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Isolation, Purification, and Identification of Taxol and Related Taxanes from Taxol-Producing Fungus *Aspergillus niger* subsp. *taxi* S

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The content of taxol in the bark of *yews* is very low, and this is not affordable from the environmental point of view. Thus, it is a necessity to look for alternative sources of taxol production to solve its supply. Currently, a large portion of the taxol in the market comes from chemical semi-synthesis, but the semi-synthetic precursors such as baccatin III and 10-deacetyl-baccatin III are extracted from needles and twigs of yew trees. Taxol-producing fungi as a renewable resource is a very promising way to increase the scale of taxol production. Our group has obtained a taxol-producing endophytic fungus, *Aspergillus niger* subsp. *taxi* HD86-9, to examine if *A. niger* can produce the taxanes. Six compounds from the fermentation broth of strain HD86-9 were isolated and identified by ^{1}H NMR, ^{13}C NMR, and ESI-MS. The results showed that the six compounds included four taxane diterpenoids (taxol, cephalomannine, baccatin III, and 10-deacetyl-baccatin III) and two non-taxane compounds (β-sitosterol and flavonoid isovitexin). The study verified that the taxanes can be produced by the *A. niger*, which is very important to taxol production via chemical semi-synthesis. Additionally, the finding is potentially very significant to solve the taxol semi-synthetic precursors extracted from needles and twigs of *yew* trees, and the precursor production can be easily increased through the culture condition optimization, genetic breeding, and metabolic engineering of the *A. niger*.

Keywords: Endophytic fungi, *Aspergillus niger* subsp. *taxi*, taxol, taxane diterpenoids, normal and reverse-phase chromatography

Introduction

Paclitaxel (Taxol) is a promising antitumor agent with poor water solubility. Taxol, the first of a new class of microtubule stabilizing agents, has been hailed by the National Cancer Institute as the most significant advance in chemotherapy of the past 30 years. Taxol was isolated in the early 1960s from the bark of *Pacific yew* (*Taxus brevifolia*; family Taxaceae), one of the geographical varieties of *yew* [1]. Taxol was obtained in a pure form in 1969 and its structure was published in 1971, after many complexities due to its low concentration and structure complexities [1]. Taxol is a diterpenoid pseudoalkaloid having molecular formula $C_{47}H_{51}NO_{14}$, corresponding to a molecular weight of 853 Da. Taxol is a white to off-white crystalline powder. It is highly lipophilic, insoluble in water, and melts at around 216–217°C. The UV spectral λ_{max} is 273 nm, and the IR spectrum displays a broad peak in the range of 3,336–3,436 cm^{-1}.

Although the extraction of taxol has increased yields to 0.04% (w/w), four trees have to be sacrificed to produce 2 g of the drug for the chemotherapy of one patient [2]. Since the content of taxol in this original source is very low, this is not affordable from the environmental point of view. Thus, there is a need to look for alternative sources of taxol production to solve its supply problem. Among them, total synthesis of taxol was achieved but is far from being commercially cost-efficient [3]. An appropriate solution,

thereby, seems to be a semi-synthesis, which involves extraction of taxol from needles and twigs of more abundant English *yew* trees, or Chinese red bean *yew* trees [4, 5]. The semi-synthesis is a process of joining the side chains to natural taxol precursors like baccatine III, 10-deacetyl-baccatine III, 10-deacetylpaclitaxel (10-DAT), cephalomannine, 7-epi-10-deacetylpaclitaxel (7-epi-10-DAT), and 7-xyl-10-deacetylpaclitaxel (7-xyl-10-DAT) isolated from plant sources [6–9]. Although this approach and subsequent plant cell culture-based production efforts have reduced the need for harvesting the *yews*, the production of taxol still heavily depends on inefficient plant-based processes, with accompanying limitations on productivity [10]. In addition, these production methods also constrain the number of taxol derivatives that can be synthesized in the search for more efficacious drugs [11]. Recently, many studies have demonstrated that taxol-producing endophytic fungi fermentation is a feasible and efficient approach to the production of taxol [10].

