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Microbial transformation of 10-deacetylbaccatin III (10-DAB) by *Curvularia lunata* and *Trametes hirsuta*

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Abstract

The microbial transformation of 10-deacetylbaccatin III (10-DAB) (**1a**) and 13-DeBAC (**4b**) was investigated. *Trametes hirsuta* induced 13-oxidation of 10-DAB to give (**4a**) in high yield, whereas incubation with *Curvularia lunata* resulted in the isolation of the 7-*epi*-10-DAB (**2**) and the 7-*epi*-10-oxo-10-DAB (**3**). 13-DeBAC (**4b**) was biotransformed into compounds (**4a**) and (**4c**) by *Alternaria alternata*.

Keywords: Taxanes; 10-DAB; 13-DeBAC; Biotransformations; Fungi

1. Introduction

Paclitaxel (Taxol), is an approved drug for the treatment of ovarian cancer, breast cancer, non-small-cell lung cancer and AIDS-related Kaposi's sarcoma [1]. Taxol has generated additional interest because of its novel mechanism of action as an inhibitor of the depolymerization of microtubules [2].

At present, the major source of Taxol and analogs is semisynthesis [3] from the advanced taxoid pathway intermediate 10-deacetylbaccatin III (10-DAB) (**1a**), which is obtained by isolation in suitable yield (0.1% dry weight) from needles of wild or cultivated *Taxus baccata* and *Taxus yunnanensis* [1].

An interesting and alternative approach to produce new taxanes, is the bioconversion with bioagents; unfortunately, few studies of the metabolism of taxol and other taxoids by microbial systems have been reported so far. Microorganisms, such as filamentous fungi, are known to carry out regio- and stereoselective hydroxylations and oxidations of baccatin III [4] and similar terpenes [5–8].

The literature, also reports some works on reactions of hydroxylation and oxidation of peracetylated natural taxoids performed by fungi [9–11].

Encouraged by these promising studies and having at hand 10-DAB (**1a**), Baccatin III (**1c**) and 13-DeBAC (**4b**), we have carried out an extensive screening of microorganisms, in order to examine their abilities to transform these compounds in analogs with improved polarity.

This paper describes microbiological transformations of 10-DAB (**1a**) by whole growing cultures of *Curvularia lunata* and *Trametes hirsuta*; (**1a**) was also transformed by other microorganisms (see Table 1).

The fermentation methods, the isolation and the structural identification of the metabolites are also reported.

2. Experimental

2.1. General experimental procedures

Mass spectra were obtained with a Finnigan-MATT-TSQ 70 eV and Bruker-Esquire 3000 spectrometers; NMR spectra were measured on a Bruker spectrometer operating at 400 MHz with Me_4Si as internal standard. Flash-column chromatography was performed on Merck silica gel; TLC and PLC with Merck HF_{254} silica gel. The purity of products was checked by TLC, NMR and MS and deemed sufficient for the purpose of structural determination. 10-DAB **1a**, Baccatin III **1c** and 13-DeBAC **4b** were kindly provided by Indena S.p.A-Milan.

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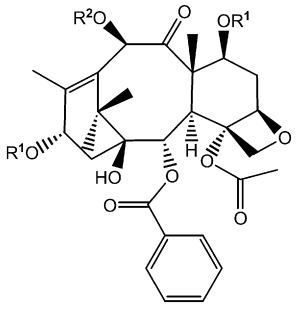
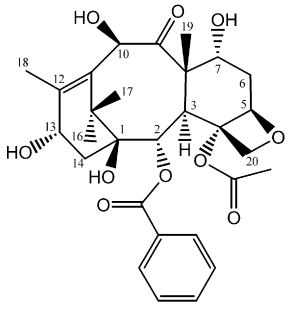


