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Microbial Transformation of Taxoids: Selective Deacetylation and Hydroxylation of 2α,5α,10β,14β-Tetra-acetoxy-4(20),11-taxadiene by the Fungus *Cunninghamella echinulata*

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Abstract: The fungal genus *Cunninghamella*, in particular, *Cunninghamella echinulata* AS 3·1990 selectively transformed 2α,5α,10β,14β-tetra-acetoxy-4(20),11-taxadiene (**1**) to give 6α,10β-dihydroxy-2α,5α,14β-triacetoxy-4(20),11-taxadiene (**2**) in moderate yield (33%), along with 6β,10β-dihydroxy-2α,5α,14β-triacetoxy-4(20),11-taxadiene (**3**), 10β-hydroxy-2α,5α,14β-triacetoxy-taxadiene (**4**), 10β-hydroxy-4β,20-epoxy-2α,5α,14β-triacetoxy-tax-11-ene (**5**) as minor products.
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The novel diterpenoid taxol, a promising antitumor agent, was first isolated by Wall and his collaborators from the bark of *Taxus brevifolia*.[1] The exciting therapeutic profile of this compound, combining with its limited availability, has made it the subject of intensive synthetic and cell culture investigations. The most promising options for large scale production of taxol are likely semisynthesis and cell culture.[2]

It seems that cell cultures are potential means of discovering interesting compounds that have not been previously isolated from intact plants. Recently a series of new taxoids with oxygen substituent at C-14 have been isolated from stem barks and roots of *Taxus yunnanensis* and *Taxus chinensis*.[3-5] Interestingly, the contents of some compounds from cell cultures of *Taxus yunnaxanesis*, in particular, 2α,5α,10β,14β-tetra-acetoxy-4(20),11-taxadiene (**1**) is very high (ca. 1-2% dry biomass).[6] Unfortunately, all of these C-14 oxygenated taxoids exhibit poor cytotoxicities, presumably due to their lack of C-13 side chain and oxetane ring which are necessary for tubulin binding.[7]

The biotransformation of taxol is an important area of study because a knowledge of its mammalian metabolism is an essential feature of its clinical pharmacology. The metabolism of taxol in rats has been reported, and several metabolites were isolated from rat bile and their structures were identified.[8-9] In human, the metabolites of taxol are different from those of rats. 6α-Hydroxytaxol is the major human metabolite of taxol and its structure has been determined.[10-12] Furthermore, the biotransformation of taxol and other taxoids offers potential pathways to the preparation of chemically inaccessible metabolites. Unfortunately, few studies of the metabolism of taxol and other taxoids by microbial systems have been reported so far. The first example is site-specific enzymatic hydrolysis of taxanes at C-10 and C-13.[13]

Biotransformation of diterpenes has been studied as means of producing new compounds of pharmaceutical and agricultural potential.[14] Encouraged by these promising studies and the high content of compound **1** from cell cultures of *T. yunnaxanesis*, we have carried out an extensive screening of microorganisms in order to examine their abilities to biotransform **1**.

RESULTS AND DISCUSSION

All of the fungal strains used were shown in experimental section. Of the cultures examined, several strains of the genus *Cunnighamella* could convert **1** and exhibited similar patterns of polar metabolites of **1** as evidenced by TLC and HPLC: *C. echinulata* AS 3·1990, *C. echinulata* AS 3·2000, *C. echinulata* AS 3·2474, *C. elegans* AS 3·1207, *C. elegans* AS 3·2033, *C. elegans* AS 3·2477.

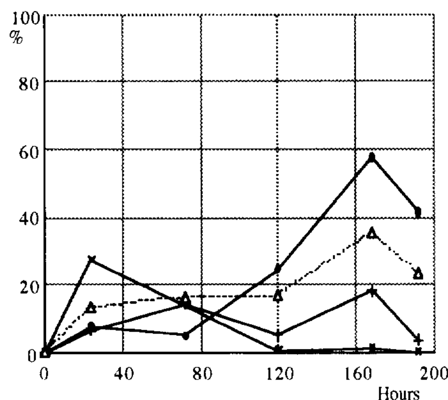
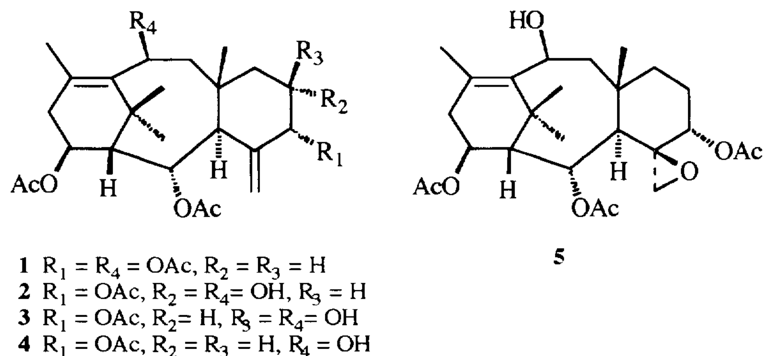


