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The microbiological hydroxylation of some 2,9-dioxygenated clovanes by *Mucor plumbeus*

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Abstract

The fungus, *Mucor plumbeus*, has been shown to hydroxylate 9α- and 9β-hydroxy and 9-oxo-2β-methoxyclovane at C-6 and to reduce the 9-ketone stereospecifically to afford the 9β-alcohol.

Keywords: *Mucor plumbeus*; Microbiological hydroxylation; Sesquiterpenoids; Clovanes

1. Introduction

The Jones model (Browne et al., 1973; Holland, 1982) for rationalizing the results of the microbiological hydroxylation of the steroids envisages a triangular relationship between the binding sites and a site of hydroxylation in a hydrophobic portion of the molecule. The 2,9-dioxygenated clovanes **1** which are readily obtained (Collado, Hanson & Macias-Sanchez, 1998) by cyclization of the sesquiterpenoid, caryophyllene oxide **2**, possess two potential binding sites on rings A and C and a hydrophobic region on ring B. The hydroxylation of these rigid polycyclic sesquiterpenoids was therefore of interest in the context of developing similar models to rationalize sesquiterpenoid microbiological hydroxylation (Fraga, 1998; Furstoss & Lamare, 1990).

2. Results and discussion

9α-Hydroxy-2β-methoxyclovane **3**, obtained from caryophyllene oxide **2**, was oxidized with chromium trioxide to the 9-ketone **6** which was reduced with sodium borohydride to give the 9β-alcohol **7** (Collado, Hanson & Macias-Sanchez, 1996). These compounds were used as substrates with *Mucor plumbeus*.Incubation of 9α-hydroxy-2β-methoxyclovane **3** with *M. plumbeus* for 5 days gave 6β,9α-dihydroxy-2β-methoxyclovane **4** as the major metabolite. The location of the new secondary alcohol at C-6 (δ_C 68.6, δ_H 3.90) followed from the downfield shifts in the ^{13}C NMR spectrum (see Table 1) for the signals assigned to C-5 ($\Delta\delta$ 6.6 ppm) and C-7 ($\Delta\delta$ 10.2 ppm) when compared to their position in the substrate. The stereochemistry of the alcohol followed from the multiplicity of the C**H**OH signal in the ^1H NMR spectrum and from nOe experiments. The signal was a doublet of doublets of doublets (J 11.6, 11.1 and 6.6 Hz) corresponding to two diaxial and one axial:equatorial coupling. Irradiation of the β-oriented methyl group signal (H-14) gave an nOe enhancement of 5% H-5 (δ_H 1.31) but produced no effect at δ_H 3.90. On the other hand irradiation of the α-oriented methyl group (H-13, δ_H 1.04) gave an nOe enhancement of 6.5% to the signal at δ_H 3.90. Hence, the hydroxyl group had the 6β-configuration. The known (Aebi, Barton & Lindsey, 1953) 2β,9α-dihydroxy-clovane 5 was a minor metabolite.

Incubation of 9β-hydroxy-2β-methoxyclovane **7** with *M. plumbeus* gave four metabolites which were separated by chromatography. The first metabolite to be

