Microbiological Transformation of Manoyl Oxide Derivatives by *Mucor plumbeus*

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Biotransformations of jhanol (18-hydroxymanoyl oxide) (2), jhanidiol (1β,18-dihydroxymanoyl oxide) (3), and 1-oxo-jhanol (1-oxo-18-hydroxymanoyl oxide) (4) by the fungus *Mucor plumbeus* have been studied. In the incubation of 2 there exists a preference for hydroxylation at C-2(α) (8) and C-6(β) (9–11) and, to a lesser degree, at C-1(α) (7), C-11(α) (6), and C-11(β) (5 and 10). In the second substrate (3), the presence of a 1β-hydroxyl group inhibits 6β- or 11-hydroxylation. Epoxidation of the vinyl group constitutes the main reaction, with the positions 2α (14) and 3β (15) being hydroxylated. In the incubation of 4, there was a preference for 6β-hydroxylation (21) or epoxidation of the vinyl group (22). Other hydroxylations observed were at the 2α (19), 2β (20), 3α (23), 5β (24), and 11β (18) positions.

In a continuation of studies of the microbiological transformation of diterpenoids with fungi we have examined the biotransformation of three manoyl oxide derivatives with *Mucor plumbeus* (Mucoraceae), a fungus with a low substrate specificity. The purpose was to obtain substances with functionality similar to that of forskolin1 (1). In previous work we studied incubation of the same compounds with Gibberella fujikuroi,2 a fungus that, despite possessing high substrate specificity, produces an enantiomeric derivative of manoyl oxide. The fungus *M. plumbeus* has been used previously for biotransformations, for example, with sesquiterpenes possessing the cedrane3 and armadendran4 skeletons and with diterpenes of the labdane type.5–7 In the latter case, the main compounds isolated were hydroxylated at ring A.

Results and Discussion

The substrates used were jhanol (2), jhanidiol (3), and 1-oxo-jhanol (4). The first two diterpenes had been isolated from *Eupatorium jhanii*,8 a plant that grows in the Andean region of Venezuela, and the last was a synthetic sample obtained chemically from 3 or microbiologically from 2.2 The incubation of 2 led to the isolation of compounds 5–11. The substances 5–7 had been obtained in the incubation of this compound with *G. fujikuroi*2 and were identified by direct comparison.

The structure of 2α,18-dihydroxymanoyl oxide (8) was given to one of the metabolites, on the basis of the following considerations: HRMS showed a peak at *m/z* 307.2273, formed by the loss of a methyl group from the molecular ion. Thus, the molecular formula of this product was C20H20O5, indicating that a new oxygen had been introduced into the molecule of 2. This oxygen function was a secondary alcohol, because in the 1H NMR spectrum a proton geminal to a hydroxyl group appeared as a triplet of triplets at 3.99. The multiplicity of this resonance cannot only be explained by a 2J-hydrogen, with two adjacent methylene groups, in which the triplet of triplets arises from the equivalent coupling observed with H-1(α) and H-3(α) (J = 11.2 Hz) and with H-1(β) and H-3(β) (J = 4.5 Hz). Thus, the alcohol must be situated at C-2 with an α-stereochemistry, a conclusion that was confirmed by the 13C NMR data (Table 1).

Another compound obtained in this feeding was 9. Its HRMS was in accordance with the molecular formula C20H18O4, indicating that two new oxygen atoms had been introduced into 2 during the fermentation. The 1H NMR spectrum showed two protons geminal to hydroxyl functions. One of these resonated at 4.08 as a triplet of triplets, in a manner similar to that observed in 7, which indicated that this hydroxyl must be situated at the 2α position. The hydrogen geminal to the second alcohol group

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appeared as a broad singlet at $\delta = 4.48$. This could be explained by the presence of this hydroxyl group at various positions such as C-1(a), C-3(a), C-6(b), or C-12(a). The C-6(b) position was indicated in light of the $^{13}C$ NMR data (Table 1) and then confirmed by acetylation of this compound, leading to the 2a,18-diacetate (9a). The 6β-hydroxy is stereochemically hindered, which in this type of molecule only occurs at this position. Hence, the structure of this metabolite was resolved as 2a,6β,18-trihydroxymanoyl oxide (9).