Taxus species are hosts to endophytic fungi, defined as organisms that inhabit plants without causing disease. *Taxus* endophytic fungi have been shown to synthesize taxol with a second potential biosynthetic route for this protective metabolite [12–14]. A variety of endophytic fungi that produce taxol have been isolated from different plant species [15–21]. Since 1993, we have screened samples from the inner bark (phloem-cambium) and xylem of *Taxus cuspidata* and isolated five new endophytic fungal species that can produce taxol. These fungi are *Nodulisporium sylviforme* [22], *Pleurocytospora taxi* [23], *Alternaria taxi* [24], *Botrytis* [25], and *Aspergillus niger* subsp. *taxi* [26]. However, the levels of taxol production by these fungi are too low to be practical for commercialization.

Although many studies have described that taxol can be produced by endophytic fungi, most of these studies have not addressed the presence of other bioactive compounds extracted from endophytic fungi, such as taxol precursors baccatine III, 10-deacetylbaccatine III, 10-DAT, cephalomannine, *etc*. Nowadays, precursors for semi-synthesis of taxol are mainly extracted from the needles of various *Taxus* species. In addition, the content of taxanes in various *Taxus* species is very low [27]. Lots of methods have been tried to address this issue, including searching for the analogs of taxol and alternate precursors for taxol semi-synthesis. Some studies have found that a few taxol-producing fungi can also produce taxanes such as baccatin III, 10-DAT, and cephalomannine [28–30]. Thus, to examine if the taxol-producing fungus *A. niger* subsp. *taxi* HD86-9 can also produce the taxanes, we isolated and purified compounds from the fermentation broth of strain HD86-9, and the structure of the isolates was identified by ^{1}H NMR, ^{13}C NMR, and ESI-MS. The study indicated that the taxol and taxanes can be produced by the *A. niger* subsp. *taxi* taxol-producing fungus. No reports are found on the taxanes produced by *A. niger* currently. Additionally, the finding is potentially very significant for taxol production via semi-synthesis, which solves the problem of the taxol semi-synthetic precursors having to be extracted from needles and twigs of *yew* trees.

Materials and Methods

Strain

Aspergillus niger subsp. *taxi* HD86-9, a taxol-producing endophytic fungus with a taxol output of 273.46 μg/l, was isolated from *Taxus cuspidata* Sieb. et Zucc [26]. *A. niger* subsp. *taxi* HD86-9 was deposited in the China Center for Type Culture Collection (CCTCC M 206137, Beijing, China).

Preparation and Isolation of Fermentation Broth from Strain HD86-9

After the activation of strain HD86-9 on potato dextrose agar medium composed of peeled and sliced potato 200 g/l, glucose 10 g/l, and agar 15 g/l at 28°C for 3 days, it was transferred to S-7 fermentation medium composed of sodium acetate 1.0 mg/l, zinc sulfate 2.5 mg/l, cupric nitrate 1.0 mg/l, magnesium sulfate 3.6 mg/l, ferric chloride 2.0 mg/l, manganese chloride 5.0 mg/l, lime nitrate 6.5 mg/l, sodium benzoate 10 mg/l, glucose 1.0 g/l, sucrose 6.0 g/l, fructose 1.0 g/l, and peptone 1.0 g/l and cultured at 28°C with centrifugation at 38 ×*g* for 12 days. The fermentation broth was extracted with an equal volume mixture of ethyl acetate and methanol (v:v = 1:1) for 3 h. The organic phases were extracted and collected by a separating funnel. The organic solvents were evaporated under reduced pressure using a rotary evaporator at 45°C to give 1.45 g of dry sample.

Isolation and Purification of Taxol and Related Taxanes from Strain HD86-9

Isolation by normal-phase chromatography. Firstly, the sample was fully dissolved in chloroform, and silica gel (80–100 mesh) was added to the sample solution and stirred for several minutes. After the solvent evaporated, the resulting solid was ground into a powder that can be subjected to column chromatography. The prepared 1.45 g sample was loaded onto a silica gel column (2.5 × 60 cm, 200–300 mesh silica gel), and eluted with gradient chloroform-methanol system (99:1, 98:2, 87:3, 90:10, 60:40) at a flow rate of 1.0 ml/min. The silica gel column was eluted with 2–3 column volumes of each gradient, and fractions of 5–10 ml were collected in tubes. A total of 237 fractions were collected and analyzed by thin-layer chromatography (TLC) for rapid qualitative detection. A 100 μg/ml solution of taxol was used as the standard for TLC analysis. The TLC plates were developed in a solvent

