

## Production, Purification, and Characterization of Taxol and 10-DABIII from a new Endophytic Fungus *Gliocladium* sp. Isolated from the Indian Yew Tree, *Taxus baccata*

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**We have isolated endophytic fungi from the Indian yew tree, *Taxus baccata*, and then screened for taxol production. Out of the 40 fungal cultures screened, one fungus *Gliocladium* sp. was found to produce taxol and 10-DABIII (10-deacetyl baccatin III). These compounds were purified by TLC and HPLC and characterized using UV-spectroscopy, ESI-MS, MS/MS, and proton NMR. One liter of *Gliocladium* sp. culture yielded 10 µg of taxol and 65 µg of 10-DABIII. The purified taxol from the fungus showed cytotoxicity towards cancer lines HL-60 (leukemia), A431 (epidermal carcinoma), and MCF-7 (breast cancer).**

**Keywords:** Taxol, *Taxus baccata*, endophytic fungi, *Gliocladium* sp., 10 DAB III

Paclitaxel (Taxol), a tubulin-binding diterpenoid, was first isolated from the Pacific yew tree *Taxus brevifolia* [27]. Because of its ability to bind specifically to  $\beta$ -tubulin and its cytotoxicity at lower concentrations, it is being used for the treatment of several classical tumors [1, 8–10, 20]. In comparison with other antineoplastic agents such as Vinca alkaloids and colchicine, its binding dynamics with tubulin are peculiar; the former enhances microtubule disassembly whereas taxol promotes assembly and stabilization [19]. Owing to the complex structure and limited supply of Taxol, scientists have been posed with the difficulty of finding an alternative potential source of this compound [6]. A number of methods for the production of Taxol including tissue culture of *Taxus* sp. [13], extraction from endophytic fungi [21], and chemical synthesis [17] have been reported. However, these procedures are either low yielding or very complex and tedious.

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India has a large wealth of medicinal plants with abundance of *Taxus baccata*, hence a screening programme was initiated to isolate endophytic fungi from *Taxus baccata* that produce taxol. Forty slow-growing, few nonsporulating, and uncommon endophytic fungi were isolated from *Taxus* bark, stem, and leaves (needles) and brought to pure culture state. In this communication, the production, purification and characterization of Taxol and its useful precursor 10-deacetyl baccatin III (10-DABIII) from an endophytic fungus identified as *Gliocladium* sp. is reported. Our report for the first time discovers *Gliocladium* sp. as an endophyte of *Taxus baccata*, which is otherwise usually isolated as a mycoparasite or as a saprophyte [2]. There are several reports of bioactive molecules including nematicidal compounds isolated from this genus [14], but our studies revealed that the fungus is not only established as an endophyte, but is also successful in mimicking the host chemical diversity.

### MATERIALS AND METHODS

A novel endophytic fungus *Gliocladium* sp. having optimum growth at pH 7 and 25°C was isolated from *Taxus baccata* tree growing in West Bengal, India. This fungus was maintained on potato dextrose agar (PDA) slants at 15°C.

#### Production and Isolation of Taxol from *Gliocladium* sp.

Production of Taxol by the fungus was studied by a two-stage fermentation procedure.

In the first stage, a 500 ml Erlenmeyer flask containing 100 ml modified mycological medium was inoculated with agar plugs, containing mycelium from 7-day-old slants and incubated at 25–27°C on a rotary shaker (240 rpm) for 5 days. This culture was used as seed culture for the second stage. For Taxol production, 10 ml of seed culture was transferred to 500-ml flasks containing 100 ml of modified S7 medium and incubated at 25–27°C for 21 days as stationary culture (second stage). As reported earlier, modified mycological medium

and S7 medium were used for maximum production of Taxol [21]. After 3 weeks of incubation, the culture was harvested, lyophilized to dryness, and extracted with three equal volumes of chloroform:methanol (9:1) each time. The extracts were pooled, concentrated at 40°C *in vacuo*, and subjected to thin-layer chromatography (TLC) on silica gel G (0.5 mm thickness) using a chloroform:methanol (7:3) mobile phase. The TLC plates when sprayed with anisaldehyde-sulfuric acid reagent or vanillin sulfuric acid reagent showed dark blue spots that later turned to gray [26]. The putative Taxol was partially purified on silica gel column pre-equilibrated with chloroform, and eluted with a gradient of chloroform:acetonitrile (chloroform 100, 75:25, 50:50, 25:75, 100% acetonitrile). Fractions containing compounds with an  $R_f$  value similar to that of the standard Taxol were pooled and subjected to preparative TLC on a 0.25-mm thick (20 cm×20 cm) silica plate and developed in solvent system chloroform:acetonitrile (7:3). The putative Taxol band was eluted with chloroform:methanol (9:1).

