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Microbial transformation of 7-*epi*-10-deacetylbaccatin III to 10-deacetylbaccatin III

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ABSTRACT

The microbial transformation of 7-*epi*-10-deacetylbaccatin III (7-*epi*-10-DAB III) to 10-deacetylbaccatin III (10-DAB III) was studied. In this report, seven microorganisms were found to be able to realize the transformation at yields from 20.0% to as high as 70.8%. The optimized conditions such as the solvent, pH value of the medium, the microorganisms, transformation time, and substrate concentration were investigated.

1. Introduction

Paclitaxel (Taxol) was first isolated from the bark of *Taxus brevifolia* in 0.02% yield [1], however, the harvest of the yew bark is devastating to yew trees and the supply issue of Taxol was a tremendous obstacle that slowed the development of Paclitaxel into market. A lot of efforts have been made to increase the supply of Paclitaxel including isolation from other natural sources, total synthesis, semi-synthesis from 10-deacetylbaccatin III (10-DAB III will be used thereafter), etc. The potential of semi-synthesis was first recognized by Potier and co-workers, who found that 10-DAB III was a relatively abundant taxane in the needles of *Taxus baccata* L., the European yew, with a yield of 0.02% [2]. The isolated yield could be improved to 0.1% [3]. More significantly, 10-DAB III is easier to isolate from yew needles than Taxol and the needles are easily regenerated. 10-DAB III seems to be an easily and permanently accessible Taxol precursor. The first semi-synthesis of Taxol from 10-deacetylbaccatin III was achieved by Denis et al. in 1988 [3].

Because of the highly regio- and stereo-selectivity of biotransformation, various microorganisms were used for the transformation of taxanes in an attempt to search for new potent anticancer agents. The reactions usually happened to taxanes were hydroxylation [4–7], oxidation [5], acetylation [8], epimerization [9] as well as the impressive rearrangement of taxanes [6,10].

7-*epi*-10-Deacetylbaccatin III (7-*epi*-10-DAB III will be used thereafter) is one of the taxanes that can be produced by actinomycetes [11] or recovered from conifers [12] and *Taxus* species albeit in a very low yield. In our previous work on taxanes, we have made great effort to increase the production of 10-DAB III, including the chemical modification of the taxanes with the core structure of 10-DAB III. Although most of the 10-DAB III (about 80%) remained intact, small part (about 20%) of it was transformed into 7-*epi*-10-DAB III during the chemical reaction and purification process (unpublished results). It is very difficult to transform 7-*epi*-10-DAB III to 10-DAB III by chemical method and 7-*epi*-10-DAB III is usually considered as a by-product. In this report, to make use of 7-*epi*-10-DAB III, 7-*epi*-10-DAB III was converted to 10-DAB III by microbial transformations.

2. Experimental

2.1. General

NMR spectra were measured on a Bruker Avance DRX-500 spectrometer operating at 500 MHz with Me_4Si as internal standard. The analytical HPLC was carried out on a Waters™ 600 Controller and Pump, with Waters™ 996 Photodiode Array Detector and a GraceSmart RP 18 column (5 μm, 4.6 mm × 250 mm). The mobile phase consisted of 55% methanol and 45% water. The flow rate was 1 ml/min. The column temperature was set at 25 °C. The detection wave-length was 230 nm. The injection volume was 20 μl. The retention times for 10-DAB III and 7-*epi*-10-DAB III were 7.2 min and 11.4 min, respectively. Analytical TLC was carried out on Silica gel GF254 plates (Qingdao Oceanic Chemicals, China), and the

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visualization of TLC plates was performed by spraying with 10% H_2SO_4 in methanol followed by heating.

2.2. Substrate

The substrate 7-*epi*-10-DAB III was obtained from Beijing Fei-SiDe(First) Bio-pharmaceutical Development Co. Ltd. 7-*epi*-10-DAB III is the by-product during the production of 10-DAB III from other taxanes by chemical method and was purified to obtain the purity of more than 99% [17].