The more polar metabolites 10 and 11 were obtained as their acetates by acetylation and chromatography of the fraction containing them. The structure of 6β,11β,18-trihydroxymanoyl oxide (10a) was assigned to the first alcohol. The molecular formula of its 11β,18-diacetate (10a) was C$_{39}$H$_{52}$O$_{9}$, indicating that the parent triol 10a was C$_{23}$H$_{35}$O$_{8}$. Its $^1H$ NMR spectrum showed a proton geminal to a hydroxyl group at $\delta = 4.37$ as a broad singlet. The chemical shift and multiplicity of the resonance were similar to those observed for the 6α-H in compound 9a, indicating the existence in 10a of a 6α-alcohol that was not acetylated under usual conditions. A proton geminal to an acetoxy group, which was assigned to C-11(β), also appeared in this spectrum. It resonated as a doublet at $\delta = 5.54$. Double resonance experiments permitted observation of the couplings of this carbonyl with the hydrogens of the C-12 methylene ($J = 4.6$ Hz) and with that of the C-9 methine ($J = 3.2$ Hz). These $^1H$ NMR data were similar to those observed for 11β-acetoxymanoyl oxide. Finally, the structure of the diacetate (10a) was confirmed by assignment of its $^{13}C$ NMR spectrum (Table 1).

The second metabolite, also obtained as the acetate, was 14β,15,18-triacetate (11a), which had a molecular formula of C$_{39}$H$_{52}$O$_{9}$, indicating that the compound obtained in the feeding was the tetracetol 10. Thus, the nonacetylated hydroxyl was assigned to the 6β-position for the reason given previously for 9 and 10. In the $^{1H}$ NMR spectrum, the disappearance of the double bond and the observation of three hydrogens situated on two adjacent carbon-bearing alcohols indicated that one of the alcohols was primary and the other secondary. These facts permitted us to assign the remaining two hydroxyls to C-14 and C-15, with the stereochemistry at C-14 remaining undefined.

The incubation of jhanidol (3) with M. plumbus led to the isolation of 12-16. Compound 18 has been obtained in the biotransformation of this same substance (3) by G. fujikuroi. The least polar metabolite isolated from the feeding of 3 was the epoxide 12, which was a single stereoisomer. The $^1H$ NMR spectrum of this metabolite showed no vinylic hydrogens but had those indicative of an epoxide at C-14,15. The two H-15 protons resonated as two doublets at $\delta = 2.68$ ($J = 5.1, 2.9$ Hz) and $2.71$ ($J = 5.1, 3.9$ Hz), while H-14 appeared as a double doublet at $\delta = 2.87$ ($J = 3.8, 2.9$ Hz). Consequently, the structure 1β,18-dihydroxy-14β,15-epoxymanoyl oxide (12) was assigned. Epoxidation of 3 with m-chloroperbenzoic acid led to a mixture of stereoisomers in a 6:4 ratio. The pure stereoisomer observed in the biotransformation was identical with that formed, in a lesser proportion, in the chemical epoxidation.

The metabolite 13, obtained in this feeding of 3, was isolated together with 14 as their triacetates 13a and 14a by acetylation and chromatography of the fractions containing them. Structure 13a was assigned by comparing its $^1H$ NMR spectrum with that of 3a. The hydrogens of the double bond disappeared, being substituted by those of a -CH$_2$-CH$_2$OAc group. The two protons of this 15-acectoxyethylene group resonated as a triplet at $\delta = 4.19$ ($J = 7.3$ Hz). This structure was confirmed by assignment of the $^{13}C$ NMR spectrum (Table 1), thus, the parent triol must be 1β,15,18-trihydroxymanoyl oxide (13).

The molecular formula of triacetate 14a (C$_{39}$H$_{52}$O$_{9}$), corresponding to an alcohol C$_{29}$H$_{52}$O$_{8}$, indicated that a new oxygen had been introduced. This must be a secondary alcohol, because a new hydrogen geminal to an acetoxy group appeared in the $^{1H}$ NMR spectrum of 14a, resonating as a double doublet at $\delta = 2.3, 4.8$ Hz. These data are characteristic of a proton geminal to an β-equatorial acetate at C-3, C-7, or C-12. In light of the $^{13}C$ NMR spectrum (Table 2), the acetoxy group was placed at C-3β. Therefore, the structure of 13β,18-trihydroxymanoyl oxide (14) is assigned to the corresponding triol.