mixture of chloroform:methanol (7:1), and the chromogenic agent was 10% sulfuric acid/ethanol. The standard appeared as a deep purple spot on the stained TLC plate. The fungal taxol and its analogs were identified by comparison with the standard taxol on the TLC plates. The compound possessing the same or similar R_f value to the standard taxol was considered as taxol or its analog. On the basis of the TLC analyses, taxol and its analogs were detected in factions from the gradient eluents chloroform:methanol = 98:2 and chloroform:methanol = 87:3, and there was no desired product in fractions from the other three gradient eluents. The fractions having the same R_f value were combined. After evaporation of the solvent under reduced pressure, five components (A, B, C, D and E) with different R_f values were obtained.

Column chromatography of component A. The component A was dissolved in petroleum ether and subjected to normal-phase column chromatography (1.5 × 25 cm, 100–160 mesh silica gel). The column was eluted with a gradient eluent of petroleum ether and acetone (10:1, 8:1, 6:1, 5:1, 4:1, 2:1) at a flow rate of 1.0 ml/min. A total of 60 fractions were collected and analyzed by TLC analysis. The fractions having the same R_f value were combined for concentration. The fractions from eluent petroleum ether and acetone (6:1) showed a red spot on stained TLC, and the fractions from eluent petroleum ether and acetone (4:1) appeared as a dark purple spot. After evaporation of the solvent under reduced pressure, coarse crystals were precipitated from these two fractions. These coarse crystals were repeatedly washed with acetone and recrystallized at 4ºC to yield white needle-like crystals. After drying under vacuum for 24 h, compound I and compound II were obtained as white needles. The purity of isolated compounds was determined using high performance liquid chromatography (HPLC; Waters 2695, USA) analysis. The analytic sample was prepared by dissolving 0.5 mg of compound in 10 ml of HPLC-grade acetonitrile. An analytic column (C_{18}, 4.6 × 250 mm, 5 μm) and gradient elution were used for the analysis. The mobile phase was acetonitrile-water at a flow rate of 1.0 ml/min. The volume of sample injected was 10 μl, and the sample was detected at 227 and 254 nm by a dual wavelength detector. The calculated purity of each compound was more than 95% as determined by HPLC analysis.

Column chromatography of component B. The component B was dissolved in *n*-hexane and subjected to normal-phase column chromatography (2.0 × 40 cm, 100–160 mesh silica gel). The column was eluted with a gradient eluent of *n*-hexane and ethyl acetate (90:10, 70:30, 55:45, 50:50, 100%) at a flow rate of 1 ml/min. A total of 140 fractions were collected and analyzed by TLC. The fractions from eluent *n*-hexane and ethyl acetate (55:45) showed a dark purple spot, suggesting this fraction could be terpenes. Compared with the taxol standard, this fraction had a different R_f value. This fraction was analyzed by HPLC using a C_{18} column (4.6 × 250 mm, 5 μm), acetonitrile:water = 47:53 as the mobile phase at a flow rate of 1.0 ml/min, and a detection wavelength at 227 nm. Compared with the taxol standard, this fraction had a different peak retention time on HPLC, indicating this compound could be a taxol analog rather than taxol. After normal-phase chromatography, fractions having the same R_f value were combined. As a result, five new components (Fr.B1, Fr.B2, Fr.B3, Fr.B4, and Fr.B5) were obtained from component B. Fr.B3 was further purified by semi-preparative high performance liquid chromatography and detected at wavelength 227 nm. The mobile phase was acetonitrile-water (55:45) at a flow rate of 3.0 ml/min, and the injection volume was 5 μl. Fractions were collected based on peak retention time. After the purification by semi-preparative HPLC, the compound III was obtained with a purity greater than 97%.