Fig. 1 Time-course of metabolite production from **1** (0.2 g/L) by *C. echinulata*, (derived from HPLC determinations in the incubation medium): x——x, **1**; •——•, **2**; +——+, **4**; Δ·····Δ, other products.



C. echinulata AS 3·1990 seemed to be the most efficient strain and it produced a major polar product in 55% yield (measured by HPLC) after 7 days incubation (Fig. 1), along with several other more polar metabolites than **1**. So it was selected for preparative scale transformation of **1**. However, when **1** was incubated with the other fungal strains examined, generally no metabolites were detected by TLC and HPLC. Large amount of substrate **1** was recovered from the supernatant and the mycelium.

C. echinulata was employed in the preparative scale study for characterizing the converted products. After 7 days incubation of **1** with *C. echinulata*, we isolated four pure metabolites of **1** from the supernatant. On the

basis of spectroscopic analysis, particularly, mass spectra and NMR techniques, the main metabolite was identified as 6α,10β-dihydroxy-2α,5α,14β-triacetoxy-4(20),11-taxadiene (**2**), a 6α-hydroxylated derivative of **1**, along with 10β-deacetylation at C-10. The other three minor metabolites were identified as 6β,10β-dihydroxy-2α,5α,14β-triacetoxy-4(20),11-taxadiene (**3**), 10β-hydroxy-2α,5α,14β-triacetoxy-taxadiene (**4**), 10β-hydroxy-4β,20-epoxy-2α,5α,14β-triacetoxy-tax-11-ene (**5**), respectively.

Table 1 ^{1}H-NMR Data of compouds **2-5** ($CDCl_3$, 500 MHz)

Carbon	**2**	**3**	**4**	**5**
1	1.92 (d, 2.2)	1.89 (d, 2.3)	1.88 (d, 2.2)	1.67 (s)
2	5.37 (dd, 6.6, 2.5)	5.35 (m)	5.36 (dd, 6.5, 2.2)	5.34 (dd, 3.8, 1.5)
3	2.96 (d, 6.4)	2.89 (d, 6.4)	2.93 (d, 6.4)	2.55 (d, 3.8)
5	5.04 (d, 1.6)	5.42 (d, 3.9)	5.28 (t, 2.9)	4.20 (brs)
6	3.89 (m)	3.92 (ddd, 4.1, 5.5, 11.8)	1.80 (m)	1.30 (m), 1.86 (m)
7	1.47 (d, 15.8), 1.99 (dd, 4.5, 15.0)	1.26 (d, 5.9) 1.53 (dd, 5.3, 13.0)	1.25 (m), 1.96 (m)	1.26 (m), 2.04 (m)
9	1.69 (dd, 15.2, 5.7), 2.26 (dd, 11.7, 14.9)	1.72 (dd, 14.8, 5.9) 2.33 (dd, 12.4, 14.9)	1.53 (dd, 14.9, 5.6), 2.44 (dd, 11.8, 14.8)	1.60(dd, 14.9, 5.5), 2.33 (dd, 11.7, 14.8)
10	5.05 (m)	5.07 (dd, 5.6, 11.7)	5.11 (dd, 5.6, 11.7)	5.10 (dd, 5.6, 11.7)
13	2.43 (dd, 4.5, 19.3), 2.80 (dd,18.9, 9.2)	2.44 (dd, 3.8, 18.9) 2.78 (dd, 18.9, 9.3)	2.43 (m), 2.80 (dd,18.9, 9.3)	2.53 (dd, 3.8, 21.3), 2.63 (dd, 20.3, 9.1)
14	5.01 (dd, 9.2, 5.0)	5.00 (dd, 9.3, 4.9)	5.00 (dd, 9.2, 4.8)	5.16 (dd, 9.1, 4.9)
16	1.72 (s)	1.71 (s)	1.72 (s)	1.67 (s)
17	1.19 (s)	1.19 (s)	1.19 (s)	1.19 (s)
18	2.19 (s)	2.23 (s)	2.16 (s)	2.14 (s)
19	1.05 (s)	0.87 (s)	0.84 (s)	1.07 (s)
20	5.41 (s), 4.97 (s)	5.36 (s), 4.94 (s)	5.27 (s), 4.85 (brs)	2.25 (d, 5.2), 3.67 (d,5.2)
2-OAc	2.02 (s)	2.04 (s)*	2.02 (s)	1.98 (s)*
5-OAc	2.05 (s)	2.05 (s)*	2.04 (s)	2.06 (s)*
14-OAc	1.94 (s)	1.99 (s)	1.98 (s)	1.98 (s)

