Microbial Transformation of 10-Deacetyl-7-epitaxol and 1β-Hydroxybaccatin I by Fungi from the Inner Bark of *Taxus yunnanensis*

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Fungi present in the inner bark of the yew tree *Taxus yunnanensis* were studied for the capability to biotransform natural taxoids. On the basis of screening, three fungi, *Microsphaeropsis onychiuri*, *Mucor* sp. and *Alternaria alternata*, were revealed to be able to selectively hydrolyze and epimerize two taxoids, 10-deacetyl-7-epitaxol (**1**) and 1β-hydroxybaccatin I (**2**), previously isolated from the bark of the same yew species. After preparative-scale incubation of 10-deacetyl-7-epitaxol with *M. onychiuri* and *Mucor* sp., three metabolites were isolated and characterized as 10-deacetylbaccatin V (**3**), 10-deacetyltaxol (**4**), and 10-deacetylbaccatin III (**5**). Incubation of 1β-hydroxybaccatin I with *A. alternata*, on the other hand, gave 5-deacetyl-1β-hydroxybaccatin I (**6**), 13-deacetyl-1β-hydroxybaccatin I (**7**), and 5,13-dideacetyl-1β-hydroxybaccatin I (**8**).

Being interested in the biotransformation of natural products, we were inspired by the discoveries of two endophytic fungi in *Taxus* spp.[1, 2] and a strain of bacteria isolated from soil[3] that can produce Taxol[4] and other taxoids to study their biotransformation. Indeed, biotransformation of Taxol in human tissues gave hydroxylated and epimerized products,[5,6] whereas investigations on the microbial transformation of taxoids[7−10] resulted in the detection of two site-specific taxoid hydrolytic enzymes from two strains of *Nocardioides* bacteria; selective deacetylation and hydroxylation, as well as epoxidation, were also reported by microbial transformation of taxoids by the fungi *Cunninghaamella echinulata*, *C. elegans*, and *Aspergillus niger*.

As taxoids are a series of complex natural compounds with potential bioactivity, their structural modification by the fungi from the yew tree may be of some value. We report here the microbial transformation of 10-deacetyl-7-epitaxol (**1**) and 1β-hydroxybaccatin I (**2**), two taxoids that are more abundant than Taxol in the bark of *Taxus yunnanensis*, by three fungi isolated from the inner bark of the same yew species.

About 80 strains of fungus from the inner bark of *Taxus yunnanensis* Cheng et L. K. Fu were screened for their ability to biotransform **1**, with *Microsphaeropsis onychiuri* (Punith) Morgan-Jones and *Mucor* sp. being chosen for preparative-scale transformation in order to obtain the metabolites. Accordingly, three biotransformation products **3**, **4**, and **5** were obtained from both the two fungi cultures.

The ^{1}H NMR spectra of compounds **3** and **5** showed that both are hydrolysis products that have lost their C-13 side chains, and FABMS and FDMS confirmed that they had the same molecular weight of 544. They were identified, respectively, as 10-deacetylbaccatin V and 10-deacetyl baccatin III, with their ^{1}H NMR data being identical to that in the literature.[11] The FABMS of compound **4** showed a protonated molecular ion peak at *m*/*z* 812, which indicates a molecular weight of 811, the same as the starting compound **1**, and was shown to be 10-deacetyltaxol, as confirmed by comparing its ^{1}H NMR spectrum with that previously reported.[12]

It was noted several years ago[11,12] that partial epimerization at C-7 of 10-deacetyltaxol and cephalomannine occurred under neutral and mild basic conditions. This facile epimerization noted by the early investigators was suggested to occur as a result of a reversible retro-Aldol reaction involving ring opening at the bond between C-7 and C-8. It was also suggested that the 7α-hydroxyl epimers of the series (e.g., 7-epitaxol) were more stable than their 7β epimers because of the existence of intramolecular hydrogen bonding of the 7α-hydroxyl group to the acyl-oxygen of the 4α-acetate group. The results of our investigation show that the enzyme system in these two fungus cultures may have some special activity in promoting epimerization at C-7. This finding may lead us to develop a method for using the byproduct in Taxol purification to produce the starting material 10-deacetylbaccatin III and 10-deacetyltaxol for the semisynthesis of Taxol.