#### TLC, UV, and HPLC

Purity of the isolated compound was checked on TLC in different solvent systems such as (A) chloroform:acetonitrile (7:3); (B) chloroform:methanol (7:1), and (C) ethylacetate:2-propanol (95:5), and spots were detected using anisaldehyde-sulfuric acid reagent or vanillin sulfuric acid reagent [26]. The absorption maximum of the purified compound was determined by a Shimadzu PC 101 spectrophotometer. Sample was dissolved in HPLC-grade methanol and spectral data were collected over a 200 to 450 nm range. The purity and characteristics of putative Taxol were determined by HPLC using a C18 Symmetry column (Waters). Sample was taken in 10- $\mu$ l chloroform, injected in the HPLC column, and gradient elution was performed using 25% to 95% acetonitrile at a flow rate of 0.5 ml/min. A dual-wavelength recorder set at 227 and 254 nm was used to detect the compounds eluting from the column [16].

#### ESI-MS, MS/MS, and <sup>1</sup>H NMR

Molecular mass of the purified compound was determined by an M/S Applied Biosystems API QSTAR pulsar (ESI-MS) mass spectrometer. Samples for the analysis were dissolved in HPLC-grade methanol:water:acetic acid in the ratio of 50:50:0.1. Samples were then analyzed by infusion method/(injected into MS) at a flow rate of 5  $\mu$ l/min and at an IS voltage of 3,800 V in TOF mode. Spectra from a range of  $m/z$  500 to 900 Da were obtained. <sup>1</sup>H NMR analysis was carried out on a Bruker AV 400 spectrophotometer at 400 MHz. The NMR spectrum was measured with a spectral width of 8223.68 and data were acquired into 32 K data points. An acquisition time of 1.9 s and a relaxation delay of 1.0 s were used. Samples were then dissolved in CDCl<sub>3</sub> (deuteriochloroform) and scanned overnight.

#### Cytotoxicity of Fungal Taxol

Cytotoxicity of the compound on cell lines HL-60 (leukemia), A431 (epidermal carcinoma), and MCF-7 (breast cancer) was determined by MTT assay [25]. The cell suspension at a concentration of 1×10<sup>4</sup> cells/ml was added to 96-well microtiter plates. Culture media used for HL60, MCF 7, and A431 were RPMI 1640, MEM, and DMEM, respectively. Plates containing culture media and test compound were incubated overnight for HL60, 7–8 days for MCF, or 7 and 4 days for A431, at 37°C, 5% (v/v) CO<sub>2</sub>, and 95% humidity. All the samples were taken in triplicates. Ten  $\mu$ l of MTT reagent (5 mg/ml) was added to each well and cells were incubated for 1 h

at 37°C. At the end of this period, 200  $\mu$ l of acidified isopropanol was added and plates were incubated for 4 h to solubilize the purple formazan crystals produced. Absorbance was measured at 490 nm with a Beckman Coulter spectrophotometer.

#### Immunoassay and Quantification of Taxol

Taxol in the crude extracts was qualitatively and quantitatively determined by competitive inhibition enzyme immunoassay (CIEIA) using an immunoassay kit (TA01) purchased from Hawaii Biotechnology, Hawaii. The crude extracts were dissolved in methanol and centrifuged to remove the insoluble materials.