*Interchangeable assignment

FAB mass spectra analysis of **2** showed an $[MH]^+$ fragment peak at m/z 479, consistent with the molecular formula of $C_{26}H_{38}O_8$. The ^{1}H-NMR spectrum of **2** was similar to that of **1**, but only three OAc signals were observed. The C-10 proton signal shifted upfield 0.9 ppm as compared with that of **1**, strongly suggesting a free hydroxyl group at C-10 in **2**. The ^{1}H-NMR spectrum of **2** further showed that the resonances corresponding to H-6α or 6β (δ 1.8) in **1** had disappeared, and one oxygen-bearing methine signal appeared at δ 3.89, suggesting an insertion of a hydroxyl group at C-6 position in **2**. The connectivities of protons in **2** were determined by analysing ^{1}H-^{1}H COSY spectrum. The 1H multiplet at δ 3.89 was assigned to C-6 methine

proton, based upon its couplings with the H-5β doublet at δ 5.04 (J= 1.6 Hz), the H-7 doublet at δ 1.47 (J= 15.0 Hz) and the H-7 doublet of doublets at δ 1.99 (J= 4.5 and 15.0 Hz). Both H-7 signals were geminally coupled (J= 15.0 Hz). All resonances observed in the ^{1}H-NMR spectrum of compound 2 were assigned and listed in Table 1.

Table 2 ^{13}C-NMR Data of compounds **2-5** ($CDCl_3$, 125 MHz)

Carbon	**2**[15]	**3**	**4**	**5**
1	59.03	59.04	59.21	59.06
2	70.33	70.61	70.73	69.77
3	42.37	41.96	36.86	36.63
4	138.92	140.27	142.50	59.51
5	81.61	80.51	78.41	78.44
6	70.15	69.13	28.96	24.47
7	41.53	41.08	33.99	33.05
8	39.49	38.00	37.50	38.51
9	47.64	46.61	47.25	47.71
10	67.46	67.25	67.44	67.43
11	138.67	138.84	138.79	139.40
12	132.26	132.43	132.39	133.31
13	39.52	39.24	39.55	39.63
14	70.71	70.73	70.79	70.16
15	37.51	37.46	37.50	38.36
16	25.41	25.37	25.44	25.49
17	32.09	32.13	32.13	31.77
18	21.55	21.13	21.07	21.16
19	25.41	23.45	22.56	22.75
20	120.42	119.59	116.88	50.12
O$\underline{CO}$CH$_3$	169.99	170.98	169.97	169.16
O$\underline{CO}$CH^3	169.92	170.00	169.97	169.16
O$\underline{CO}$CH^3	169.47	169.90	169.75	169.16
OCO$\underline{CH_3}$	21.40	21.65	21.80	21.49
OCO$\underline{CH_3}$	21.35	21.38	21.45	21.26
OCO$\underline{CH_3}$	21.05	21.38	21.39	21.70

HETCOR analysis was used to assign carbon signals for all proton-bearing carbons. The downfield signal at δ 70.15 was assigned to the C-6 methine as it could be correlated with the 1H multiplet at δ 3.89. The resonance for C-10 at δ 67.46 correlated exactly with the corresponding signal at δ 5.05. A full assignment of ^{13}C-NMR resonances was realized (Table 2), which confirmed the structure elucidation of **2**.[15]

The stereochemistry of the 6-hydroxyl group was determined from the NOE experiment. Recently, the stereochemistry of 6α-hydroxytaxol, the principal human metabolite of taxol was determined based upon the NOE difference spectra.[11-12] For compound **2**, the NOE between H-6 and Me-19 was produced when Me-19 was irradiated. Furthermore, when H-6 was irradiated, an enhancement of H-5 was produced. These data showed that the 6-hydroxyl group was introduced by *C. echinulata* in **2** at α-position.