2.3. Microorganisms, culture and screening procedures

Seven strains: *Pseudomonas oleovorans* AS1.1641, *Pseudomonas putida* AS1.1003, *Pseudomonas fluorescens* AS1.33, *Nocardia corallina* AS4.1037, *Bacillus cereus* AS1.126, *Bacillus mycoides* AS1.182, *Bacillus megaterium* AS1.127 were used in the experiment. All the microbes were purchased from Institute of Microbiology, Chinese Academy of Sciences (IM/CAS), Beijing, China.

Cultures were grown according to the standard two-stage fermentation protocol. Screening experiments were performed in conical Erlenmeyer flasks (250 ml) containing 100 ml of sterile beef-protein medium (beef-protein medium, Peptone 10.0 g/l, beef extract 3.0 g/l, NaCl 5.0 g/l, the pH value was adjusted to 7.0 before autoclaving for 20 min at 121 °C).

Stage I cultures were inoculated with microorganism obtained from freshly grown agar slants. 3–5% inoculums from stage I cultures were used to initiate stage II cultures. The stage II cultures were incubated for 2 days before receiving 1 mg of substrate in 0.1 ml of acetone. The cultures were further incubated for 7 days on a rotary shaker at 170 rpm at 27 °C. The cultures were then harvested and extracted three times with equal volumes (50 ml) of CH_2Cl_2 and evaporated to dryness under reduced pressure. Two controls of culture with microorganism but without substrate and culture with substrate but without microorganism were run synchronously with the fermentation and work-up in the same way.

The fermentations were analyzed by TLC and HPLC analysis with pure samples as reference.

2.4. pH value kinetics of the B. mycoides AS1.182 growth period

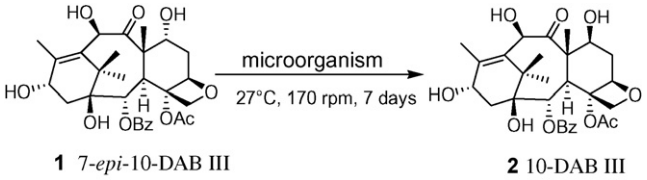
To each of the seven Erlenmeyer flasks (250 ml) containing 100 ml of sterile beef-protein medium was added 3–5% inoculums from stage I cultures. Flasks were incubated at 27 °C on a rotary shaker at 170 rpm. The pH value of one flask was measured at 24 h interval.

2.5. The influence of medium pH value on biotransformation rate

To each of the three Erlenmeyer flasks (250 ml) containing 100 ml of sterile beef-protein medium (with pH values 6.5, 7.5, and 8.5, respectively) was added 1 mg of substrate in 0.1 ml of acetone. The flasks were incubated at 27 °C on a rotary shaker at 170 rpm for 7 days. The extraction and HPLC checking procedure was the same as above.

2.6. Preparative biotransformation of 7-epi-10-DAB III to 10-DAB III

B. mycoides AS1.182 was grown in four 1000 ml Erlenmeyer flasks each containing 500 ml of liquid beef-protein medium. The flasks were incubated at 27 °C on an orbital shaker (170 rpm). After 48 h incubation, a total of 200 mg of 7-*epi*-10-DAB III **1**, dissolved in acetone (2 ml), was evenly distributed among 4 flasks containing stage II cultures (final concentration 0.1 g/l). After 10 days, the cultures were harvested and extracted three times with equal volumes of CH_2Cl_2 and evaporated to dryness under reduced pressure to give a brown oil.



1 7-*epi*-10-DAB III **2** 10-DAB III

Scheme 1. Microbial transformation of 7-*epi*-10-DAB III.

The crude residue (250 mg) was purified by column chromatography on silica gel with a stepwise elution with petroleum ether/EtOAc (from 3:1 to 1:2). The fractions containing the product were further purified by recrystallization in acetonitrile to afford a white solid metabolite **2** (117.8 mg, 58.9%). 63 mg (31.5%) of the substrate (7-*epi*-10-DAB III) was recycled.