In brief, this assay was performed in a 96-well microtiter plate coated with Taxol-protein coating antigen. The plate was blocked with 1% (w/v) BSA in PBS. After washing, the solid-phase-bound Taxol was incubated with samples and Taxol standard and a specific anti-Taxol monoclonal antibody. The Taxol in the sample competes with solid-phase-bound Taxol for binding to the monoclonal antibody. The monoclonal antibody bound to the solid-phase-bound Taxol was detected by an alkaline phosphatase-conjugated second antibody and alkaline phosphatase substrate, *p*-nitrophenyl phosphate; the inhibition of color development was proportional to the concentration of free Taxol present in the samples. The amount of Taxol in each sample was calculated from an inhibition curve made by using different concentrations of standard Taxol supplied with the kit. This technique was used to screen for Taxol in each of the fungal extracts. The assay is sensitive to about 1 ng/ml.

#### Production and Isolation of 10-DABIII from *Gliocladium* sp.

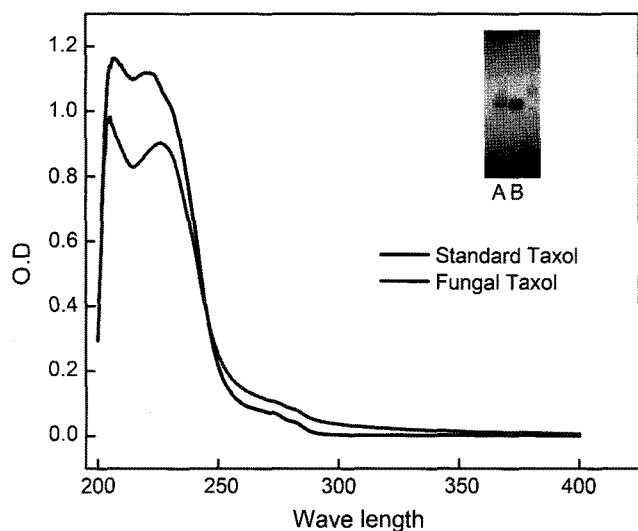
Production of 10-DABIII by the fungus was studied by a two-stage fermentation procedure as described above for Taxol production. The crude extract obtained was subjected to silica gel chromatography and eluted with a gradient of chloroform:acetonitrile as described earlier. Fractions showing 10-DABIII on TLC were pooled and subjected to further fractionation on preparative TLC using chloroform:acetonitrile (7:3) as the solvent phase. The homogeneity of 10-DABIII was checked using HPLC, and the molecular mass was determined by ESI-MS. 10-DABIII from 1 l of culture was estimated by immunoassay kit TA04 as described for Taxol estimation.

## RESULTS

#### Taxol from Culture of Fungus *Gliocladium* sp.

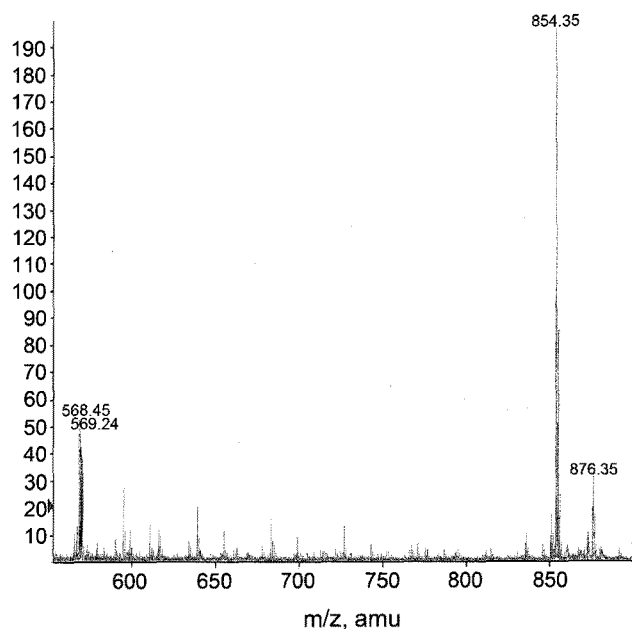
The crude fractionated sample from the silica gel column with 100% chloroform and chloroform:acetonitrile (75:25) showed partially purified compound having chromatographic properties similar to that of the standard Taxol in three different solvent systems A, B, and C on TLC. Partially purified Taxol obtained from preparative TLC showed a single dark blue spot that later turned to gray (with  $R_f$  value 0.5) when sprayed with the anisaldehyde-sulfuric acid reagent/vanillin-sulfuric acid reagent [3]. The UV absorption analysis showed a peak with absorption maxima at 227 nm (Fig. 1).

