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NATURAL PRODUCTS

Isolation of an endophytic fungus producing baccatin III from *Taxus wallichiana* var. *mairei*

Jian Zaiyou · Meng Li · Xu Guifang · Zhou Xiuren

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Abstract The objective of this study was to isolate endophytic fungi producing baccatin III from yew for the purpose of baccatin III and paclitaxel manufacture. Surface sterilized bark of *Taxus wallichiana* var. *mairei* was used as source material with potato dextrose agar culture medium for isolation of endophytic fungi. Fungal cultures were extracted with a mixture of chloroform/methanol (1:1, v/v) and the baccatin III in the extracts was determined and authenticated with LC–MS. An endophytic fungus that produced baccatin III was identified by ITS rDNA and 26S D1/D2 rDNA sequencing. A total of 192 endophytic fungal strains were isolated from *T. wallichiana* var. *mairei*. Only one of the 192 strains produced baccatin III and it was identified as *Diaporthe phaseolorum*. The productivity of this strain cultured in PDA culture medium was 0.219 mg/l. The isolated endophytic fungus produced baccatin III at a relatively high level and shows promise as a producing strain for baccatin III and paclitaxel manufacture after strain improvement.

Keywords Baccatin III · Endophytic fungus · *Diaporthe phaseolorum* · *Taxus wallichiana* var. *mairei*

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J. Zaiyou (✉) · M. Li · X. Guifang · Z. Xiuren
Henan Institute of Science and Technology, Hualan road, Xinxiang, Henan, China
e-mail: jian19732004@126.com

Introduction

Paclitaxel has high activity as an anticancer agent and is widely used in hospitals and clinics. Paclitaxel is a naturally occurring chemical component that was first identified from the bark of yew trees, but is present at low levels [21]. In recent years, stands of yew trees have been destroyed or seriously damaged by harvesting to extract paclitaxel. In addition to the yew trees themselves, some fungi endophytic to yew have been found to produce paclitaxel [1, 2, 5, 10, 11, 16–18, 22], but the levels of production are too low to be useful for commercialization. Baccatin III, 10-deacetyl baccatin III, and related compounds are the precursors of paclitaxel in paclitaxel synthesis. The procedure to synthesize paclitaxel artificially from baccatin III is relatively straightforward (transfer an aminophenylpropanoyl group to C-13 of baccatin III by an *O*-(3-amino-3-phenylpropanoyl) transferase and then benzamidate and hydroxylate C-2′ of the side chain with *N*-debenzoylpaclitaxel *N*-benzoyltransferase and a taxoid side chain hydroxylase) [3, 4, 6, 8, 19]. In addition to paclitaxel, baccatin III is also present in yew, but again at very low levels. While there are numerous reports of endophytic fungi that produce paclitaxel, only a few endophytic fungi producing baccatin III have been found [7, 20]. To find endophytic fungi that can produce baccatin III, we isolated a large number of endophytic fungal strains from *Taxus wallichiana* var. *mairei* (Lemée et H. Lév.) L. K. Fu et Nan Li distributing in Taihang Mountain in Henan Province of China. Among these endophytic fungal strains, one strain that can produce baccatin III was isolated and production was authenticated with LC–MS. This discovery is potentially very significant for paclitaxel manufacture and environmental protection of yew trees.

Materials and methods

Materials

Instruments

Instruments used were HPLC instrument, Shimadzu GC-2010, LCQ Advantage LC-MS, Lectronic analytic balance (precision: 0.0001), Ultrasonator, Rotary evaporator, Superclean bench and Shake culture box.

Reagents

Reagents used were methanol (AR), ethanol (AR), acetonitrile (HPLC grade), potato dextrose agar (PDA) culture medium (prepared by us at that time), potato dextrose broth (PDB) culture medium (prepared by us at that time). Standard baccatin III (99.5 %) was purchased from Sigma-Aldrich (St. Louis, MO, USA.).

Materials

Fresh bark was collected from branches of *Taxus. wallichiana* var. *mairei* (Lemée and H. Léveillé) L. K. Fu and Nan Li, trees growing in the Taihang mountains, Henan province, China in August, 2011. The branches were 2–10 cm in diameter.