Isolation by reversed-phase chromatography. Since the fraction Fr.B4 and component C had the same color and R_f value on TLC, the fraction Fr.B4 and component C were combined and subjected to reversed-phase column chromatography (2 × 30 cm). The column was eluted with a gradient acetone-water system (20 %, 40%, 52%, 60%, and 100%) at a flow rate of 3.0 ml/min. A total of 120 fractions were collected and analyzed by TLC. No desired product was detected in the fractions from eluent of 20% and 40% acetone content. Next, we focused on component D. Based on the results of TLC analysis, we isolated a compound that had an identical R_f value and stain color on TLC compared with those of the taxol standard. This isolated compound was further identified and confirmed by HPLC analysis. An HPLC column (C_{18}, 4.6 × 250 mm, 5 μm) was used, and the sample was detected at the wavelength of 227 nm. The mobile phase was acetonitrile:water = 47:53 at a flow rate of 1.0 ml/min. The result of HPLC analysis showed the isolated compound and the standard taxol had an identical peak retention time. Thus, the isolated compound IV was confirmed as taxol. Another compound, V, which appeared as a blue spot on stained TLC, was also isolated from the component D by reversed-phase chromatography. Based on the result of TLC analysis, we inferred that compound V might be an analog of taxol. Finally, compounds IV and V were further purified by semi-preparative HPLC followed by recrystallization at 4°C to provide white needle crystal compounds IV and V.

Next, we purified component E by reversed-phase chromatography. The sample was loaded on a reversed-phase column (2 × 30 cm) and eluted with gradient methanol-water (20%, 40%, 60%, and 100%) at a flow rate of 3 ml/min. A total of 100 fractions were collected and analyzed by TLC. A desired product, which appeared as a large and dark spot on TLC, was detected in the fractions from eluent of 40% methanol. This isolated compound was named as compound VI, which was further purified by semi-preparative HPLC (C_{18}, 4.6 × 250 mm, 5 μm) and detected at the dual wavelength of 210 and 280 nm. The mobile phase was methanol-water at a flow rate of 1.0 ml/min. After optimizing the chromatographic conditions, we selected 38% methanol-water as the mobile phase, a flow rate of 3.0 ml/min, and the detection wavelength of 270 nm. Fractions were collected based on the peak retention time. The collected fractions were concentrated and recrystallized to give the pure product of compound VI.

Identification of Taxol and Related Taxanes from Strain HD86-9 by MS and NMR

The structure of taxol and related taxanes was confirmed with a Waters triple quadrupole tandem LC-MS system (Waters, USA). Samples were analyzed by the direct infusion electrospray ionization (ESI) method in the positive-ion mode. Samples were dissolved in acetonitrile prior to infusion into the ESI source at a flow rate of 5.0 µl/min. The MS scanning ranged from 100 to 1,000 *m/z*, and the shell gas (N_2) and assistant gas (N_2) were 65 international units (IU) and 20 IU, respectively. The discharge current was 5 µA. The evaporator and capillary temperatures were 465°C and 180°C, respectively.

Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker Avance 400 MHz NMR system. The NMR spectra were performed in $CDCl_3$ using tetramethylsilane as an internal standard.

Results

Identification of Compound I

The molecular weight of compound I was determined as 414 based on the observed molecular ion peak at *m/z* 437 $[M+Na]^+$ in ESI-MS. The molecular formula of compound I was determined as $C_{29}H_{50}O$ from the combined analysis of ESI-MS, ^1H NMR, and ^{13}CNMR data (Figs. S1A–S1C and Table S1). Based on the results from the ^1H NMR and ^{13}C NMR data of compound I and literature references [31], we determined compound I as β-sitosterol. It is a white powder and soluble in methanol. The chemical structure of β-sitosterol is shown in Fig. 1A. We obtained 3.4 mg of β-sitosterol from a 54 L fermentation broth of strain HD86-9, and the yield of β-sitosterol was 62.96 ± 7.62 µg/l.