1β-Hydroxybaccatin I is an abundant taxoid with a unique 4β,20-epoxide skeleton from *Taxus yunnanensis*. On the basis of screening, another fungus, identified as *Alternaria alternata* (Fr.) Keisel, was selected for preparative-scale transformation. Three hydrolysis products, **6**, **7**, and **8** in the yields of 3.1%, 2.8%, and 1.8%, respectively, were isolated. The ^{1}H and ^{13}C NMR spectral data of **2** and its metabolites are shown in Tables 1 and 2. Compound **6** has the same molecular composition as of **7**, that is $C_{30}H_{42}O_{13}$, on the basis of

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Table 1. ^{1}H NMR Data of Substrate **2** and Metabolites **6**, **7**, and **8** ($CDCl_3$, δppm)

H	**2**	**6**	**7**	**8**[a]
2β	5.49 d (3.6[b])	5.50 d (3.5)	5.49 d (3.5)	5.48 d (3.1)
3α	3.18 d (3.6)	3.40 d (3.5)	3.20 d (3.5)	3.34 d (3.1)
5α	4.22 t (3.0)	3.03 t (2.8)	4.23 t (2.8)	2.99 t (2.8)
6α	2.17 m	2.08 m	2.11 m	2.09 m
6β	1.74 m	1.82 m	1.76 m	1.77 m
7α	5.49 dd (12.0; 4.5)	5.61 dd (12.0; 4.6)	5.49 dd (12.0; 5.1)	5.57 dd (12.0; 4.7)
9β	6.03 d (11.1)	5.96 d (11.0)	5.99 d (11.0)	5.96 d (10.9)
10α	6.21 d (11.1)	6.23 d (11.0)	6.21 d (11.0)	6.17 d (10.9)
13β	6.08 br t (8.2)	6.00 br t (4.6)	4.81 br d (4.2)	4.61 dd (10.1; 2.8)
14α	1.88 dd (14.8; 6.4)	1.94 dd (15.4; 4.8)	1.98 m	1.99 m
14β	2.54 dd (14.8; 9.7)	2.55 dd (15.4; 10.3)	2.55 dd (15.3; 10.0)	2.56 dd (15.7; 10.1)
16-Me	1.24 s	1.13 s	1.08 s	1.03 s
17-Me	1.66 s	1.60 s	1.59 s	1.53 s
18-Me	2.23 d (1.3)	2.20 d (1.3)	2.34 d (1.1)	2.28 d (1.2)
19-Me	1.25 s	1.20 s	1.23 s	1.19 s
20A	3.55 d (5.1)	3.64 d (5.2)	3.61 d (5.2)	3.72 d (5.2)
20B	2.32 d (5.1)	2.26 d (5.2)	2.35 d (5.2)	2.31 d (5.2)
OCO*CH*$_3$	2.22 s; 2.12 s; 2.09 s; 2.06 s; 2.05 s; 2.00 s	2.11 s; 2.09 s; 2.07 s; 2.06 s; 2.00 s	2.17 s; 2.09 s; 2.07 s; 2.06 s; 2.00 s	2.10 s; 2.07 s; 2.07 s; 2.00 s

[a] Determined in $CDCl_3$ + CD_3OD. [b] The coupling constant (J) in Hz.

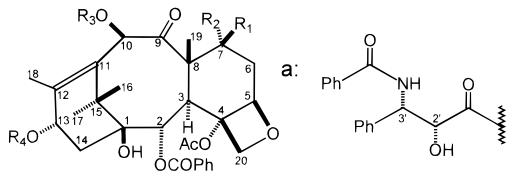
Table 2. ^{13}C NMR Spectral Data of Substrate **2** and Metabolites **6**, **7**, and **8** ($CDCl_3$, δppm)

C	**2**	**6**	**7**	**8**[a]
1	76.1	76.3	75.5	75.6
2	72.2	71.8	71.7	72.0
3	41.3	39.5	41.5	39.4
4	58.3	60.5	58.4	59.9
5	77.7	75.3	77.8	74.6
6	31.1	32.7	31.0	33.0
7	68.7	68.6	68.8	69.1
8	46.6	47.0	46.8	46.7
9	75.2	75.4	75.3	75.3
10	70.7	71.1	71.5	71.1
11	135.6	136.6	135.1	135.3
12	140.4	140.8	144.5	145.1
13	71.1	71.3	69.3	69.5
14	38.6	38.6	42.1	41.9
15	43.3	42.9	42.9	42.6
16	28.4	28.8	28.7	28.6
17	21.8	21.5	21.4	21.2
18	15.4	16.0	16.2	16.2
19	13.7	13.2	13.3	12.7
20	49.9	50.0	50.2	50.7
OCO*Me*	21.8; 20.9; 21.8; 21.7; 21.4; 20.7	21.0; 21.0; 20.9; 20.7; 20.6	21.1; 21.0; 20.9; 20.8; 20.7	20.7; 20.5; 20.4; 20.0
O*C*OMe	170.1; 169.9; 169.7; 169.3; 169.2; 169.2	169.9; 169.8; 169.7; 169.5; 169.2	169.9; 169.8; 169.5; 169.5; 169.3	170.5; 170.2; 169.9; 169.7

