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MICROBIAL TRANSFORMATION OF DITERPENES: HYDROXYLATION OF 17-ACETOXY-KOLAVENOL ACETATE BY *MUCOR PLUMBEUS*

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Key Word Index—*Mucor plumbeus*; biotransformation; diterpenes; clerodanes; kolavene derivatives.

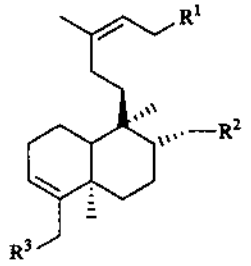
Abstract—When 17-acetoxy-kolavenol acetate was subjected to biotransformation with *Mucor plumbeus*, the substrate was deacylated and converted into 17,19-dihydroxy-kolavenol, 3,4,17-trihydroxy-3α,4β-dihydro-kolavenol, and an isomeric mixture of 13,17,19-trihydroxy-clerod-14,15-ene. The three compounds were identified as their acetate derivatives.

INTRODUCTION

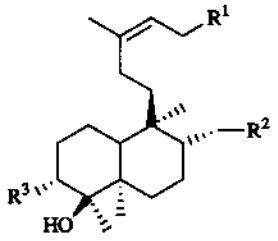
The biotransformation of steroidal molecules has been extensively investigated [1, 2]. However, limited published data on the biotransformation of diterpenes exists and the results from the biotransformation of steroids do not always translate to diterpenes [3]. For example, the fungus *Mucor plumbeus* can selectively introduce a 6β-hydroxy group into pregnanes [4]. By contrast, the diterpene sclareol was transformed into a 3β-hydroxy derivative in high yields [5]. Although this fungus is capable of oxidizing other positions in diterpenes, the preferred attack appears to be the 3 and 18 or 19 positions in labdanes. To determine how the clerodane nucleus is transformed, we decided to use 17-acetoxy-kolavenol acetate (**1a**) as a model substrate with a 3,4-double bond, an allylic methyl group at C-4 coupled with an axial methyl group at C-5. We report here the biotransformation of **1a** into three major further hydroxylated kolavene derivatives.

RESULTS AND DISCUSSION

Due to the instability of 17-hydroxy-kolavenol (**1**) and its derivatives, the substrate was added as its diacetate analogue. When **1a** was incubated at 0.5 g l^{-1} for 10 days with a stage II culture of *M. plumbeus*, no starting substrate was detected in the filtrate and less than 1% was recovered from the cell mass. The major product isolated was the mono-hydroxylated product 17,19-dihydroxy-kolavenol (**2**). The second highest yielding product was identified as 3,4,17-trihydroxy-kolavenol (**3**). The final recovered product was a mixture of isomeric 13,17,19-trihydroxy-kolavenols (**4**). After the initial ^{1}H NMR spectra of all recovered products verified the absence of any acetate groups, these unstable products were immediately acetylated and identified as their acetate derivatives.



	R^1	R^2	R^3
1	OH	OH	H
1a	OAc	OAc	H
2	OH	OH	OH
2a	OAc	OAc	OAc

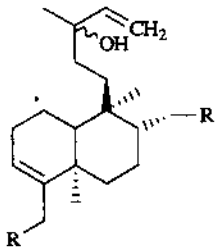


	R^1	R^2	R^3
3	OH	OH	OH
3a	OAc	OAc	OAc

Comparing the NMR data (Tables 1 and 2) of **1a** with **2a** readily demonstrated that the only change that had occurred was the introduction of an acetoxy group at C-19. In the ^{1}H NMR spectra the C-19 methyl at δ1.56 was

Table 1. ^{1}H NMR spectral data [δ (J = Hz), $CDCl_3$, 200 MHz]