Identification of Compound II

The molecular weight of compound II was determined as 544 based on the observed molecular ion peak at *m/z* 567 $[M+Na]^+$ in ESI-MS. The molecular formula of compound II was determined as $C_{29}H_{36}O_{10}$ from the combined analysis of ESI-MS, ^1H NMR, and ^{13}C NMR data (Figs. S2A–S2C and

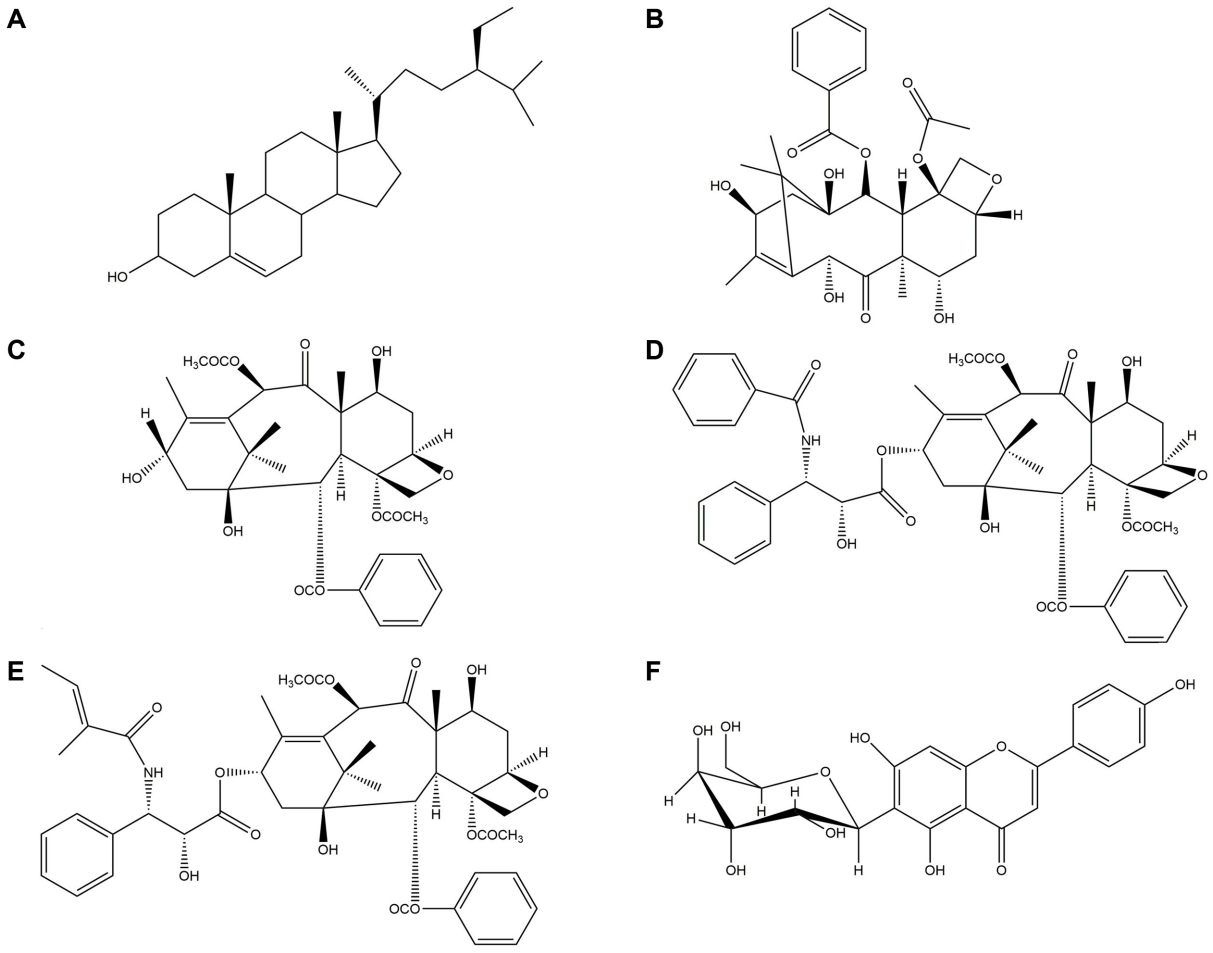

Fig. 1. Chemical structure of six compounds extracted from *Aspergillus niger* subsp. *taxi* strain HD86-9.
(**A**) β-Sitosterol; (**B**) 10-Deacetyl-baccatin III; (**C**) Baccatin III; (**D**) Taxol; (**E**) Cephalomannine; (**F**) Flavonoid isovitexin.