Isolation of endophytic fungus

The outer layer of fresh bark was removed with a sharp blade and then the bark was surface sterilized with 75 % (v/v) ethanol for 2 min, followed by a 0.1 % mercuric chloride solution for 8 min in super-clean bench. The surface-sterilized bark was washed three times with sterilized water for 1 min each and then cut into pieces (approximately 5 × 5 cm) with the aid of a flame-sterilized blade. Each piece was placed in a Petri dishes (8 cm in diameter) containing sterilized potato dextrose agar (PDA) culture medium for incubation at 21–22 °C for 4–6 days. The three blank cultures (uninoculated sterile medium) were taken as control. Hyphal tips of endophytic fungi that grew out from the bark were isolated with a flame-sterilized inoculating blade and sub-cultured on PDA plates to obtain isolated colonies. Each fungal culture was checked for purity.

Screening of endophytic fungi producing baccatin III

Submerged fermentation of endophytic fungi

Three to four agar plugs (approximately 4 mm in diameter) containing mycelia were inoculated into 250-ml culture flasks containing 100 ml of potato dextrose broth (PDB) with incubation at 120 rpm and 21–22 °C for 4 days, and then subcultured (5 ml of fungal liquid culture into a 250-ml culture flask containing 100 ml of PDB) with incubation at 120 rpm and 21–22 °C for 8 days. Three blank cultures (uninoculated sterile medium) were taken as control.

Preparation of extract

Fungal cultures were filtered with filter paper and the mycelia were ground in a mortar with quartz sand. The ground mycelia were dried at 45 °C, weighed, and then extracted with 30 ml of chloroform/methanol (1:1, v/v) in an ultrasonic bath for 30 min. The extraction was repeated (the mixture was filtered and the residue was extracted once again) and the pooled filtrates were evaporated to dryness under reduced pressure at 40 °C in a rotary vacuum evaporator. The dry residue was dissolved in 30 ml chloroform and then back-extracted with 30 ml of water. The organic phase was collected, evaporated to dryness at 40 °C in a rotary vacuum evaporator, and the dry residue was dissolved in 5 ml of methanol and filtered with a 0.45-μm filter. This filtrate was referred to as mycelial extract.

Filtered spent culture medium was evaporated to dryness under reduced pressure at 70 °C in a rotary vacuum evaporator and then was extracted using the same method described for ground mycelia. This filtrate was referred to as spent medium extract.

Filtered spent culture medium from yet another culture flask was evaporated to dryness under reduced pressure at 70 °C using a rotary vacuum evaporator and then was mixed with dried ground mycelia from the same culture flask and the mixture was extracted using the same method described for ground mycelia. This filtrate was referred to as whole culture extract.

Determination of baccatin III

Fungal baccatin III in the extracts was determined with high-performance liquid chromatography (HPLC). The HPLC column used was a Shimadzu C_{18} reverse-phase column (5 μm, 250 × 4.6 mm). The volume of extract injected was 10 μl, and then elution was performed with a gradient mobile phase consisting of acetonitrile and water. The content of acetonitrile in the gradient mobile phase varies as below (v/v): from 27 to 30 % in 0–15 min, 30 to 37 % in 15–30 min, 37 to 42 % in 30–40 min, 42 to 47 % in 40–60 min, and 47 to 48 % in 60–68 min. The flow rate was 1 ml/min at a temperature of 35 °C. A variable wavelength recorder set at 228 nm was used to detect ingredients eluted from the column. Standard baccatin III solutions were prepared at 0.0005, 0.001, 0.004, 0.01, 0.02,

and 0.05 mg/ml, respectively, analyzed according to the above HPLC method and peak areas of baccatin III were used to prepare a standard curve relating peak area of baccatin III to its content, using SPSS (Statistical Product and Service Solutions, Chicago, IL, USA). The content of fungal baccatin III in extracts was computed according to chromatogram peak areas using the standard curve.

Spectroscopic analysis of extracts

Collected fungal baccatin III samples from HPLC column during the retention time (begin at 32.5 min and end at 33.5 min). Electrospray ionization-tandem mass spectrometry (ESI–MS/MS) analysis was conducted on fungal baccatin III samples. ESI was used as the ion source, with scanning in the negative ion mode. The range of molecular weights scanned was 50–2,000 amu. The sample was injected with a 0.2 μl/min spray flow and a spray voltage of 5.0 kV using N_2 as the atomization gas and auxiliary gas. The temperature of the capillary cone was 350 °C.