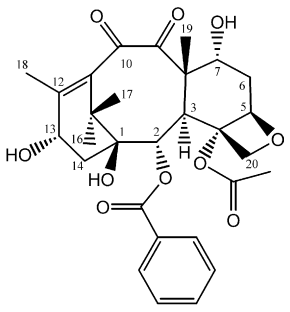
(1a) $R^1 = R^2 = H$

(1b) $R^1 = R^2 = Ac$

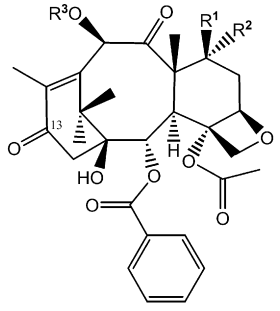
(1c) $R^1 = H$; $R^2 = Ac$



(2)



(3)



(4a) $R^1 = OH$; $R^2 = H$; $R^3 = H$

(4b) $R^1 = OH$; $R^2 = H$; $R^3 = Ac$

(4c) $R^1 = H$; $R^2 = OH$; $R^3 = Ac$

2.2. Culture and screening procedures

Cultures were grown according to the standard two-stage fermentation protocol. Screening experiments were performed in conical Erlenmeyer flasks (300 ml) containing 100 ml (fungi) or 50 ml (bacteria) of sterile medium CSB (per litre): corn steep 10 g, glucose 30 g, $NaNO_3$ 2 g, K_2HPO_4 2 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.02 g.

Stage I cultures were inoculated with a water-spore suspension obtained from freshly grown agar slants. Flasks were incubated at 24 °C (fungi) or 28 °C (bacteria) on a rotary shaker at 250 rpm.

After 72–96 h (fungi) or 48 h (bacteria), a 3–5% inoculum from stage I cultures was used to initiate stage II cultures, which were incubated for 48–72 h before receiving 10 mg of substrates in 100 μl of DMSO as substrate and incubated as before.

Table 1
Screening for the metabolism of 10-deacetylbaccatin III (10-DAB) **1a** and 13-DeBAC **4b**

Microorganism	Strain[a]
Absidia coerulea	CBS 102.28
Absidia coerulea	CBS 104.28
Absidia coerulea	ATCC 38187
Absidia cuneospora	ATCC 24693
Acinetobacter calcoaceticus	ICRM 89.01
Alternaria alternata	ICRM 42.94
Aspergillus niger	ICRM 4.85
Aspergillus ochraceus	ATCC 18500
Bacillus megaterium	ICRM 196.01
Beauveria bassiana	CBS 209.27
Botryodiplodia malorum	CBS 134.50
Botrytis cinerea	ICRM 73.01
Chaetomium cochliodes	ATCC 10195
Cunninghamella echinulata var. echinulata	ATCC 9244
Curvularia lunata	CBS 215.54
Diaporthe celastrina	CBS 139.27
Epicoccum sp.	ICRM 56.96
Fusarium equiseti	CBS 219.63
Gibberella fujikuroi	ICRM 120.01
Mucor circinelloides Mucor hiemalis	ATCC 1207a ICRM 122.01
Mucor plumbeus	CBS 129.41
Ophiobolus herpotrichus	CBS 240.31
Penicillium adametzii	ATCC 10407
Pseudomonas fluorescens	ICRM 30.89
Rhizopus oryzae	ATCC 11145
Streptomyces griseus Streptomyces catenulae	CBS 905.68 CBS 679.68
Trametes hirsuta	ICRM 140.01

[a] ATCC, American Type Colture Collection; CBS, Centraalbureau voor Schimmelcultures; ICRM, Istituto di Chimica del Riconoscimento Molecolare-Milan, Italy.

Culture controls consisted of fermentation blanks in which microorganisms were grown under identical conditions but without substrate. Substrate controls consisted of sterile medium containing the same amount of substrate and incubated under the same conditions. The fermentations were sampled periodically by TLC analysis, with pure samples as reference. Metabolites **2** and **3**, were reproducibly produced by fermentations of *C. lunata* while metabolite **4a** was produced by cultures of *T. hirsuta* using 10-DAB as substrate. Metabolite **2** was also produced by cultures of *B. bassiana*, *P. fluorescens*, *Epicoccum* sp., *A. alternata*, *C. echinulata*, *O. herpotrichus*, *S. catenulae*, *B. cinerea*, *A. coerulea*, *M. hiemalis*, *P. adametzii*, *M. plumbeus*, *M. circinelloides*, *F. equiseti* i *S. griseus*; the first six produced also compound **3**.