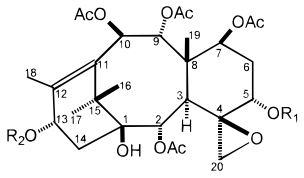
[a] Determined in $CDCl_3$ + CD_3OD.

analysis of their FABMS and ^{13}C NMR spectra. The major ^{1}H NMR spectral differences between **6** and **7** from **2** is that of the C-5 proton in **6** and the C-13 proton in **7**. The signal of the C-5 proton of **6** shifts from δ 4.22 (t, J = 3.0 Hz) to δ 3.03 (t, J = 2.8 Hz), and the C-13 proton of **7** shifts from δ 6.08 (br t, J = 8.2 Hz) to δ 4.81 (br d, J = 4.2 Hz). The upfield shifts of about 2 ppm for C-5 in metabolite **6** and for C-13 in **7** were also observed in their ^{13}C NMR spectra. These data lead to the deduction that metabolites **6** and **7** are 5-deacetyl-1β-hydroxybaccatin I and 13-deacetyl-1β-hydroxybaccatin I, respectively. Though the former compound was supposedly first isolated from the leaves and stems of *T. yunnanensis*,[13] its structure was problematic. On comparison with a fuller investigation,[14] it is reasonable to suggest that 5-deacetylbaccatin I [13] is, in fact, 7-deacetyl-1β-hydroxybaccatin I. The signals of the highly shielded C-5β proton at δ 4.22 when 5α-hydoxyl is acetalyted and at δ 3.03 when the acetyl is absent is observed in substrate **2** and metabolite **6**. Hence, the structures of metabolites **6** and **7** have not been previously reported.

The composition of metabolite **8** is $C_{28}H_{40}O_{12}$ on the



(1) R_1=H, R_2=OH, R_3=H, R_4=a
(3) R_1=H, R_2=OH, R_3=H, R_4=H
(4) R_1=OH, R_2=H, R_3=H, R_4=a
(5) R_1=OH, R_2=H, R_3=H, R_4=H



	R_1	R_2
(2)	Ac	Ac
(6)	H	Ac
(7)	Ac	H
(8)	H	H

basis of FDMS and ^{13}C NMR spectral data. When we compared its ^{1}H NMR spectrum with that of the substrate **2** and metabolites **6** and **7**, we found that both the C-5 and the C-13 protons are shifted upfield. This observation leads us to the conclusion that the structure of metabolite **8** is 5,13-dideacetyl-1β-hydroxybaccatin I, also a new compound.

It has been suggested that the epoxide-bearing taxoids, such as substrate **2**, are important intermediates in the biosynthesis of Taxol-like taxoids.[15] What we are interested in is whether the endophytic fungi in *Taxus* spp. contribute to the biotransformation of the epoxide-bearing taxoids into the more bioactive oxetane-bearing analogues. The results from this experiment reveal that the activity of *A. alternata* does not appear to contribute to the formation of oxetanes. The enzyme systems of this fungus, which produce site-specific hydrolysis of the C-5 and the C-13 acetates, may be quite different from those of the plant, inasmuch as no C-5 or C-13 acetyl-free taxoids with a 4,20-epoxide have been isolated from *Taxus* spp.

Experimental Section

General Experimental Procedures. The ^{1}H NMR (400 MHz) spectra and ^{13}C NMR (100 MHz) were obtained in $CDCl_3$ on a Bruker ARX-400 spectrometer. FABMS were obtained using VG ZabSpec mass spectrometer, whereas FDMS employed a MAT 90 mass spectrometer. Analytical and preparative TLC were carried out on Si gel GF_{254} plates (Qingdao Oceanic Chemical, China), the developing system used was a $CHCl_3$−MeOH (94:6) solution, and visualization of plates was performed using 10% H_2SO_4 EtOH solution. For column chromatography, the absorbent used was Si gel H (300−400 mesh, Qingdao Oceanic Chemical, China).

Endophytic Fungi Isolation and Identification. The fungi used in these biotransformations were three of about 80 fungi isolated from the inner bark of *T. yunnanensis* Cheng et L. K. Fu obtained in Yunnan, China. These organisms were isolated by treating the yew bark pieces (1 cm × 1 cm) with 70% (V/V) EtOH and placing on potato dextrose agar (PDA). After several days, individual hyphal tips of the various fungi were transferred to new PDA plates. This process was repeated 3−5 times, and more than 80 different fungi were isolated. *Microsphaeropsis onychiuri* (Punith.) Morgan-Jones, *Mucor* sp., and *Alternaria alternata* (Fr.) Keissl were identified by Professor Yinglan Guo of the Institute of Microbiology, Chinese Academy of Sciences.

10-Deacetyl-7-epitaxol (1). 10-Deacetyl-7-epitaxol was isolated from the bark of *T. yunnanensis* Cheng et L. K. Fu as reported previously.[16]

1β-Hydroxybaccatin I (2). 1β-hydroxybaccatin I was also isolated from the bark of *T. yunnanensis* Cheng et L. K. Fu. Its structure was confirmed by spectral methods.