C	1a	2a	3a	4Aa	4Ab
3	5.17 *br s*	5.59 *br s*	4.70 *t* (2.5)	5.58 *t* (3.4)	
14	5.31 *br t* (7.0)	5.31 *br t* (7.2)	5.29 *br t* (7.6)	5.92 *dd* (14.0, 8.7)	5.85 *dd* (14.0, 8.7)
15	4.54 *d* (3.5)	4.56 *d* (3.5)	4.53 *d* (3.6)	5.22 *dd* (10.0, 0.7)	5.21 *dd* (10.0, 0.7)
				5.04 *dd* (6.2, 0.9)	5.03 *dd* (6.2, 0.7)
16	1.68 *s*	1.67 *s*	1.68 *s*	1.29 *s*	1.26 *s*
17	3.76 *dd* (9.6, 4.2)	3.76 *dd* (9.6, 4.2)	3.74 *dd* (11.5, 4.1)	3.64 *dd* (9.6, 4.1)	3.56 *dd* (9.6, 4.1)
	4.19 *dd* (7.5, 2.0)	4.19 *dd* (7.5, 2.0)	4.16 *dd* (7.4, 2.0)	4.35 *dd* (6.3, 1.0)	4.35 *dd* (6.3, 1.0)
18	0.77 *s*	0.79 *s*	0.77 *s*	0.79 *s*	0.78 *s*
19	1.56 *d* (0.7)	4.51 *s*	1.16 *s*	4.50 *s*	4.50 *s*
20	0.99 *s*	1.07 *s*	1.06 *s*	1.07 *s*	1.07 *s*
OAc	2.05 *s* (×2)	2.05 *s* (×2)	2.02 *s* (×2)	2.06 *s*	2.06 *s*
		2.06 *s*	2.03 *s*	2.05 *s*	2.05 *s*



	R
4a, b	OH
4Aa, b	OAc

absent and replaced with a singlet for two protons at δ4.51 coupled with an additional acetate singlet at δ2.03. In addition to the extra acetate group, in the ^{13}C NMR spectra the major difference was the C-19 singlet shifting to δ64.8 and verified as a -CH_2O- grouping by DEPT.

The EI mass spectra of **1a** and **2a** were very similar and the fragmentation pattern was almost identical to the previously reported mass spectra obtained for 19 kolavenol derivatives [6]. In both cases, the $[M]^+$ was not observed, but the highest observed peak was at m/z 375 and 433, respectively, corresponding to $[M-15]^+$. The other observed peaks are due to the loss of acetic acid and the previously reported fragmentation scheme [6].

Compound **3a** was formed from **1a** by transhydroxylation of the 3,4 double bond or epoxidation followed by *trans*-ring opening. The NMR data were instrumental in the identification of **3a**. The DEPT ^{13}C NMR clearly established the new hydroxylated signals as –CHO– at δ76.8 and a quaternary –C–O– at δ75.2. The disappearance of the double bond at δ126.2, 142.6 and the addition of another acetate group added further proof to the assigned structure. In the ^{1}H NMR spectrum the H-3 signal occurred as a broadened triplet at δ4.70 with the largest observed coupling constant of 2.5 Hz indicating an equatorial hydrogen corresponding to an axial acetoxy group. The C-19 methyl was shifted to δ1.16 indicating an axial methyl or an equatorial hydroxyl group at C-4 [7]. Therefore, **3a** was assigned the structure as drawn. The highest observed peak in the EI mass spectrum was at m/z 406 $[M-HOAc]^+$. The remainder of the spectrum

Table 2. ^{13}C NMR data (δ, $CDCl_3$, 50.3 MHz)