With *A. alternata* as bioagent, 13-DeBAC **4b** gave compounds **4a** and **4c**.

2.3. Preparative biotransformation of 10-DAB *1a* to metabolites *2* and *3* by C. lunata

Seventy-two hour-old stage I cultures were transferred in thirty-five 300 ml Erlenmeyer flasks each containing 100 ml of CSB liquid medium. The flasks were incubated at 28 °C on an orbital shaker (250 rpm). After 48 h, a total of 350 mg of 10-DAB **1a**, dissolved in DMSO (3.5 ml), was evenly distributed among 35 flasks containing stage II cultures (final concentration

0.01 g/l). After 2 weeks, the cultures were harvested and filtered. The filtrate obtained was extracted two times with equal volumes of EtOAc/MeOH (100:1) and evaporated to dryness under reduced pressure, to give a brown oil.

The crude residue (260 mg) was purified by column chromatography on silica gel with a stepwise elution with CH_2Cl_2/isopropanol from 30:1 to 10:1. The fractions obtained, were further purified on preparative plates (PLC) in CH_2Cl_2/diethylether/isopropanol (10:1:0.5) as eluent to afford metabolites **2**, 21 mg (8%) and **3**, 23 mg (8.7%); unreacted 10-DAB 50 mg (20%). Compound **2** was isolated as a white solid, with the same mass (Maldi) (M^+, 544) of the starting material; ^{1}H NMR ($CDCl_3$) δ: 8.12, 7.62 and 7.50 (5H, m, ArH), 5.70 (2H, d, J = 7.3 Hz, H-2), 5.50 (1H, s, H-10), 4.96 (1H, dd, J = 8.5 and 4.0 Hz, H-5), 4.88 (1H, m, H-13), 4.41 and 4.39 (2H, d, J = 8.8 Hz, H_2-20), 4.04 (1H, J = 7.3 Hz, H-3), 3.67 (1H, m, H-7), 2.5–2.2 (4H, m, H_2-6 and -14), 2.38 (3H, s, OAc), 2.00 (3H, brs, H_3-18), 1.71 (3H, s, H_3-19), 1.08 (3H, s, H_3-16), 1.07 (3H, s, H_3-17). Selected NOEs ($CDCl_3$): {H-2} enhanced H_3-17 (1%) and H_3-19 (1.5%), {H-10} enhanced H-3 (3%) and H_3-18 (2.5%), {H_3-19} enhanced H-2 (9%), H-7 (6%) and H_2-20 (3.5%).

Compound **3**-white solid, mp 158–160°; FABMS *m/z* 543 (MH^+), 525, 504, 487, and 443; ^{1}H NMR ($CDCl_3$) δ: 8.12, 7.63 and 7.50 (5H, m, ArH), 5.82 (1H, brd, J = 7.3 Hz, H-2), 4.92 (1H, m, H-5), 4.91 (1H, m, H-13), 4.60 (1H, d, J = 11.5 Hz, OH-7), 4.42 and 4.31 (2H, brd, J = 8.7 Hz, H_2-20), 4.10 (1H, brd, J = 7.3 Hz, H-3), 3.82 (1H, ddd, J = 11.5, 6.0 and 3.0 Hz, H-7), 2.5-2.2 (4H, m, H_2-6 and -14), 2.37 (3H, s, OAc), 1.94 (3H, brs, H_3-18), 1.69 (3H, s, H_3-19), 1.08 and 1.07 (6H, s, H_3-16 and -17); ^{13}C NMR ($CDCl_3$) δ: 208.4 (C-9); 189.2 (C-10); 172.5 ($MeCO_2$); 167.1 (Ar-CO_2); 146.3 and 129.2 (C-12 and -11); 140.6, 133.9, 130.1 and 128.8 (ArC); 82.5 (C-5); 81.2 (C-4); 79.0 (C-1); 77.3 (C-7); 77.2 (C-20); 74.9 (C-2); 67.5 (C-13); 57.3 (C-8); 39.8 (C-15); 39.6 (C-3); 38.8 (C-14); 35.3 (C-6); 26.3 (C-17); 22.5 ($MeCO_2$); 22.1 (C-16); 15.0 (C-19); 14.6 (C-18).