FABMS of compound **3** showed an elemental composition of $C_{26}H_{38}O_8$, which had the same molecular formula as compound **2**. By comparing its ^{1}H-NMR spectrum with that of **2**, it was clear that the structure of **3** was essentially similar to that of **2**, which was confirmed by their ^{13}C-NMR spectra data. The assignments of the ^{1}H-NMR spectra data were established by analysis of the ^{1}H-^{1}H COSY, which indicated that **3** had the same oxygenation pattern as **2**. On the basis of the spectra evidence described above, the structure of **3** was thereby established as 6β-hydroxy epimer of **2**. In the NOE difference spectra, neither NOE between H-6 and Me-19 nor betweent H-5 and H-6 was observed as expected.

FAB mass measurement ($[MH]^+$ at m/z 463) of **4** was consistent with the molecular formula $C_{26}H_{38}O_7$. The ^{1}H-NMR spectrum of **2** was similar to that of **1**, but only three OAc signals were observed. The C-10 proton signal shifted upfield from δ 6.06 to 5.11 indicating an OH, not an OAc group present at C-10. Therefore, the structure of **2** is 10-deacetyl derivative of **1**, which was also supported by ^{13}C-NMR signal at δ 67.44. The assignements of ^{1}H and ^{13}C-NMR spectra data of **4** were simplified by direct comparison with those of **1**-**3**.

FAB mass mesurement and elemental analysis of **5** were corresponding to the molecular formula $C_{26}H_{38}O_8$. The ^{1}H-NMR spectrum of **5** showed only three acetyl signals and the H-10 signal was 0.96 ppm upfield shift from δ 6.06 to 5.10, suggesting the presence of a free hydroxyl group at C-10. The ^{1}H-NMR spectrum further indicated the disappearance of the signals corresponding to the C-20 exocyclic methylene protons at δ 4.86 and 5.27 in **1** and the appearance of two doublets at δ 2.25 and 3.67 (J= 5.2 Hz) assigned to the C-20 methylene protons of the oxirane bridge, which were supported by the carbon resonances at δ 59.51 and 50.12 assigned to C-4 and C-20, respectively. These assignments were consistent with those for some other taxane epoxides.[16] The configuration of the 4,20 epoxide was deduced to be β, by comparison with the precedent taxane oxiranes.[17-18] The ^{1}H-NMR spectrum showed the downfield (0.23 ppm) of methyl 19 and the upfield of the proton 5β (1.09 ppm) due to their proximities to the 4-oxygen substituent further supported the configuration of 4β,20 epoxide in **5**.

The results obtained in this study indicated that the taxane **1** could be stereospecifically hydroxylated at the 6-position, along with deacetylation at the C-10 position by the fungus *C. echinulata*. The main product **2** was obtained in 33%. Unfortunately, the metabolite **2** showed poor antitumor activity. Interestingly, 6α-hydroxytaxol as the major human metabolite of taxol was recently isolated and identified by two different groups in USA.[10-11] The hydroxylation of taxol in human liver was stereospecific and was catalyzed by cytochrome P-450.[11] Hence, we suggested the hydroxylation of **1** by *C. echinulata* could be catalyzed by cytochrome P-450 as many other hydroxylation reactions performed by this fungus.

The 4(20)-oxirane ring in taxane diterpenes was considered to be derived biogenetically from compounds with an exocyclic methylene group at C-4 by epoxidation of the double bond.[19] The production of the minor metabolite **5**, a 4β(20)-epoxide derivative from **1** suggested the epoxidases in *C. echinulata* could epoxidize the exocyclic double bonds in taxanes and also provided a proof for the biogenetic hypothesis of the taxane with a 4(20)-oxirane ring.

EXPERIMENTAL SECTION

General

Melting points were determined on a Yanaco apparatus and uncorrected. Optical rotations were measured with Perkin-Elmer 241 polarimeter. IR spectra were obtained on a Perkin-Elmer 683 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Brucker AM-500 spectrometer (^{1}H-NMR, 500 MHz; ^{13}C-NMR, 125 MHz) in $CDCl_3$with TMS as the internal standard. Chemical shifts (δ) are given in parts per million (ppm). Coupling constants (J) are given in hertz (Hz). FAB mass spectra were measured on a VG Zabspec mass spectrometer and CI mass spectra were performed on a VG ZAB-2f mass spectrometer. Macrosporous styrene resin DA-201 (10-40μ) was purchased from Agriculture Chemical Factory of Tianjing, China. Si gel F254 and silica gel (200-300 mesh) were obtained from Qingdao Marine Chemical Factory, China.