Identification of endophytic fungi

The identification of the baccatin III producing endophytic fungus was done by Taihegene Biotechnology Co. Ltd using 26S rDNA D1/D2 sequence PCR and ITS Sequence PCR.

Results

Altogether, 192 endophytic fungal strains were isolated from *T. wallichiana var. mairei*. The retention time of a peak seen in the chromatograms of spent culture medium extracts prepared from isolate no. 79 was identical with that of standard baccatin III (Fig. 1). No peak was found at that retention time in the chromatograms of spent culture medium extracts from any of the other 191 strains or from any blank culture samples. Electrospray ionization-tandem mass spectrometry (ESI–MS/MS) analysis was then carried out on a presumptive baccatin III sample prepared from fungal isolate no. 79. In the mass spectrum of the fungal baccatin III sample, there are molecules or ions with the same molecular weight (or W + H) as that of molecules or ions in the mass spectrum of baccatin III standard. The mass spectrometry analysis of the fungal baccatin III sample confirmed that there is baccatin III in the spent PDB culture fluid of isolate no. 79 (Fig. 2). The baccatin III concentrations detected in extracts of the whole culture, the mycelium, and the spent culture medium from isolate no. 79 were 0.00438 mg/ml,

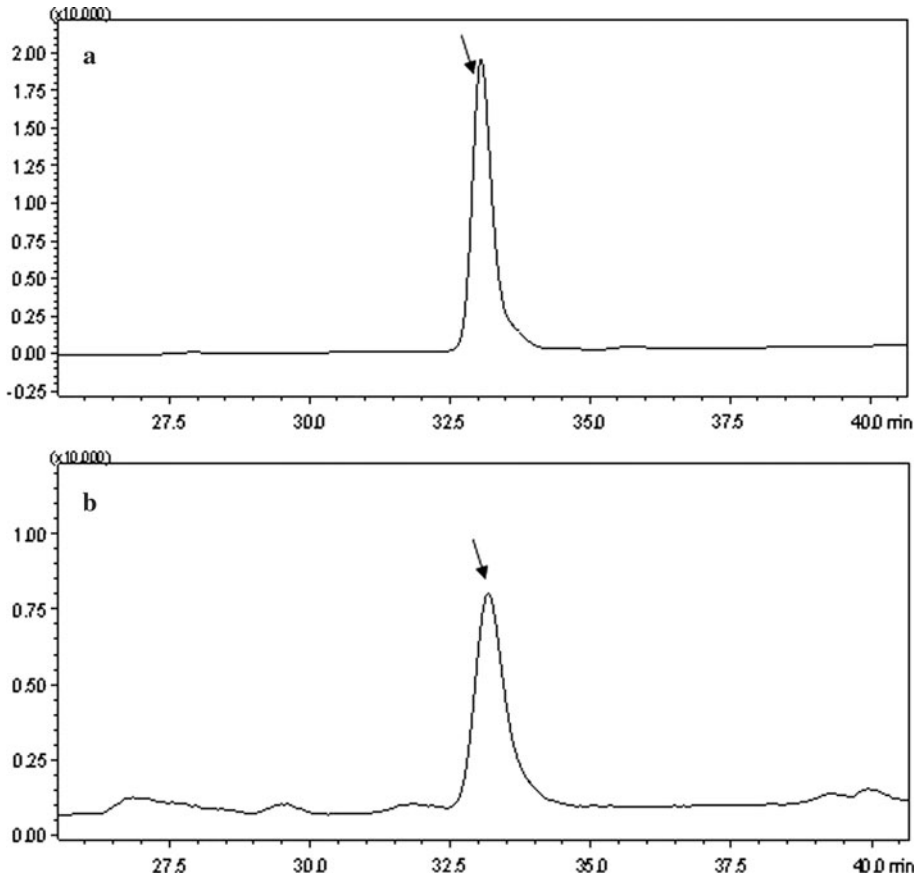

Fig. 1 HPLC of baccatin III standard (**a**) and baccatin III in extraction from mixture of mycelia and spent culture medium (**b**)

0.00071 mg/ml, and 0.00386 mg/ml according to the standard curve (Table 1). Therefore, the baccatin III content of whole PDB culture and of spent culture medium from isolate no. 79 is 0.219 mg/l and 0.193 mg/l, respectively. The baccatin III content of dry mycelium is 0.014 mg/g.