2.4. Preparative biotransformation of 10-DAB **1a** to metabolite **4a** by T. hirsuta

T. hirsuta was grown in 300 ml Erlenmeyer flasks containing 100 ml of liquid medium CSB. The flasks were incubated at 24 °C on a orbital shaker (180 rpm). After 5 days, a 5% inoculum stage I culture was transferred in twenty 300 ml flasks containing 100 ml of the same medium. Stage II cultures were incubated at 28 °C on an orbital shaker (250 rpm) for 72 h before receiving the substrate. 10-DAB **1a** (300 mg), dissolved in DMSO (2 ml), was evenly distributed among 20 flasks containing stage II cultures (final concentration 0.015 g/l).

After 48 h, the substrate was completely consumed and the cultures were harvested and filtered. The filtrate was subsequently extracted two times with equal volumes of EtOAc/MeOH (100:1) to give 250 mg of a white solid. After chromatography on a silica gel column with a stepwise elution with CH_2Cl_2/isopropanol from 40:1 to 25:1, 133 mg of metabolite **4a** (44%) were obtained. Compound **4a** is a solid, mp 192 °C, mass spectrum (EIMS), *m/z* 542 (42%), 525 (28) and 105 (100); ^{1}H NMR (acetone-d_6) δ: 8.12, 7.67 and 7.53 (5H, m, ArH), 5.73 (1H, brd, J = 6.9 Hz, H-2), 5.48 (1H, brd J = 3.0 Hz, H-10), 4.94 (1H, dd, J = 9.6 and 2.1 Hz, H-5), 4.57 (1H, brd, J = 3.0 Hz, OH-10), 4.43 (1H, brd, J = 7.0 Hz, OH-7), 4.34 (1H, s, OH-1), 4.32 (1H, m, H-7), 4.20 and 4.16 (2H, brd, J = 8.1 Hz, H_2-20), 4.03 (1H, d, J = 6.9 Hz, H-3), 3.11 and 2.76 (2H, d, J = 19.9 Hz, H_2-14), 2.48 (1H, ddd, J = 14.5, 9.6 and 6.8 Hz, H-6a), 2.15 (3H, s, OAc), 2.06 (3H, s, H_3-18), 1.83 (1H, ddd, J = 14.5, 10.8 and 2.1 Hz, H-6b), 1.72 (3H, s, H_3-19), 1.21 and 1.19 (6H, s, H_3-16 and -17).

2.5. Preparative biotransformation of **4b** to metabolites **4a** and **4c** by A. alternata

Cultures of *A. alternata* were incubated as described above for (Section 2.3); **4b**, 13-DeBAC (200 mg) was dissolved in DMSO and added to cultures; after 2 weeks growth, the filtrate was extracted with EtOAc and the residue (80 mg) was chromatographed to obtain metabolites **4a**, 5 mg and **4c**, 15 mg. Compound **4c** was obtained as a solid, mass spectrum (EIMS), *m/z* 584 (M^+), 524, 420, 343 and 105; ^{1}H NMR ($CDCl_3$) δ: 8.10, 7.66 and 7.51 (5H, m, ArH), 6.95 (1H, s, H-10), 5.82 (1H, brd, J = 7.5 Hz, H-2), 4.93 (1H, brdd, J = 9.0 and 3.8 Hz, H-5), 4.55 (1H, d, J = 11.0 Hz, OH-7), 4.42 and 4.33 (2H, brd, J = 8.6 Hz, H_2-20), 4.02 (1H, brd, J = 7.5 Hz, H-3), 3.70 (1H, ddd, J = 11.0, 4.0 and 2.5 Hz, H-7), 3.05 and 2.69 (2H, d, J = 20.0 Hz, H_2-14), 2.38 and 2.26 (2H, m, H_2-6), 2.27 and 2.26 (6H, s, 2xOAc), 2.00 (3H, s, H_3-18), 1.63 (3H, s, H_3-19), 1.23 and 1.22 (6H, s, H_3-16 and -17).