Table S2). According to the results of the ^{1}H NMR and ^{13}C NMR data and literature references [32], we determined compound II to be 10-deacetyl-baccatin III, which is white needle in form and soluble in acetone. The chemical structure of 10-deacetyl-baccatin III is shown in Fig. 1B. We obtained 16.3 mg of 10-deacetyl-baccatin III from a 54 liter fermentation broth of strain HD86-9, and the yield of 10-deacetyl-baccatin III was 301.85 ± 29.85 μg/l.

Identification of Compound III

The ESI-MS *m/z* $[M+H]^+$ ion peak was observed at 587, and the $[M+Na]^+$ ion peak was observed at 609, indicating that the molecular weight of the compound III was 586. The ESI-MS, ^{1}H NMR, and ^{13}C NMR data of compound III in $CDCl_3$ can be seen in Figs. S3A–S3C and Table S3. Compared with the spectrum of taxol, the typical AB system peaks at H-9 and H-10 disappeared, indicating the presence of a ketone group at the C-9 position. Based on the results from the ^{1}H NMR and ^{13}C NMR data and literature references [33], we determined compound III to be baccatin III with a molecular formula of $C_{31}H_{38}O_{11}$, and it is white needle-like crystals and soluble in acetone. The chemical structure of baccatin III is shown in Fig. 1C. We obtained 21.8 mg of baccatin III from a 54 liter fermentation broth of strain HD86-9, and the yield of baccatin III was 403.70 ± 24.12 μg/l.

Identification of Compound IV

The ESI-MS *m/z* molecular ion $[M+H]^+$ peak was observed at 854, and the $[M+Na]^+$ ion peak was observed at 876, indicating that the molecular weight of compound IV is 853. The ESI-MS, ^{1}H NMR, and ^{13}C NMR data of compound IV in $CDCl_3$ can be seen in Figs. S4A–S4C and Table S4. Based on the results from ^{1}H NMR and ^{13}C NMR data of compound IV and literature references [34], we determined that compound IV was taxol with a molecular formula of $C_{47}H_{51}NO_{14}$, and it is a white powder. The chemical structure of compound IV is shown in Fig. 1D. We obtained 16.7 mg of taxol from a 54 liter fermentation broth of strain HD86-9, and the yield of taxol was 309.36 ± 31.46 μg/l.

Identification of Compound V

ESI-MS *m/z*: The presence of a molecular ion peak at 832 $[M+H]^+$ indicated that the molecular weight of compound V was 831. The ESI-MS, ^{1}H NMR, and ^{13}C NMR data of compound V in $CDCl_3$ can be seen in Figs. S5A–S5C and Table S5). Compound V contains two phenyl rings. According to the results and and literature references [33], we determined that compound V was cephalomannine, and it is a white powder, with the molecular formula $C_{45}H_{53}NO_{14}$. The chemical structure of compound V is shown in Fig. 1E. We obtained 14.0 mg of cephalomannine from a 54 L fermentation broth of strain HD86-9, and the yield of cephalomannine was 259.26 ± 17.34 μg/l.

Identification of Compound VI

ESI-MS *m/z*: The molecular ion peak was observed at 433 $[M+H]^+$, indicating that the molecular weight was 432. The calculated degree of unsaturation was 12. The results of testing by ferric chloride, hydrochloric acid/magnesium, and Molich's reagent were all positive, indicating that compound VI was a flavonoid glycoside. The ESI-MS, ^{1}H NMR, and ^{13}C NMR data of compound IV in $CDCl_3$ can be seen in Figs. S6A–S6C and Table S6. According to the results and literature references [35], we determined that compound VI was the flavonoid isovitexin, and it is a white powder, with molecular formula $C_{45}H_{53}NO_{14}$. The chemical structure of compound VI is shown in Fig. 1F. We obtained 3.2 mg of flavonoid isovitexin from a 54 L fermentation broth of strain HD86-9, and the yield of flavonoid isovitexin was 59.26 ± 8.31 μg/l.