Compound **2** was obtained as a solid. The purity of the product was checked by TLC and HPLC. The structure of the isolated product was determined by comparing its NMR spectra with authentic samples of 10-DAB III.

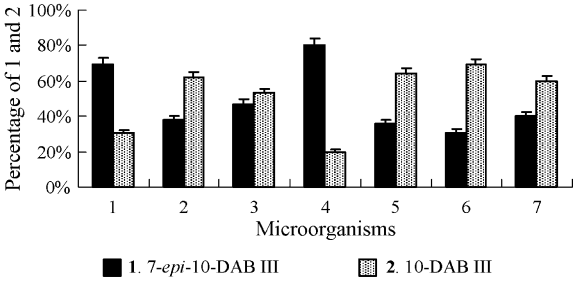
3. Results and discussion

One of the main drawbacks in biotransformation of 7-*epi*-10-DAB III is the low solubility of the substrate in water, which diminishes reaction rates and overall productivity. Different solvents were investigated to improve the solubility of the substrate 7-*epi*-10-DAB III. Acetone was found to be the best solvent and was used in all of the experiments.

Seven strains: *P. oleovorans* AS1.1641, *P. putida* AS1.1003, *P. fluorescens* AS1.33, *N. corallina* AS4.1037, *B. cereus* AS1.126, *B. mycoides* AS1.182, *B. megaterium* AS1.127 were able to directly convert 7-*epi*-10-DAB III into 10-DAB III as the single product (Scheme 1). This is the first successful direct conversion of 7-*epi*-10-DAB III to 10-DAB III by microorganism. This finding may open a new way to make use of the 7-*epi*-10-DAB III and thus to increase the yield of the important intermediate 10-DAB III. From the data of screening (as shown in Fig. 1), *B. mycoides* AS1.182 has the highest transformation rate (as high as 70.8% in HPLC checking) and was used for the next optimization experiment.

A series of experiments to optimize the conditions of microbial transformation were investigated, including the pH value of the medium, culture time and concentration of substrate, etc.

Since the kinetics of reaction during incubation is greatly influenced by pH value, the pH effects on the reaction were also



1. *Pseudomonas oleovorans* AS1.1641, 2. *Pseudomonas putida* AS1.1003, 3. *Pseudomonas fluorescens* AS1.33, 4. *Nocardia corallina* AS4.1037, 5. *Bacillus cereus* AS1.126, 6.*Bacillus mycoides* AS1.182, 7. *Bacillus megaterium* AS1.127

Fig. 1. Effect of microorganisms on the biotransformation rate (27 °C, 170 rpm, 7 days).

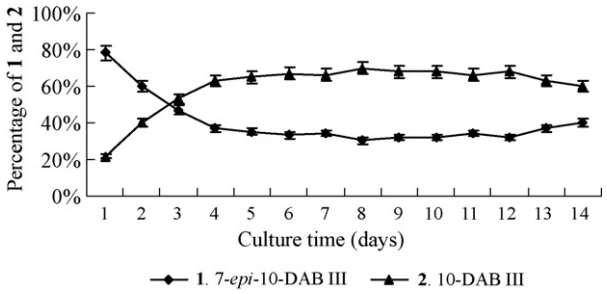


Fig. 2. Effect of culturing time on the biotransformation rate of 7-*epi*-10-DAB III by *Bacillus mycoides* AS1.182 (27 °C, 170 rpm).

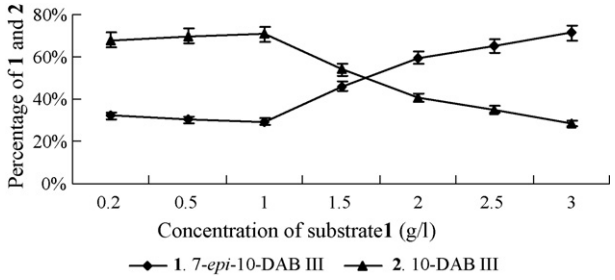


Fig. 3. Effect of substrate concentration on the biotransformation rate of 7-*epi*-10-DAB III by *Bacillus mycoides* AS1.182 (27 °C, 170 rpm, 7 days).

investigated systematically with *B. mycoides* AS1.182 as the example.

