,5α,10β,14β-tetraacetoxy-4(20),11-taxadiene (IV) at the end of plant cell culture of *Taxus chinensis*.

Batch No.	Paclitaxel [ppm]	Comp. **I** [ppm]	Comp. **IV** [ppm]
1	19.7	78.2	133.0
2	38.9	111.0	137.4
3	42.2	38.2	33.4
4	73.4	101.6	22.3
5	110.3	107.0	38.7
6	119.9	141.5	39.7
7	153.3	81.4	50.3
8	166.0	105.7	83.7
9	173.1	134.9	61.7

oxidation, for example cytochrom 450, and the Baccatin III is combined with sidechain-methylester (*N*-benzoyl-3-phenyl-isoserine-methylester) to paclitaxel, successively.

A cell culture of *T. chinensis* was established to produce compound **IV** at a 2.6% (dry weight) yield.[6] Yield of compound **IV** showed ca. 75% through the whole procedure of purification from the biomass of *T. chinensis* in a large-scale process. 2α,5α,10β,14β-Tetraacetoxy-4(20),11-taxadiene (**IV**) is obviously a physiologically active substance which has a NGF activity and the effect of enhancing the activity of NGF.[8] At C-14 the oxygenated taxoides such as compound **IV** exhibited poor cytotoxicities, presumably due to the lack of C-13 side chain and oxetane ring which are necessary for the tubulin binding.[11] However, a precise NGF like activity of the compound **IV** has to be investigated further in detail.

Compounds **II** and **V** were also previously described to be synthesized with a β-lactam into paclitaxel analogues, which were potent cytotoxic agents useful for reversing or inhibiting the tumor growth.[12]

The separated diterpene compounds **I, II,** and **V** could be applied as cytotoxic agents after the semi-synthesis into paclitaxel analogues, while compound **IV** could be applied as an NGF substance.

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