Compound **4a** from *A. alternata* was identical by TLC and ^{1}H NMR to a sample obtained by fermentation of 10-DAB with *T. hirsuta*.

3. Results and discussion

Some biotransformations of taxanes by filamentous fungi have been reported in the literature. Interesting results have been obtained with *Absidia coerulae* and different strains of *C. echinulata*. It has been demonstrated that these fungi are capable to perform efficient reactions of hydroxylation, oxidation and reduction on specific sites and rearrangement reactions of taxoids [4–8].

We started a microbial transformation program with microorganisms screened for their ability to hydroxylate or oxidize substrates (see Table 1). Because fully acetylated taxadienes were hydroxylated in position 13 and 14 by the fungus *Absidia coerulea* [9–11], we started working on peracetate of 10-DAB **1a**, **1b**, but our efforts were unsuccessful.

Subsequently we selected 13-DeBac **4b** as a starting material but with *S. griseus*, *S. fluorescens*, *Epicoccum* sp., *A. alternata*, *B. bassiana*, *M. plumbeus*, *O. herpotrichum*, *D. celastrina*, we obtained only the isomerization at C-7 with the formation of compound **4c**.

In the case of *A. alternata* and *G. fujikuroi* we evidenced the loss of the 10-Ac group to obtain compound **4a**.

While baccatine III **1c** was biotransformed with our microorganisms principally into 10-DAB **1a**, for the above reasons we continued to work on 10-DAB. A specific enzymatic hydrolysis of the acetyl group at C-10 and C-13 has been reported by action of *Nocardia* sp. [12].

Twenty-nine microbial organisms (see Table 1) were initially screened: among the cultures screened, fifteen were found to be able to convert the compound **1a**.

From the TLC analysis results, *C. lunata* and *T. hirsuta* were found to be the most potent strains. Therefore, these two strains were selected to transform **1a** in a preparative scale in order to improve the yields of products for their characterisation.

C. lunata transforms compound **1a** to metabolites **2** and **3**; ^{1}H NMR data of **2** were very similar to those exhibited for **1a**, the only relevant difference being the presence of a resonance at δ 3.67 attributed to H-7β in place of the signal at δ 4.28 exhibited by **1a** for H-7α.; the retro-aldol epimerization of the 7-hydroxy group from a β-equatorial to an α-axial orientation is typical of Baccatin III derivatives [13].

Compound **3**, exhibits a molecular peak of two units less than for compound **2**; ^{1}H and ^{13}C NMR spectra of **3** were very similar to those published for the semi-synthetic product 10-dehydrobaccatin V [13]. In particular, with respect to the starting compound **1a**, the NMR data indicated the presence of an additional carbonyl group (C-10) in place of the CHOH moiety and an upfield shift of ca. 0.5 ppm attributed to the epimerization at C-7.

T. hirsuta transforms 10-DAB into metabolite **4a** in a good yield (44%). The ^{1}H NMR spectrum shows the disappearance of the proton in 13 position. The ^{1}H NMR spectrum of **4a** paralleled that exhibited by a sample of 13-DeBAC **4b**, the only relevant difference being the upfield shift of ca. 1 ppm of H-10 and the absence of the acetyl signal of the 10-Ac group. *A. alternata transforms* **4b** into metabolites **4a** and **4c**. The ^{1}H NMR spectrum of **4c** was very similar to that exhibited by **4b**, the only difference being the upfield shift of ca. 0.6 ppm of H-7 because of epimerization at C-7. Compound **4a** from *A. alternata* was identical by TLC and ^{1}H NMR to a sample obtained by fermentation of 10-DAB with *T. hirsuta*.

In conclusion, we report here for the first time, a biotransformation of 10-DAB by redox enzymes of some filamentous fungi with the formation of compounds **2** and **3**.

Another remarkable result is the good yield (44%) in the formation of compound **4a** by the action of *Trametes hirsuta* (Basidiomycetae) and the deacetylation process on C-10 for Baccatin III. These results show the difficulty for the enzymes of bioagents to attack functional groups in the core of the taxane molecules.

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