The pH value of the medium was adjusted to 7.0 before incubation. After the addition of the *B. mycoides* AS1.182 seeds, the pH value increased from 7.03 to 8.26 during the first day and stayed at 8.26 to 8.50 for the next 6 days during fermentation. The result indicated that the transformation occurred in the weak basic conditions during the incubation.

To evaluate whether the epimerization resulted from the effect of pH values or enzymatic transformation, the substrate 7-*epi*-10-DAB III (**1**) was incubated in the medium with 3 different pH values (6.5, 7.5 and 8.5, respectively) without microbes for 7 days. At pH 6.5, there was no transformation. At pH 7.5, the transformation rate is about 1%. At pH 8.5, 5% of substrate was transformed. These transformation rates were much lower than that of the microbial transformation of the substrate (7-*epi*-10-DAB III) by *B. mycoides* AS1.182, which gives a much higher yield (70.8% by HPLC checking and 58.9% by isolated yield). These results clearly showed that the basic condition contributed a very small part of the transformation only and the microorganisms were the main contributors.

The effect of culturing time on the biotransformation of 7-*epi*-10-DAB III was studied. *B. mycoides* AS1.182 was grown in 14 250 ml Erlenmeyer flasks containing 100 ml of liquid beef-protein medium. The flasks were incubated at 27 °C on an orbital shaker (170 rpm). After 48 h, a total of 280 mg of 7-*epi*-10-DAB III **1**, dissolved in acetone (2.8 ml), was evenly distributed among 14 flasks containing stage II cultures (final concentration 0.2 g/l). The fermentation was sampled every day by HPLC analysis. The conversion rates increased dramatically in the first 4 days, but remained stable at about 65% after 5 days (Fig. 2). The optimized culturing time could be 5 days.

The effect of the substrate concentration was studied with initial concentration of 7-*epi*-10-DAB III from 0.2 g/l to 3 g/l. There is no significant difference in the transformation yield when the substrate concentration below 1 g/l. The transformation rate decreased significantly when the substrate concentration was above 1 g/l (as shown in Fig. 3).

The C-7 hydroxyl group was one of the most accessible functional groups on the taxane ring, and was subjected to epimerization. The 7-epitaxol and other *R*-epimers were determined as the more stable form than the *S*-epimers in alcohol and other mixed solvents [13]. Some biotransformations of 10-DAB III have been reported in the literatures [8,14,15]. 10-DAB III could be transformed to 7-*epi*-10-DAB III by *Curvularia lunata* CBS 215.54 [15]. However, the transformation of 7-*epi*-10-DAB III to 10-DAB III by microbes has never been reported previously.

In this report, the more stable epimer 7-*epi*-10-DAB III was converted to its less stable isomer 10-DAB III by microorganisms which seems contradictive to the results from the chemical transformations [16]. This is a very interesting finding. It was indicated that the enzymatic system of the microbes has the ability to stabilize the 7-*S*-epimers. This enzymatic system may also exist in the *Taxus* species, because there is much high yield of 10-DAB III than that of 7-*epi*-10-DAB III in the natural yew trees. More investigations are needed to understand the mechanism of this new discovery.

4. Conclusion

We reported for the first time the microbial transformation of 7-*epi*-10-DAB III to 10-DAB III in high yield. The optimization of conditions for this transformation was investigated. The substrate 7-*epi*-10-DAB III was dissolved in acetone at a concentration of 1 g/l and was transformed in neutral medium for 5 days by *B. mycoides* AS1.182. The yield of 10-DAB III by this biotransformation is 70.8% by HPLC checking and 58.9% after isolation.

Acknowledgements

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