Substrate

2α,5α,10β,14β-Tetra-acetoxy-4(20),11-taxadiene (**1**) was isolated from the cell cultures of *Taxus yunnanensis* in ca. 1-2%. dry biomass.[6] Its structure was fully characterized on the basis of the spectral methods.[4-5]

Microorganisms

Microorganisms were obtained from Institute of Microbiology, Academia Sinica (AS) and Institute of Antibiotic (IA), the Chinese Academy of Medical Sciences, Beijing, China. During preliminary screening studies, ten genera (23 species) of fungi were used, which included *Adsidia* (*A. coerula*), *Aspergillus* (*A. niger* AS 3·3883 and *A. niger* AS 3·4523), *Beauveria* (*B. sp.* AS 3·3575), *Cunnighamella* (*C. blakesleana* AS 3·910, *C. echinulata* AS 3·953, *C. echinulata* AS 3·1990, *C. echinulata* AS 3·2000, *C. echinulata* AS 3·2474, *C. elegans* AS 3·1207, *C. elegans* AS 3·2033, *C. elegans* AS 3·2477), *Curvularia* [*C. lunata* AS 3·3589, *C. lunata* AS 3·4381(NRRL 2380)], *Gibberella* (*G. fujikuroi* IA Gib-1), *Mortierella* (*M. isabellina* AS 3·3410), *Mucor* (*M. griseo-cyanus* AS 3·2725, *M. plumbeus* AS 3·201, *M. rouxianus* AS 3·3447), *Penicillum* (*P. soinulosum* AS 3·149), *Rhizopus* (*R. arrhizus* AS 3·2744, *R. japonicus* AS 3·1218, *R. nigricans* AS 3·2050).

Culture and general biotransformation procedure

Microorganisms used in this study were maintained on potato dextrose agar slants and freshly subcultured before using in transformation experiments. The microorganism was inoculated into 250-mL Erlenmeyer flasks, each containing 50 mL of culture medium (g per litre: glucose, 30; cornsteep, 10; K_2HPO_4, 2; KH_2PO_4, 1; $NaNO_3$, 2; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.02. Sterilization in an autoclave 30 min at 115°C and cultured at 27°C on a reciprocal shaker (80 rpm). After 48 h of incubation, 0.5 mL of a 200 mg/L solution of **1** in acetone (containing Tween 80: 0.05 mL/mL) was added to the culture. The incubation allowed to continued for further 8-10 days. Culture controls consisted of fermentation blanks in which organisms were grown under identical conditions without substrate. Substrate controls consisted of steril medium containing the same amount of substrate and incubated under the same conditions. Samples (1ml) of substrate containing fermentation culture were withdrawn at various interval and extracted with 0.5 ml of CH_2Cl_2, concentrated CH_2Cl_2 extracts were spotted on TLC which was developed by petroleum ether -acetone (7:3). Taxoids were detected by spraying with a 10% H_2SO_4 solution, followed by heating. The extract was also measured by analytical HPLC using a C_{18} (0.46 cm x 25 cm) column to follow the course of the biotransformation. Elution

was achieved with MeOH-H_2O (70:30) with a flow rate of 1.0 mL/min. UV-detection was performed at 227 nm.

For preparative scale biotransformations, five Erlenmeyer flasks (250 mL), each containing 50 mL of the same medium was inoculated with freshly obtained spores from slants of *C. echinulata* and incubated in the same conditions. After 24 h incubation, each 50 ml of culture was used to inoculated 5-L Erlenmeyer flasks (x5) containing 1 L of the same medium. After 48 h of growth on a reciprocal shaker (80 rpm) at 27°C, 150 mg of **1** dissolved in 10 ml of acetone (containing Tween 80: 0.05 mL/mL) was added to each flask. During the incubation, pH was maintained at 6.0 with 20% HCl.