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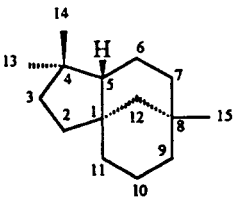
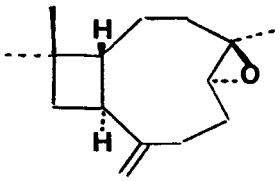
isolated from the column was 6α,9β-dihydroxy-2β-methoxyclovane **8**. The location of the new hydroxyl group at C-6 (δ_C 68.9, δ_H 4.41) followed from the downfield shifts of the signals assigned to C-5 ($\Delta\delta$ 3.7 ppm) and C-7 ($\Delta\delta$ 6.4 ppm) compared to the starting material. However the coupling constants of the new C**H**OH signal (doublet of doublets of doublets, J 6.1, 5.1 and 4.8 Hz) differed from the 6β-alcohol 10. Spin decoupling experiments revealed that the signal at δ_H 4.41 was coupled to H-5 (δ_H 1.57, J 5.1 Hz) and hence the metabolite was assigned the structure **8** with a 6α-hydroxyl group. Further elution gave the known (Aebi et al., 1953) 2β,9β-dihydroxyclovane **9** which was identified by its ^{1}H and ^{13}C NMR spectra. The major metabolite was 6β,9β-dihydroxy-2β-methoxyclovane **10**. The location of the additional hydroxyl group at C-6 (δ_C 68.3, δ_H 3.70) followed from changes to the position of the C-5 ($\Delta\delta$ 6.1 ppm) and C-7 ($\Delta\delta$ 6.2 ppm) resonances compared to the starting material. The stereochemistry of the C-6 alcohol followed from the multiplicity of the C**H**OH resonance and the magnitude of the coupling constants (J 11.3, 10.9 and 7.5 Hz) which corresponded to two diaxial and one axial:equatorial coupling. Selective decoupling experiments established that one of the diaxial couplings (J 11.3 Hz) was with H-5 (δ_H 1.12). Furthermore nOe experiments based on irradiation of the α-oriented methyl group signal (H-13) at δ_H 0.93 gave enhancements to the H-2α signal (δ_H 3.29,6.3%) and the H-6α signal (δ_H 3.70, 7.2%). The final metabolite was the related triol 2β,6β,9β-trihydroxyclovane **11** which was identified by its ^{1}H and ^{13}C NMR spectra. The downfield shifts for C-5 ($\Delta\delta$ 6.0 ppm) and C-7 ($\Delta\delta$ 6.3 ppm) located the additional hydroxyl group at C-6. The new C**H**OH ^{1}H NMR signal at δ_H 3.68 overlapped with that of H-2α. Nevertheless it was possible to identify H-5 (δ_C 57.4, δ_H 1.13) from the ^{1}H:^{13}C correlation spectrum and to show that this possessed a diaxial coupling (J 10.8 Hz) to H-6. Furthermore irradiation of the H-13 signal (δ_H 0.91) gave an nOe enhancement (10.4%) to the multiplet which was assigned to H-2α and H-6α.

Incubation of 9-oxo-2β-methoxyclovane **6** with *M. plumbeus* gave the same four metabolites **8**–**11** that had been obtained from the 9β-alcohol. This suggested that stereospecific microbial reduction of the ketone had taken place prior to hydroxylation. This reduction follows the typical pattern of microbial reductions leading to 'S' alcohols (Acklin, Prelog, Schenker, Serarevic & Walter, 1965).

These hydroxylations show an interesting triangular relationship between the binding groups and the hydroxyl group which has been inserted in the hydrophobic region of the molecule. These transformations provide access to regions of the clovane skeleton which are difficult to functionalize by chemical means.



1



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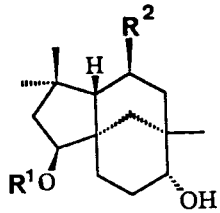
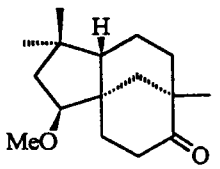
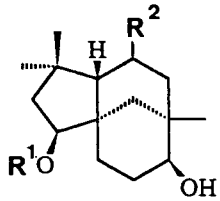


3 R^1 = Me, R^2 = H
4 R^1 = Me, R^2 = OH
5 R^1 = R^2 = H



6



7 R^1 = Me, R^2 = H
8 R^1 = Me, R^2 = α-OH, β-H
9 R^1 = R^2 = H
10 R^1 = Me, R^2 = α-H, β-OH
11 R^1 = H, R^2 = α-H, β-OH

3. Experimental

3.1. General experimental methods

^{1}H NMR spectra were recorded in deuteriochloroform at 300 or 500 MHz. ^{13}C NMR spectra were determined at 75 MHz. IR spectra were recorded as nujol mulls. Chromatography was carried out on silica, Merck 9385. Light petroleum refers to the fraction, b.p. 60–80°C. Extracts were dried over anhydrous sodium sulfate.