The homogeneity of the purified compound was confirmed by HPLC analysis, which showed a single symmetrical peak with a retention time of 25.5 min on the C18 symmetry



**Fig. 1.** UV absorption spectrum of standard Taxol and fungal Taxol.

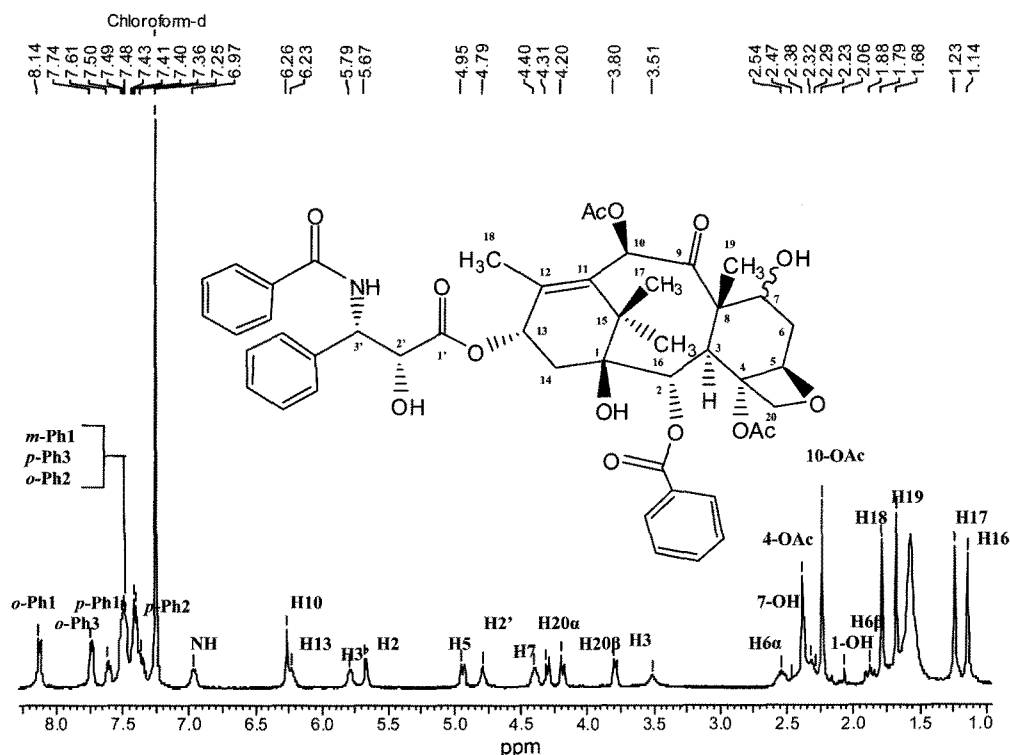
The inset shows the TLC profile of fungal Taxol purified from culture broth along with standard Taxol on silica gel G using a chloroform: acetonitrile (7:3) solvent system. A: purified fungal Taxol; B: Standard Taxol. Detection: Vanillin-sulphuric acid.



**Fig. 2.** ESI-MS spectrum of the  $m/z$  854 (M+H) and  $m/z$  876 (M+Na) ions of fungal Taxol.

column. Electrospray ionization mass spectrometry yielded a major ion at  $m/z$  854 and at  $m/z$  876. Apart from the major ions, a fragment ion at  $m/z$  569 was also seen (Fig. 2). In MS/MS, fragment ions at  $m/z$  286 and at  $m/z$  570 were seen, but the relative abundance of the same was very low.