The most polar metabolite (16) did not yield a molecular ion in its MS, but gave one at m/z 323.2240 (C$_{29}$H$_{52}$O$_{8}$) formed by loss of water and a methyl group. The two oxygen atoms introduced during the feeding must be situated at C-14 and C-15, because the hydrogen signals of the double bond disappeared in its $^1H$ NMR spectrum, being substituted by those of another three coupled protons, now on carbon-bearing oxygens. These signals were over-
leaped at $\delta 3.70$ and were resolved in the corresponding spectrum of the triacetate $16a$, which showed the H-14 as a doublet at $\delta 5.00$ ($J = 9.0, 2.5$ Hz) and the two H-15 signals as another pair of double doublets at $\delta 4.09$ ($J = 11.5, 9.0$ Hz) and 4.43 ($J = 11.9, 2.5$ Hz). Hence, the corresponding alcohol possessed the structure $15\alpha,15\beta,15\gamma$-trihydroxyxanomol oxide (16). Assignments of the $^{13}$C NMR spectra of 16 and 16a are given in Table 2.

Incubation of 1-oxo-15-hydroxyxanomol oxide (4) led to compounds 17–25. Metabolites 18, 19, 21, 22, and 24 had been previously obtained in the biotransformation of 4 with the fungus G. fujikuroi. The least polar product was 20, which had the molecular formula $C_{20}H_{26}O_{8}$. Its $^1H$ NMR spectrum showed the resonance of a hydrogen geminal to a new hydroxyl group, which was assigned to C-2 in light of the disappearance in this spectrum of the characteristic methylene group adjacent to the 1-oxo group and assignment of the $^{13}$C NMR data (Table 2). The stereochemistry assigned to this hydroxyl group was determined as follows. The most stable conformation of ring A, obtained using molecular mechanics calculations, was a boat, showing a difference of about 6 kcal/mol from that of a chair. Therefore, the observed coupling constants between H-2 and the two H-3, 14.1 and 6.1 Hz, indicated a $\beta$-equatorial stereochemistry for the hydroxyl group. On the other hand, the spectroscopic data of this product were similar to those of its $2\alpha$-epimer (19), obtained in this feeding and also in the incubation of 4 with G. fujikuroi. Consequently, the structure of 1-oxo-2$\beta$,18-dihydroxyxanomol oxide (20) was given to this compound.

Another substance isolated was 23, which was an isomer of 24. Comparing the $^1H$ NMR spectrum of 23 with that of the substrate 4, a new hydrogen geminal to a hydroxyl group appeared. This alcohol was assigned to C-3(\alpha) on the basis of the following considerations: (a) the least energy conformation was determined as a boat by molecular mechanics calculations; and (b) the $3\beta$-proton resonated as a double doublet at $\delta 3.93$ ($J = 12, 5.2$ Hz), indicating an axial stereochemistry. This hydrogen is coupled with the characteristic signals of H-2, which are deshielded by the adjacent 1-oxo group. The location of the hydroxyl group was confirmed by the formation of an $\alpha,\beta$-unsaturated carbonyl group during treatment with $\text{Ac}_2\text{O}$–pyridine, which led to the dehydrated product 25. Thus, the structure of 1-oxo-3$\alpha$,18-dihydroxyxanomol oxide (23) was given to this substance. The assignment of its $^{13}$C NMR spectrum is given in Table 2.

Finally, the structure of 1-oxo-14,15,18-trihydroxyxanomol oxide (17) was assigned to the most polar metabolite on the basis of the following considerations. The peak at m/z 321.2062, observed in its MS, was formed from the molecular ion by loss of water and a methyl group. This compound gave a triacetate (17a), the $^1H$ NMR spectrum of which showed signals of a $-\text{CH(OAc)}_2-\text{CH}_2\text{OAc}$ group. Thus, the two H-15 protons appeared as a pair of double doublets at $\delta 4.14$ ($J = 11.7, 9.0$ Hz) and 4.46 ($J = 11.7, 2.4$ Hz), while the H-14 resonated as another double doublet at $\delta 4.99$ ($J = 9.0, 2.4$ Hz). The corresponding alcohol 17 is formed in the incubation by epoxidation of the vinyl group to give 22 and opening of the oxirane ring by nucleophilic attack of water from the culture medium.

The results of the biotransformation of jhanol (2) indicated that there is a preference for hydroxylation at C-2(\alpha) or C-6(\beta) and, to a lesser extent, at C-1(\alpha) or C-11(\alpha or $\beta$), while the presence of a $\beta$-hydroxyl group in jhanidiol (3) inhibits the 6$\beta$- or the 11-hydroxylation, the epoxidation of the vinyl group appearing as the main reaction. Moreover, the positions 2$\alpha$ and 3$\beta$ were also hydroxylated. In the case of the 1-oxo-jhanol (4), there exists preference for 6$\beta$-hydroxylation or epoxidation of the vinyl group. Other hydroxylations observed were at the 2$\alpha$, 2$\beta$, 3$\alpha$, 3$\beta$, or 11$\beta$ positions.