C	1a	2a	3a	4Aa	4Ab
1	26.6	26.5	27.2	27.1	27.1
2	17.7	17.5	16.3	17.4	17.4
3	120.5	126.2	76.8	126.4	126.3
4	144	142.6	75.2	142.4	142.3
5	38	38	41.1	35.4	35.3
6	36	35.4	31.3	35	35
7	22.2	22	21.3	22.6	22.6
8	40.7	40.7	40.5	40.7	40.6
9	37.8	37.5	37.9	37.7	37.5
10	46.3	46.1	40.1	46	46
11	32.7	32.7	32.8	31.5	31.5
12	36.4	36.5	36.6	31.9	31.7
13	142.7	142.3	142.6	73	73
14	118	118.1	118	145.6	144.5
15	61.3	61.4	61.3	112	111.5
16	16.7	16.8	16.7	29.6	28.8
17	66.2	66	66	66.3	66.2
18	19.8	22	16.7	21.9	21.9
19	17.9	64.8	21.4	64.9	64.9
20	19.2	19.2	19.2	19.2	19.2
MeCO					
CO-1′	171.3	173	171.3	171.4	171.4
Me-2′	21	21.2	21.2	21.2	21.2
CO-1″	171	172	171	170.9	170.9
Me-2″	21	21	21	21.1	21
CO-1‴		171	170.1		
Me-2‴		21	21		

was similar to the previously reported schematic with a base peak at *m/z* 187, which was identical to **2a**.

The isomeric mixture of **4Aa** and **b** had NMR spectral features in common with ring A of **2a**, indicating the formation of an acetoxy group at C-19. The rest of the molecule had spectral features in common with sclareol, resulting from solvolysis of the hydroxyl group at C-15, migration of the double bond and neutralization of the carbonium ion formed at C-13, probably with water of the medium. This resulted in the formation of the classical ^{1}H NMR patterns for the C-14 and C-15 hydrogens as presented in Table 1 [8]. Likewise, the C-16 methyl signal was shifted from δ1.68 to 1.29. The fact that both the *S* and *R* configurations at C-13 are present can easily be distinguished by the doubling of the signals in the NMR spectra. The data from the ^{13}C NMR of this mixture match very well with the previously reported data for model labdanes, including sclareol [9]. Due to the instability of this mixture, no parent peak was detected in the EI mass spectrum, but the fragmentation pattern was very similar to the other kolavenol derivatives, including the base peak at *m/z* 187. Therefore, the assumed structures as drawn fit the NMR data extremely well and these assignments are supported by the published literature.

In the case of kolavenols, *M. plumbeus* appears to preferentially hydroxylate at the C-19 position and to attack the 3,4 double bond. These results indicate that this microorganism's oxidative biochemistry is designed for the A ring in diterpenes. The presence of other functional groups in the molecules can redirect some of this biochemical activity as in the case of manool [10]. Also, the first step in the biotransformation of **1a** appeared to be deesterification followed by oxidation. Due to the instability of these compounds, the yields may be higher than the recovered amounts indicate. Additional reactions in the presence of the aqueous medium may explain the large number of relatively low yielding additional products present in the filtrate. Nevertheless, when **1a** was subjected to identical conditions in the absence of the fungus, almost 80% of the substrate was recovered unreacted.

EXPERIMENTAL

For general experimental conditions, see refs [10, 11] and references within.

Isolation of compound **1a**. The CH_2Cl_2 extract obtained from *Vanclevea stylosa* was taken up in Et_2O and partitioned with 5% Na_2CO_3. The aq. layer was then neutralized with HCl and reextracted with Et_2O to give acids I. This crude complex mixture was hydrolysed with $NaHCO_3$. After the hydrolysis was completed, the aq. mixture was extracted with Et_2O, which was then washed with $NaHCO_3$ followed by H_2O until the pH was neutral. To stabilize the neutrals, the mixture was acetylated in the usual manner followed by CC to recover **1a** as the major component. EIMS: *m/z* (rel. int.): 375 (12) [M $-$ Me]$^+$, 330 (29) [M $-$ HOAc]$^+$, 315 (12), 301 (9), 270 (6), 255 (23), 202 (10), 199 (8), 189 (41), 188 (25), 187 (93), 185 (18), 173 (20), 171 (11), 161 (12), 159 (33), 157 (11), 149 (6), 147 (19), 145 (35), 95 (base), 93 (46), 91 (33), 83 (11), 81 (57), 79 (37), 77 (14), 69 (26), 67 (35), 57 (9), 55 (41) and 53 (15).