The  $^1\text{H}$  NMR spectrum showed well-resolved signals and distributed in the region from 1.0 to 8.5 ppm. The three proton signals caused by the methyl group and acetate groups were seen in the range of 1.0 to 2.5 ppm, which include H16, H17, H18, H19, 10-OAc, 4-OAc, H14 $\alpha$ ,



**Fig. 3.**  $^1\text{H}$  NMR spectrum of fungal taxol.

H14 $\beta$ , 1-OH, 7-OH, H6 $\alpha$ , and H6 $\beta$ . Multiplets caused by certain methylene groups were also seen in this range. Protons in the taxane skeleton and the side chain, such as H10, H13, H3', H2, H5, H2', H7, H20 $\alpha$ , H20 $\beta$ , and H3, including NH and 2'-OH, were observed in the region between 2.5 and 7.0 ppm, and the aromatic proton signals caused by the C-2 benzoate, C-3' phenyl, and C-3' benzamide groups such as *o, m, p*-Ph1, *o, m, p*-Ph2, and *o, m, p*-Ph3 appeared in the region between 7.0 and 8.5 ppm (Fig. 3). The culture produced 10  $\mu$ g/l Taxol. Approximately 58% inhibition of cell proliferation was observed with 30  $\mu$ M fungal Taxol against HL-60 the leukemia cell line, 46% with 3.65  $\mu$ M fungal Taxol against the MCF-7 breast cancer cell line, and 53% with 3.65  $\mu$ M fungal Taxol against the A431 epidermal carcinoma cell line.

#### 10-DABIII from Culture of *Gliocladium* sp.

Fungal 10-DABIII was isolated from a 14-day-old culture. The crude fractions eluted with chloroform:acetonitrile (50:50 and 75:25) showed compounds similar to the standard 10-DABIII. The purified compound showed a single bluish violet spot when sprayed with vanillin-sulphuric acid and had an  $R_f$  value of 0.2. ESI-MS analysis showed molecular ions at  $m/z$  545 and at  $m/z$  567 (Fig. 4) [5] and the yield was 65  $\mu$ g/l culture.

#### DISCUSSION

Studies on the isolation of the taxanes produced by *Gliocladium* spp. indicate that the fungus produces taxanes, 10-DABIII

and Taxol, in the culture broth *in vitro*. TLC, HPLC, and other analyses of the extracts from culture broth confirm this conclusion. In addition to 10-DABIII and Taxol, this fungus also produces a few unidentified taxanes, although in trace amounts. To the best of our knowledge, this is the first report of isolation of 10-DABIII and Taxol from *Gliocladium* sp. isolated from the Indian yew tree *Taxus baccata*.

However, Wang *et al.* [26] and others [22] have isolated taxanes from different endophytic fungal species (*Taxomyces andreanae*, *Pestalotiopsis*, *Pestalotia*, *Fusarium*, *Alternaria*, and others) obtained from *Taxus* sp. that are common to Europe, Asia, and North America. These observations support the present finding. This suggests that the ability to synthesize 10-DABIII, Taxol, and their derivatives resides not only in the fungal strain isolated from *Taxus* sp. of the above-mentioned countries but also from endophytic fungi of *Taxus baccata* growing in India. There are several reports of *Gliocladium* sp. identified as mycoparasites and producing various biologically active molecules [2, 11, 12, 14]. But in our studies, for the first time, we have isolated the fungus as a Taxol-producing endophyte from *Taxus baccata*. The discovery of such a mycoparasitic fungus being an endophyte, which has a very close association with the host, might help us to understand the evolutionary aspects of gene transfer between the host and its endophyte, as many of these endophytes mimic the host chemical diversity. The ability of the fungus to produce Taxol can be correlated, as the necessary precursor molecules, such as sodium benzoate, sodium acetate, biotin, and pyridoxal required for the formation of the taxane ring