After further 8 days incubation, the mycelium was removed by filtration through celite. The supernatant passed through a macrosporous polystyrene resin then was eluted with ethanol. After evaporation of ethanol in vacuo, the aqueous layer was continously extracted with ethyl acetate. After evaporation of the acetyl acetate in vacuo, 1.5 g of crude residue was obtained, which was chromatographed on a silica gel column. Elution with petroleum ether-acetone-ethyl acetate (90:8:2) gave unchanged substrate **1** (60 mg). Elution with petroleum ether-acetone-ethyl acetate (85:13:2) gave **4** (68 mg) followed by a mixture of **4** and **5** (24 mg, examined by TLC), and pure product **5** (23 mg). Elution with petroleum ether-acetone-ethyl acetate (70:28:2) afforded **2** (210 mg). Elution with petroleum ether-acetone (65:35) afforded a mixture of **2** and **3** contaminated by some unidentified products (157 mg), which was further separated by silica gel chromatography, using CH_2Cl_2-acetone as eluent to give pure **2** (22 mg) and impure **3** (66 mg). For further purification of **3**, impure **3** was silylated by DMBSCl (10 equiv) in DMF/imidazole which gave a pure product **6** after purification by flash chromatography, and then **6** was treated with Bu_4NF in THF to afford pure **3** (40 mg) after flash chromatography. In addition, the dry mycelium was extracted with acetone. After evaporation of the solvent, the crude residue obtained was chromatographed on a silica gel column, using petroleum ether-ethyl acetate (8:1, 6:1 and 3:1) as eluent to afford 106 mg of unchanged substrate **1**.

6α,10β-Dihydroxy-2α,5α,14β-triacetoxy-4(20),11-taxadiene (**2**). White amorphous solid, mp 215-218°C; $[\alpha]_D^{25}$ +17.5°(c= 0.083, MeOH); IR ν max (KBr) 3447, 2928, 1736, 1645, 1371, 1252, 1024 cm^{-1}; FABMS m/z 479 (17), 401 (100), 341 (30), 281 (48); CIMS m/z 461 $[MH-H_2O]^+$ (1), 419 (5), 401 (27), 341 (38), 299 (68), 281 (100); Elemental analysis C 64.67%, H 8.00%, O 27.33% (calc. for $C_{26}H_{38}O_8$ C 65.25%, H 8.00%, O 26.75%); ^1H-NMR data, see Table 1; ^{13}C-NMR data, see Table 2.

6β,10β-Hydroxy-2α,5α,14β-triacetoxy-4(20),11-taxadiene (**3**). White amorphous solid, mp 85-88°C; $[\alpha]_D^{25}$ +47.1° (c= 0.047, MeOH); IR ν max (KBr) 3449, 2928, 1736, 1645, 1373, 1246, 1026 cm^{-1}; FABMS m/z 501 $[M+Na]^+$ (36), 479 (26), 475 (54), 401 (100); Elemental analysis C 64.34%, H 8.27%, O 27.39% (calc. for $C_{26}H_{38}O_8$ C 65.25%, H 8.00%, O 26.75%); ^1H-NMR data, see Table 1; ^{13}C-NMR data, see Table 2.

10β-Hydroxy-2α,5α,14β-triacetoxy-taxadiene (**4**) -Needles, mp 184-186°C; $[\alpha]_D^{25}$ +30.7° (c= 0.0374, MeOH); IR ν max (KBr) 3472, 2926, 1744, 1703, 1383, 1240, 1030 cm^{-1}; FABMS m/z 463 $[MH^+]$ (14), 445 (6), 385 (73), 136 (100); CIMS m/z 403 $[MH-HOAc]^+$ (2), 385 (14), 361 (28), 283 (100), Elemental analysis C 66.07%, H 8.60%, O 25.33% (calc. for $C_{26}H_{38}O_7$ C 67.53%, H 8.23%, O 24.24%); ^1H-NMR data, see Table 1; ^{13}C-NMR data, see Table 2.

10β-Hydroxy-4β,20-epoxy-2α,5α,14β-triacetoxy-tax-11-ene (**5**). Needles, mp 178-180°C; $[\alpha]_D^{25}$ +88.7° (c= 0.0568, MeOH); IR ν max (KBr) 3512, 2932, 1740, 1437, 1369, 1236, 1022 cm^{-1} ; CIMS m/z 479 $[MH^+]$ (21), 419 (20), 401 (43), 359 (79), 341 (31), 299 (100), 281 (81), 269 (24); FABMS m/z 479 $[MH^+]$

(25), 419 (14), 401 (100), 359 (26), 341 (9), 299 (31); Elemental analysis C 65.73%, H 8.08%, O 26.19% (calc. for $C_{26}H_{38}O_8$ C 65.25%, H 8.00%, O 26.75%); ^{1}H-NMR data, see Table 1; ^{13}C-NMR data, see Table 2.

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