**Experimental Section**

**General Experimental Procedures.** Melting points were determined with a Reichert Thermovar apparatus and are uncorrected. IR and UV spectra were recorded in a Perkin–Elmer 1600 FT and a Varian Cary 1E spectrophotometer, respectively. $^1H$ NMR spectroscopy were recorded in CDCl$_3$ solutions at 200.13 and 500.13 MHz, with a Bruker AC-200 or a Bruker AMX2–500 spectrometer, respectively, and the $^{13}$C NMR were run at 50.32 MHz, with a Bruker AC-200 and are reported in parts per million (δ). MS were taken at 70 eV (probe) in a Shimadzu QP2000; and HRMS, in a Micromass Autospec spectrometer. Conformations of minimum energy were determined by computational methods employing the Chem X program of Chemical Design. Dry column chromatographies were made on Si gel Merck 0.02–0.063 mm.

**Organism.** The fungal strain was Mucor plumbeus CMI 119038 and was a gift from Dr. J.R. Hanson, School of Chemistry and Molecular Sciences (University of Sussex).

**Incubation Experiments.** The fungus M. plumbeus was grown in shake culture at 25 °C, in conical flasks (250 mL), each containing 50 mL of a sterile medium comprising (per L) glucose (60 g), NH$_4$NO$_3$ (0.48 g), KH$_2$PO$_4$ (5 g), MgSO$_4$ (1 g), and trace-elements solution (2 mL). The trace-elements solution contained (per 100 mL) Ca(NO$_3$)$_2$ (0.01 g), CuSO$_4$ (0.015 g), ZnSO$_4$ (0.18 g), MnSO$_4$ (0.01 g), and (NH$_4$)$_6$Mo$_7$O$_24$ (0.01 g). The substrate dissolved in EtOH was evenly distributed among the flasks after 1 day of growth. After a further 6 days, the fermentation was harvested. The mycelium was filtered, and the culture filtrate was extracted with EtOAc. The extract was dried over Na$_2$SO$_4$, the solvent evaporated, and the residue chromatographed on a Si gel column using a petroleum ether–EtOAc gradient.

**Incubation of Jhanol (2):** The substrate (2, 300 mg) in 28 conical flasks was incubated as above. Chromatography of the extract on Si gel gave: 11$\beta$,18-dihydroxyxanomol oxide (8) (5 mg); 11a,18-dihydroxyxanomol oxide (6) (2 mg); 1a,18-
dihydroxyanomyl oxide (7) (3 mg); 2α,18-dihydroxyanomyl oxide (8) (60 mg); 2α,6β,18-trihydroxyanomyl oxide (9) (32 mg); 6β,11β,18,16-trihydroxyanomyl oxide (10) (2 mg); and 6β,14β,15,18-tetrahydroxyanomyl oxide (11) (10 mg). The two last compounds (10) and (11) were identified as the 11β,18-diacetate (10a) and the 14β,15,18-triacetate (11a), by acetylation and chromatography of the fractions containing them.

2α,18-Dihydroxyanomyl oxide (8); colorless crystals (petroleum ether-$EtOH$); $m.p.$ 158-160°C; $\text{[H] NMR (500 MHz)}$ at 1.25 (4H, $d$, $J = 7.5, 8.0$ Hz, H-16), 4.62 (4H, $d$, $J = 7.5, 8.0$ Hz, H-15), 7.06 (2H, $d$, $J = 7.5, 8.0$ Hz, H-14), 7.34 (4H, $d$, $J = 7.5, 8.0$ Hz, H-13), 7.61 (2H, $d$, $J = 7.5, 8.0$ Hz, H-12), 11.7 (2H, $d$, $J = 7.5, 8.0$ Hz, H-10), 12.0 (2H, $d$, $J = 7.5, 8.0$ Hz, H-9) ppm (for $\text{CDCl}_3$); $\text{EIMS m/z [M - CH}_3\text{]+} 269$ calculated for C$_{30}$H$_{33}$O$_2$. 11β,18-Diacetate (10a), isolated as its 14β,18-diacetate by acetylation and chromatographic separation.