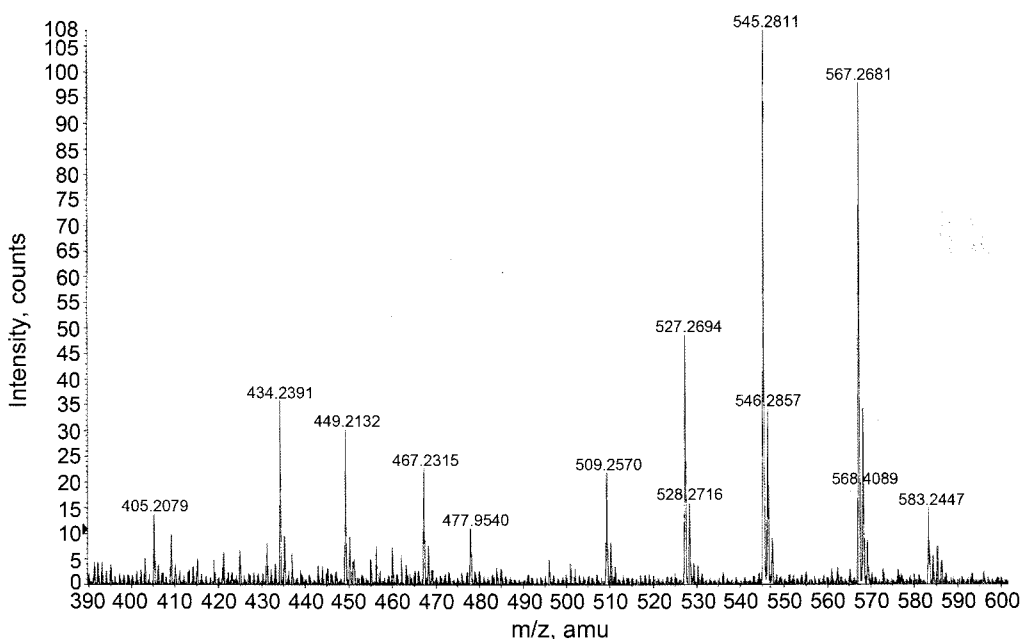


Fig. 4. ESI-MS spectrum showing ions at  $m/z$  545 and  $m/z$  567 of 10 DAB III, attributed to M+H and M+Na.

and L-phenylalanine required for formation of the side chain, were provided in the growth medium, these being essential for Taxol production as has been reported earlier [21]. Endophytic fungi producing Taxol and baccatin III in 3 weeks have been reported earlier [18], but the fungus *Gliocladium* sp. produces Taxol in 21 days; however, it does produce 10-DABIII after 14 days.

The ability of *Gliocladium* sp. to make Taxol was confirmed by isolation of a compound having chromatographic properties similar to those of standard Taxol in three solvent systems A, B, and C, and which showed a single dark bluish violet spot on TLC when sprayed with anisaldehyde reagent or vanillin-sulfuric acid reagent. Putative Taxol on HPLC C18 symmetry column showed a single symmetrical peak at retention time of 25.5 min that confirmed its homogeneity. The absorption maximum of the purified fungal Taxol was found to be at 227 nm as reported earlier [3]. In ESI-MS, molecular ions at  $m/z$  854 attributing to the  $(M+H)^+$  and at  $m/z$  876 attributable to  $(M+Na)^+$  confirmed its molecular weight to be 853 [5]. MS/MS showed fragment ions at  $m/z$  570 attributable to the taxane ring substructure and at  $m/z$  286 for the side chain substructure, which are usually seen as Taxol fragment ions [24].  $^1H$  NMR spectrum was identical with that of the standard Taxol spectrum [4]. Taxol obtained per liter of culture was estimated to be approximately 10  $\mu$ g. The concentration of Taxol produced is neither low nor high when compared with the other reports [7, 23]; however, this is the first report on Taxol from an endophyte of yew tree growing in India. The antitumor activity of Taxol was checked against the HL-60 leukemia cell line, A431 epidermal carcinoma cell line, and MCF-7 breast cancer cell line, showing 58%, 53%, and 46% with 30  $\mu$ M, 3.65  $\mu$ M, and 3.65  $\mu$ M fungal Taxol, respectively. Taxol showed less cytotoxicity on the HL-60 cell line [15]. However, in the case of MCF7 and A431, the cell-growth inhibition was significantly high even at low Taxol concentration. A number of *Taxus* spp. have been reported to produce 10-DABIII [28] and we have also isolated this compound from a 14-day-old fungal culture. ESI-MS showed a molecular ion at  $m/z$  545 and at  $m/z$  567 attributable to  $(M+H)^+$  and  $(M+Na)^+$ , inferring its mass to be 544 [5]. 10-DABIII produced was 65  $\mu$ g/l culture.

In conclusion, we have isolated an endophytic fungus *Gliocladium* sp. producing Taxol and 10-DABIII at 10  $\mu$ g/l and 65  $\mu$ g/l culture with properties and toxicity similar to that of the plant Taxol and 10-DABIII.

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