Incubation experiments. Mucor plumbeus was maintained on PDA slants. The biotransformations were run in 250 ml flasks containing 50 ml of a modified medium consisting of glucose (30 g), yeast extract (5 g), gofio (cornmeal) (6 g), NaCl (5 g), KH_2PO_4 (3 g), H_2O (1 l). The standard two-stage fermentation technique was used. Forty-eight hr after the start of stage II, each flask (60 total) was innoculated with 25 mg of **1a**, that was dissolved in absolute EtOH and Tween 80. The reaction was stopped after 168 hr and the product recovered by filtration followed by partitioning the filtrate with EtOAc. The EtOAc was removed under vacuum, and subjected to CC. The residue was extracted with Me_2CO, dried, pulverized and re-extracted with Me_2CO. The fractions were found to contain negligible amounts of **1** or **1a** and indiscernible amounts of products according to TLC.

17,19-*Diacetoxy-kolavenol acetate* (**2a**). The EtOAc extract (630 mg) was separated by CC (2 × 44 cm, 40 g silica gel) eluted with CH_2Cl_2–MeOH, 20 ml frs (24:1). Fraction 16 yielded pure **2** oil which was then acetylated in the usual manner to yield 35 mg of **2a**, (found: [M $-$ ketene]$^+$ at *m/z* 406.2689, required 406.27192 for $C_{26}H_{40}O_6 - C_2H_2O$). EIMS, *m/z* (rel. int.): 406 (2) [M $-$ ketene]$^+$, 331 (11), 328 (14) [M $-$ 2HOAc]$^+$, 313 (9), 268 (9), 253 (13), 213 (8), 211 (11), 203 (11), 187 (base), 185 (49), 173 (18), 171 (23), 169 (14), 159 (34), 157 (29), 149 (13), 147 (12), 145 (48), 143 (21), 135 (11), 133 (19), 131 (39), 121 (21), 119 (43), 117 (14), 109 (22), 107 (35), 105 (43), 95 (30), 93 (52), 91 (41), 83 (12), 81 (45), 79 (33), 77 (13), 69 (22), 67 (25), 57 (12), 55 (35) and 53 (9).

3,17-*Diacetoxy*-4-*hydroxy-kolavenol acetate* (**3a**). From column frs 33–37, **3** was isolated as an amorphous powder, after acetylation 62 mg of **3a** was obtained, (found: [M $-$ HOAc]$^+$ at *m/z* 406.26923, required 406.27192 for $C_{26}H_{42}O_7 - HOAc$); EIMS, *m/z* (rel. int.): 406 (5), 388 (2), 362 (4), 325 (20), 278 (5), 263 (46), 247 (99), 205 (57), 203 (36), 187 (base), 161 (25), 147 (26), 135 (33), 133 (29), 121 (28), 119 (18), 116 (18), 107 (32), 93 (14), 81 (20).

17,19-*Diacetoxy*-13-*hydroxy-clerod*-14,15-*ene* (**4Aa** *and* **b**). From column frs 41–46, **4a** and **b** were obtained as a highly unstable oil, which was acetylated, rechromatographed with hexane–Me_2CO (9:1) to yield 8 mg of **4Aa** and **b**. HRCI-MS (found *m/z* 328.2345, required 328.2402 for $C_{24}H_{38}O_5 - HOAc - H_2O$). EIMS, *m/z* (rel. int.): 328 (8) [M $-$ HOAc $-$ H$_2$O]$^+$, 313 (12), 253 (18), 185 (78), 171 (26), 157 (40), 147 (50), 131 (55), 119 (base), 107 (48), 93 (60), 81 (55), 71 (30) and 55 (24).

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