Transformation and Isolation Procedures with *Microsphaeropsis onychiuri*. *M. onychiuri* was grown in a flask (200 mL of medium per 500-mL conical flask). The medium was prepared by adding 20 g of dextrose to 1 L of 20% potato extract solution. The substrate (**1**, 320 mg) was dissolved in 32 mL Me_2CO−DMSO (30:2) and distributed evenly among eight culture flasks 3 days after inoculation (pH 5.33). After 9 days of incubation (25 °C, 150 rpm), the mixtures were pooled and filtered. The filtrate (1.6 L) was saturated with NaCl, extracted with EtOAc, dried over anhydrous Na_2SO_4, and evaporated to dryness at 40 °C under reduced pressure to afford a yellowish residue (170 mg). The residue was isolated by vacuum liquid chromatography (VLC) with Si gel H, using $CHCl_3$−MeOH gradient mixture as an eluting system to yield 80 mg of starting material (**1**, R_f = 0.6). Fractions 47−54, 78−83, and 86−92 were purified by preparative TLC, affording 15 mg of **3** with an R_f of 0.5, 6 mg of **4** with an R_f of 0.3, and 14 mg of **5** with an R_f of 0.2.

Compound 3: amorphous powder; FDMS *m*/*z* 544 (M^+).

Compound 4: amorphous powder; FABMS *m*/*z* 812 $(M + H)^+$.

Compound 5: amorphous powder; FABMS *m*/*z* 545 $(M + H)^+$.

Transformation and Isolation Procedure with *Mucor* sp. *Mucor* sp. was grown in a flask (200 mL of medium per 500-mL conical flask). The medium contained (per liter) tryptone 1 g, yeast extract 1 g, beef extract 1 g, and dextrose 5 g in distilled H_2O. The substrate (**1**, 400 mg) was dissolved in 40 mL of EtOH−DMF (4:1) and distributed evenly among eight culture flasks 3 days after inoculation (pH 5.48). After 9 days of incubation (28 °C, 160 rpm), the mixtures were pooled and filtered. The filtrate (1.6 L) was saturated with NaCl and extracted with EtOAc. The mycelium was washed and rinsed with EtOAc, and the EtOAc extracts of the broth and the mycelium were combined, dried over anhydrous Na_2SO_4, and evaporated at 40 °C *in vacuo*. Then 105 mg of starting substrate **1** accompanied by metabolite **4** was crystallized from the residue. The mother residue was chromatographed on Si gel H by the VLC method described earlier. Further purification by preparative TLC method yielded 6.2 mg of metabolite **3** and 16.4 mg of **5** as amorphous powders from VLC fraction 6 and fractions 11−13. Fraction 9 contained mainly metabolite **4**, and it was thus combined with impure material and purified further by preparative TLC to afford 81.2 mg of starting material **1** and 18 mg of metabolite **4** as amorphous powders.

Transformation and Isolation Procedures with *Alternaria alternata*. *A. alternata* was grown in flask (200 mL of medium per 500-mL Elenmeyer flask). The medium was prepared by adding 10 g of dextrose to 1 L of 20% potato extract solution. The substrate (**2**, 320 mg) was dissolved in 32 mL of Me_2CO−DMSO (2:1) and distributed evenly among eight culture flasks 3 days after inoculation (pH 6.08). After 12 days of incubation (28 °C, 170 rpm), the mixtures were pooled and filtered. The filtrate (1.6 L) was saturated with NaCl, extracted with EtOAc, and dried over anhydrous Na_2SO_4. The mycelium was heated to dry under 50 °C and extracted with CH_2Cl_2−MeOH (9:1). The EtOAc extract of the broth and the CH_2Cl_2−MeOH (9:1) extract of the mycelium were combined and evaporated to dryness at 40 °C under reduced pressure to afford a yellowish residue (1.84 g). The residue was isolated by the VLC method with Si gel H, using $CHCl_3$−MeOH gradient mixture as an eluting system to yield 180 mg of starting material (**2**, R_f = 0.7). Fractions 13−14, 16, and 18−20 were

purified by preparative TLC, affording 9.9 mg of **6** with an R_f of 0.5, 9.1 mg of **7** with an R_f of 0.4, and 5.5 mg of **8** with an R_f of 0.3.

Compound 6: amorphous powder; FABMS *m*/*z* 611 $[M + H]^+$, 633 $[M + Na]^+$; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2.

Compound 7: amorphous powder; FABMS *m*/*z* 633 $[M + Na]^+$; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2.

Compound 8: amorphous powder; FDMS *m*/*z* 568 $[M^+]$, 591 $[M + Na]^+$; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2.

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