Eisopodin (9), 11β,18-Diacetate (10a); colorless crystals (petroleum ether-$EtOH$); $m.p.$ 159°C; $\text{[H] NMR (200 MHz)}$ at 1.25 (4H, $d$, $J = 7.5, 8.0$ Hz, H-16), 4.62 (4H, $d$, $J = 7.5, 8.0$ Hz, H-15), 7.06 (2H, $d$, $J = 7.5, 8.0$ Hz, H-14), 7.34 (4H, $d$, $J = 7.5, 8.0$ Hz, H-13), 7.61 (2H, $d$, $J = 7.5, 8.0$ Hz, H-12), 11.7 (2H, $d$, $J = 7.5, 8.0$ Hz, H-10), 12.0 (2H, $d$, $J = 7.5, 8.0$ Hz, H-9) ppm (for $\text{CDCl}_3$); $\text{EIMS m/z [M - CH}_3\text{]+} 283$ calculated for C$_{30}$H$_{37}$O$_2$.
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215 (8), 203 (11), 199 (2); HREIMS [M]+ m/z 336.2305 (calcd for C_{25}H_{33}O_{6}, 336.2300).

Compound 25. Treatment of 23 with Ac_2O-C_6H_5N (2:1) at room temperature for 12 h and chromatography over Si gel afforded the dehydrated compound 25: UV (CHCl_3) \( \lambda_{max} \) 242 nm; \( ^1H \) NMR (200 MHz) \( \delta \) 1.09, 1.18, 1.34, 1.39 (each 3H, s), 2.04 (3H, s), 3.86, 3.99 (each 1H, d, J = 11.2 Hz, H-18), 4.94 (1H, dd, J = 9.6, 1.4 Hz, H-16), 5.16 (1H, dd, J = 17.2, 1.4 Hz, H-15), 5.83, 6.35 (each 1H, d, J = 10.2 Hz, H-2 and H-3, respectively), 5.90 (1H, dd, J = 17.2, 10.6 Hz, H-14); EIMS m/z (rel int) 360 [M]+ (1), 345 (100), 333 (6), 327 (4), 290 (3), 275 (6), 267 (19), 265 (15), 215 (6), 211 (10), 199 (2); HREIMS m/z [M]+ 360.2297 (calcd for C_{25}H_{33}O_{6}, 360.2300).

1-Oxo-14,15,18-trihydroxymanoyl oxide (17): colorless crystal (petroleum ether-EtOAc), mp 159–161 °C; IR (CHCl_3) \( \nu_{max} \) 3430, 1700 cm\(^{-1}\); \( ^1H \) NMR (500 MHz) \( \delta \) 0.97, 1.19, 1.27, 1.35 (each 3H, s), 1.99 (1H, d, J = 13.8, 10.5, 5.3 Hz, H-3), 2.31 (1H, d, dd, J = 14.0, 6.7, 5.3 Hz, H-2), 2.81 (1H, dd, J = 14.0, 10.5, 5.5 Hz, H-2), 3.28, 3.46 (each 1H, d, J = 10.7 Hz, H-18), 3.66 (2H, signals overlapped, H-14 and H-15), 3.80 (1H, dd, J = 11.3, 5.1 Hz, H-15); EIMS m/z (rel int) 321 [M - CH_3 - H_2O]+ (2), 305 (3), 293 (79), 275 (20), 257 (38), 245 (85), 227 (9), 217 (8), 201 (17), 199 (22); m/z [M - CH_3 - H_2O]+ 321.2062 (calcd for C_{25}H_{33}O_{6}, 321.2065).

Triacetate (17a): \( ^1H \) NMR (200 MHz) \( \delta \) 1.03, 1.18, 1.25, 1.30 (each 3H, s), 3.73, 3.87 (1H, d, J = 11.1 Hz, H-18), 4.14 (1H, dd, J = 11.7, 9.0 Hz, H-15), 4.46 (1H, dd, J = 11.7, 2.4 Hz, H-15), 4.98 (1H, dd, J = 9.0, 2.4 Hz, H-14); EIMS m/z (rel int) 465 [M - CH_3]+ (1), 405 (1), 387 (2), 369 (1), 345 (3), 335 (100), 317 (7), 257 (92), 239 (17), 199 (52); HREIMS m/z [M - CH_3]+ 465.2482 (calcd for C_{25}H_{35}O_{6}, 465.2488).

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References and Notes

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