The similarity between 26S D1/D2 rDNA sequence (accession numbers: KF459961) of isolate no. 79 and that of *Diaporthe* was higher than that between it and any other genus, at 99–100 %. The similarity between the ITS rDNA sequence (accession numbers: KF459962) of isolate no. 79 and that of *D. phaseolorum* was also higher than that between it and any other species, at 99 %. Therefore, isolate no. 79 was identified as *D. phaseolorum* (Cooke and Ellis) Sacc. *D. phaseolorum* grew well on PDA at 21–22 °C producing thin white hyphae and forming a radial fungal colony that clung firmly to the medium.

Discussion

The isolation of an endophytic fungus producing baccatin III is very significant to paclitaxel manufacture and resource protection of *Taxus*. There are only a few reports of the isolation of endophytic fungi producing baccatin III

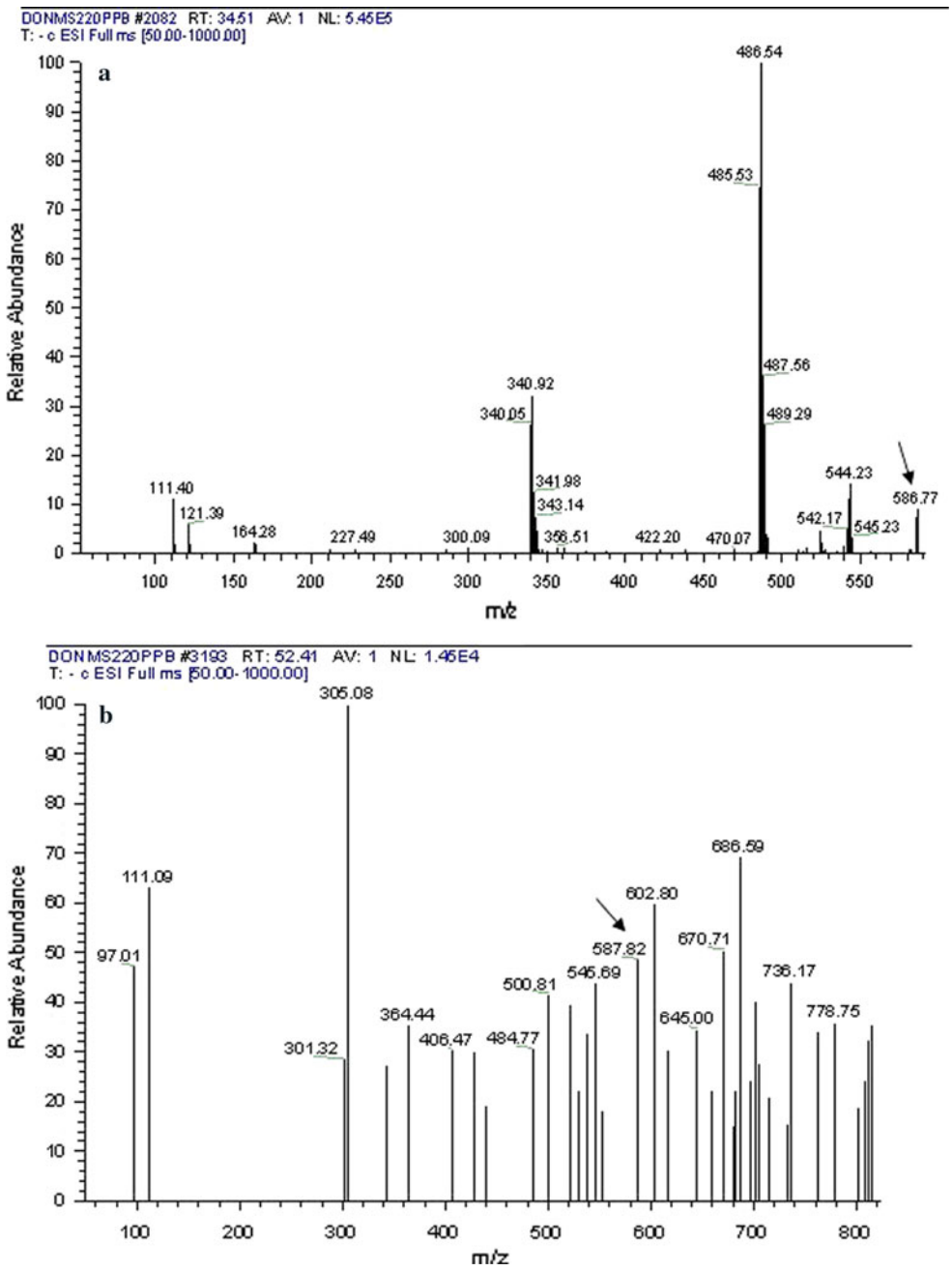

Fig. 2 MS spectra of baccatin III standard (**a**) and baccatin III in extraction from mixture of mycelia and spent culture medium (**b**)

Table 1 Standard baccatin III curve and the content of fungal baccatin III

Sample	Retention time (min)	Content (mg/l)	Peak area
Standard	32.861	0.0005	11,766
R = 0.999		0.001	27,748
		0.004	122,540
		0.01	261,996
		0.02	581,179
		0.05	1,461,443
Mixture of mycelia and spent culture medium	32.865	0.218907	139,271
Dry mycelia	32.868	0.014148 (mg/g)	16,913
Spent culture medium	32.857	0.192739	121,826

from yew, and the content of baccatin III in these isolates was low for immediate commercialization [7, 20]. Additionally, it has been reported that *E. coli*, producing endogenous acetyl-CoA and overexpressing recombinant acetyltransferase, can convert exogenously supplied 10-deacetyl baccatin III to baccatin III, but the content of baccatin III was not determined [13]. The endophytic fungus described in this study was isolated from *T. wallichiana* var. *mairei* found in the Taihang Mountains in Henan Province of China, and its product, baccatin III, was determined and authenticated with LC–MS. Although the baccatin III yield of our endophytic fungus is lower than that of cell suspension cultures of *Taxus wallichiana* as reported by Navia-Osorio et al. [14], its isolation increases the number of known species of endophytic fungi producing medicinal secondary metabolites. There are many species of endophytic fungi in