Discussion

In the present study, taxol and its taxanes from the fermentation broth of the fungus *A. niger* HD86-9 were isolated and identified. According to the results of ^{1}H NMR, ^{13}C NMR, and ESI-MS and literature references [31–35], these compounds are taxane diterpenoids (taxol, cephalomannine, baccatin III, and 10-DAB) and non-taxane compounds (β-sitosterol and flavonoid isovitexin), respectively. Cephalomannine and baccatin III have a similar typical UV spectral λ_{max} at 280 nm; the UV spectral λ_{max} of 10-DAB is 233 nm, and the FT-IR spectra demonstrate a -OH stretching frequency at 3,466 cm^{-1} [36] and three C=O stretching frequencies at 1,723, 1,711, and 1,691 cm^{-1} [37]; the UV spectral λ_{max} of β-sitosterol and flavonoid isovitexin are 270.0 and 336.9 nm, respectively [38].

These taxanes have basic 6-8-6-type taxane skeletons and common substituents, such as acetyl, benzoyl, cinnamoyl, winterstein moiety, *N*-benzoyl-3′-phenyl-isoserinyl, and *N*-benzoyl isoserinyl. Among them, acetyl is the most common substituent in taxanes and generally appears at δ2.24–2.38 as a sharp singlet in the ^{1}H NMR spectrum. Benzoyl-3′-phenyl-isoserinyl is only observed in taxol and its derivatives so far. Taxanes have characteristic signals of four tertiary methyl groups. The signal of the tertiary methyl group at 18-position is coupled with H-13β through a double bond and appears in higher field (δ1.79–2.07) as a

doublet or multiplet. On the other hand, the signals of methyl groups at the 16, 17, and 19 positions appear respectively at δ1.11–1.26, δ1.11–1.15, and δ1.67–1.69 as singlets. Olefinic proton H-10α is quite characteristic and usually appears at δ6.27–6.32 as a singlet. The signal of methane proton H-3α in taxanes appears at δ3.78–3.88 as a doublet with a coupling constant of 6.8–7.0 Hz.

The taxol pathway is the benzoylation of the taxane C-2 hydroxyl group of an advanced intermediate, whereas the third acylation reaction is considered to be the acetylation of the C-10 hydroxyl group of the advanced metabolite 10-DAB to yield baccatin III. 10-DAB and baccatin III are isolates of the Pacific yew tree and related species, and are intermediates in the biosynthesis of taxol and useful precursors for semi-synthesis of the drug. 10-Deacetylbaccatin III-10β-*O*-acetyltransferase (10-DBAT) catalyzes the conversion of 10-DAB to baccatin III in the presence of acetyl-coenzyme A in *Taxus* species. The reaction from 10-DAB to baccatin III is a key step, because the supply of taxol is currently largely sustained by semi-synthetic means in which 10-DAB isolated from yew needles is used to produce taxol. Semi-synthetic production of baccatin III involves protection of the 7-hydroxyl of 10-DAB and chemical acetylation of the 10-hydroxyl to give 7-*O*-protected baccatin III. This method involves many steps and is expensive. However, a biosynthetic approach to yield taxol by using enzyme catalysis can eliminate the complicated steps and reduce production costs. The gene encoding 10-DBAT can be strongly expressed in a soluble and active form; hence the use of a biosynthetic approach to produce baccatin III is feasible, and the gene 10-DBAT has been successfully cloned.

The introduction of taxol biosynthesis genes and expression in *Escherichia coli* was a great achievement towards genetic engineering of endophytes for taxol production [3]. Although the transformation and genetic engineering of endophytes for production of taxol remain a significant challenge, poor understanding of the regulation of the biosynthesis that is a prerequisite to enhanced production in microbes is among the limitations to the achievement. Present evolution and understanding of microbial genomics opens up the possibility to develop high-yielding genetically engineered microbes for production of taxol and its taxanes at industrial scale [3, 39]. The result revealed one important fact, and that is the presence of taxol and its taxanes in the *A. niger* were confirmed, which is very significant to solve the problem of the taxol semi-synthetic precursors having to be extracted from needles and twigs of yew trees. Currently, a large portion of the taxol in the market comes from semi-synthesis. Therefore, it is possible to increase production of taxol and taxol semi-synthetic precursors from *A. niger* through optimization of the culture condition, breeding techniques, gene engineering, and metabolic engineering, along with efficient extraction and separation methods. Taken together, the taxol-producing fungus *A. niger* subsp. *taxi* HD86-9 shows great potential in the development of biotechnology-based systems for the production of taxol and its semi-synthetic precursors.

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