Table 1
^{13}C NMR data for clovanes (determined in deuteriochloroform at 75 MHz)

Carbon	Compound							
	3	4	6	7	8	9[a]	10	11
1	44.0	45.5	44.6	43.8	47.1	45.0	44.9	44.8
2	90.0	89.3	89.0	89.3	90.2	79.7	88.5	78.7
3	44.0	44.5	43.8	42.8	44.2	48.6	44.4	47.9
4	36.8	36.7	38.2	37.0	36.0	37.5	37.2	37.1
5	50.5	57.0	50.8	51.4	55.1	51.9	57.5	57.4
6	20.4	68.6	20.4	20.2	68.9	21.1	68.3	67.8
7	33.0	43.2	33.9	31.4	37.7	32.5	37.6	37.7
8	34.6	36.5	44.3	35.2	40.1	35.9	36.7	36.5
9	75.0	74.6	216.3	78.1	74.2	77.6	77.5	77.0
10	25.8	25.6	42.6	27.2	31.9	29.3	27.5	27.5
11	26.4	26.4	32.4	27.7	29.6	28.3	31.4	30.8
12	36.4	36.4	35.6	44.0	42.2	43.1	42.4	41.7
13	25.3	24.5	24.9	25.4	27.9	25.9	24.5	24.2
14	31.2	32.7	32.0	31.3	34.4	31.9	32.7	32.5
15	28.3	28.2	26.1	28.7	29.6	30.0	28.5	28.5
OMe	58.2	58.4	57.7	58.1	57.4		58.2	

[a] In pyridine-d_5.

3.2. Fermentation conditions

Mucor plumbeus (IMI 116688) was grown on shake culture on a medium comprising (per litre): glucose (30 g), potassium dihydrogen phosphate, (2 g), magnesium sulfate (2 g), ammonium tartrate (2 g), yeast extract (1 g), calcium chloride (0.1 g), sodium chloride (1 g), iron(II) ammonium sulfate (0.1 g) and a trace elements solution (2 cm^3). The latter contained (per litre): zinc sulfate (1 g), iron(II) sulfate (1 g), cobalt nitrate (1 g), ammonium molybdate (1 g), copper sulfate (0.1 g) and manganese sulfate (0.1 g). The cultures were grown in shake culture in 250 cm^3 conical flasks each containing 100 cm^3 medium for 36 h at 25°C prior to the addition of the substrate.

3.3. Incubation of 9α-hydroxy-2β-methoxyclovane

The substrate **3** (800 mg) in ethanol (25 cm^3) was evenly distributed between 25 flasks of *M. plumbeus* and the fermentation was continued for a further 10 days. The mycelium was filtered and the broth was extracted with ethyl acetate. The extract was dried and the solvent was evaporated to give a gum which was chromatographed on silica. Elution with 10% ethyl acetate:light petroleum gave unchanged starting material (565 mg). Elution with 50% ethyl acetate:light petroleum gave 6β,9α-dihydroxy-2β-methoxyclovane **4** (86 mg) as a colourless oil (found: HREIMS 268.203; $C_{16}H_{28}O_3$ requires 268.204); ν_{max} 3583, 3387, 1053 cm^{-1}, δ_H 1.02 (3H, s, H-15), 1.04 (3H, s, H-13), 1.08 (1H, dd, *J* 13.9, 11.6 Hz, H-7β), 1.18 (3H, s, H-14), 1.31 (1H, d, *J* 11.0 Hz, H-5), 1.67 (1H, dd, *J* 13.9, 6.6 Hz, H-7α), 3.34 (1H, dd, *J* 11.5, 5.6 Hz, H-2α), 3.36 (1H, m, H-9β), 3.36 (3H, s, OMe), 3.90 (1H, ddd, *J* 11.6, 11.0, 6.6 Hz, H-6α). Elution with 60% ethyl acetate:light petroleum gave 2β,9α-dihydroxyclovane **5** (26 mg), which crystallized from light petroleum as needles, m.p. 153°C (lit. (Aebi et al., 1953), 152–153°C) identified by its ^{1}H NMR spectrum.