yew, and they produce many kinds of chemical components, including paclitaxel and 10-deacetyl baccatin III, reported to be produced at a low level by the endophytic fungus *Gliocladium* sp. [15]. The species of our newly isolated baccatin III producing endophytic fungus was identified as *D. phaseolorum* from its ITS rDNA and 26S D1/D2 rDNA sequence. A strain of *D. phaseolorum* and a strain of *Phomopsis* (anamorph of *Diaporthe*) that could produce paclitaxel have been reported previously [9, 12], but another strain of *D. phaseolorum* that could not produce paclitaxel was also reported [12]. This may be the result of a mutation or may represent strain-to-strain variation. Therefore, it is not yet clear whether all endophytic isolates of this species can produce paclitaxel. In the 192 endophytic fungal strains obtained in this study, we found only one strain that could produce baccatin III and no strains producing paclitaxel (compared with the chromatogram of standard paclitaxel). There may be paclitaxel or baccatin III producers among these strains but they were not accumulating detectable amounts of paclitaxel or baccatin III under the conditions used. These results add to our understanding of the kinds of secondary metabolites found in endophytic fungi of yew, and may provide clues as to the genetic origins of fungal metabolite production in such endophytic plant–fungus systems.

In this study, we determined the contents of baccatin III in a mixture of mycelia and spent culture medium, in the mycelia alone and in spent culture medium alone. In other studies, only the content of baccatin III in mixtures of mycelia and spent culture medium were reported [7, 20]. In our study, the content of baccatin III in spent culture medium is obviously higher than that in mycelia, indicating that most baccatin III synthesized is secreted into the culture medium. The content of baccatin III produced by this newly isolated endophytic fungus, isolate no. 79, is considerably higher (many times that of any previous reports [7, 20]) and makes this isolate a very promising candidate to be applied to baccatin III and paclitaxel manufacture after strain improvement and culture optimization.

Conflict of interest The authors of this article declare that they have no conflicts of interest.

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