3.4. Incubation of 9β-hydroxy-2β-methoxyclovane

The substrate **7** (770 mg) in ethanol (50 cm^3) was evenly distributed between 47 flasks of *M. plumbeus* and the fermentation was continued for a further 5 days. The metabolites were isolated as described above and chromatographed on silica. Elution with 20% ethyl acetate:light petroleum gave the starting material (82 mg). Further elution with 30% ethyl acetate:light petroleum gave 6α,9β-dihydroxy-2β-methoxy-clovane **8** (30 mg) as an oil (found: HREIMS, 268.203; $C_{16}H_{28}O_3$ requires 268.204), ν_{max} 3608, 3583, 3397, 1159, 1061 cm^{-1}, δ_H 1.04 (3H, s, H-15), 1.15 (3H, s, H-14), 1.24 (3H, s, H-13), 1.31 (1H, dd, *J* 14.7, 6.1 Hz, H-7β), 1.57 (1H, d, *J* 5.1 Hz H-5), 1.83 (1H, dd, *J* 14.7, 4.8 Hz, H-7α), 3.28 (2H, m, H-2 and H-9), 3.29 (3H, s, OMe), 4.41 (1H, ddd, *J* 6.1, 5.1 and 4.8 Hz, H-6β). Elution with 33% ethyl acetate:light petroleum gave 2β,9β-dihydroxyclovane **9** (111 mg) which crystallized from light petroleum as needles, m.p. 150–151°C (lit. (Aebi et al., 1953) 152–153°C), ν_{max} 3583, 3348, 1158, 1056 cm^{-1}, δ_H(pyridine-d_5) 0.80 (3H, s, H-13), 1.08 (3H, s, H-14), 1.22 (3H, s, H-15), 3.45 (1H, dd, *J* 10.9, 5.2 Hz, H-9), 4.11 (1H, dd, *J* 9.4, 6.2 Hz, H-2). Elution with 35% ethyl acetate:light petroleum gave 6β,9β-dihydroxy-2β-methoxyclovane **10** (200 mg) as an oil (found: HREIMS 268.204; $C_{16}H_{28}O_3$ requires 268.204), ν_{max} 3377, 1114, 1056 cm^{-1}, δ_H 0.93 (3H, s, H-13), 094 (3H, s, H-15), 1.07 (3H, s, H-14), 1.12 (1H, d, *J* 11.3 Hz, H-5), 3.00 (1H, dd, *J* 10.9, 4.8 Hz, H-9), 3.29 (1H, dd, *J* 11.5, 5.6 Hz, H-2), 3.64 (3H, s, OMe) 3.69 (1H, ddd, *J* 11.3, 10.9, 7.5 Hz, H-6). Elution with 70% ethyl acetate:light petroleum gave 2β,6β,9β-trihydroxyclovane **11** (50 mg) which crystallized from ethyl acetate:light petroleum as needles, m.p. 76–78°C (found: HREIMS 254.187; $C_{15}H_{26}O_3$ requires 254.188), ν_{max} 3500 (br) cm^{-1}, δ_H 0.91 (6H, s, H-13 and H-15), 1.05 (3H, s, H-14), 1.13 (1H, d, *J* 10.8 Hz, H-5), 2.98 (1H, dd, *J* 10.9, 4.8 Hz, H-9), 3.68 (2H, m, H-2 and H-6).

3.5. Incubation of 2β-methoxy-9-oxoclovane

The substrate **6** (950 mg), dissolved in a mixture of dimethylsulfoxide (44 cm^3) and ethanol (5 cm^3) was evenly distributed between 48 flasks of *M. plumbeus* and the fermentation was continued for a further 5 days. The metabolites were isolated as above and chro-

matographed on silica. Elution with 20% ethyl acetate:light petroleum gave the starting material **6** (34 mg). Further elution gave the following metabolites which were identified by their ^{1}H NMR spectra: 6α,9β-dihydroxy-2β-methoxyclovane **8** (122 mg), 2β,9β-dihydroxyclovane **9** (126 mg), 6β,9β-dihydroxy-2β-methoxyclovane **10** (193 mg) and 2β,6β,9β-trihydroxyclovane **11** (53 mg).

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