Biotransformation of the Diterpenes Epicandicandiol and Candicandiol by Mucor plumbeus

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The microbiological transformation of the diterpene epicandicandiol (1) with Mucor plumbeus gave foliol (3), sideritiol (5), and \(7\alpha,16\alpha,17,18\)-tetrahydroxy-ent-kaurane (7), while the incubation of candicandiol (2) gave \(7\alpha,9\beta,18\)-trihydroxy-ent-kaur-16-ene (10), canditiol (11), and \(7\alpha,16\alpha,17,18\)-tetrahydroxy-ent-kaurane (12). Thus, the main difference observed in both feedings, resulting from the spatial change in the orientation of the 7-hydroxyl, from axial in the substrate 1 to equatorial in 2, was the formation of a 3ε- and a 9β-hydroxylated derivative, respectively.

For several years, we have been interested in the study of the microbiological transformation of terpenoids with fungi. One of our aims was the biotransformation of ent-kaurene derivatives by Gibberella fujikuroi, the fungus that produces the gibberellin plant hormones. These studies have mainly been directed to preparing new gibberellin analogues and to obtaining information about the substrate specificity of the enzymes involved in the biosynthesis of gibberellins.1–3 In this research we have used as substrates natural ent-kaurene diterpenes or synthetic derivatives prepared from them. To continue with these studies and to prepare new substrates, we have initiated the biotransformation of ent-kaurene derivatives with Mucor plumbeus, a fungus that has been used for the functionalization of unactivated carbons in sesqui-4-terpenoids and diterpenoids.4–9 On the other hand, these biotransformations should permit the development of models to rationalize diterpenoid microbiological hydroxylation by M. plumbeus. In this context, we have previously carried out incubations of labdane diterpenes with this fungus.12–14

In this work we describe the results of the biotransformation of the two ent-kaurene derivatives, epicandicandiol (7/\(\beta\),18-dihydroxy-ent-kaur-16-ene) (1) and candicandiol (7\(\alpha\),18-dihydroxy-ent-kaur-16-ene) (2), which are epimeric at C-7. Thus, we can also determine whether a spatial change in the orientation of the hydroxyl group at this carbon, from \(\beta\)-axial in the first to \(\alpha\)-equatorial in the second, has any effect on the biotransformation.

Compounds 1 and 2 had been obtained from Sideritis candidicans5,6,16 and other species of this genus.17 We have assigned the \(^1\)H and \(^13\)C NMR spectra of these substrates utilizing 2D NMR data (COSY, HSQC, and HMBC). The carbon spectra of 1 and 2 are included in Table 1. Previously we had reported the spectrum of 1 in deuterated pyridine.18 Italian and Turkish authors have reported the isolation of candicandiol (2) from Sideritis huber-morathii,19 but its \(^13\)C NMR spectrum was different from that of 2. Therefore, the structure had been erroneously assigned. We have now identified it with epicandicandiol (1). Its spectrum, conveniently reassigned, was identical with that of 1.

Results and Discussion

The incubation of epicandicandiol (1) with M. plumbeus for 6 days afforded three hydroxylation products, 3, 5, and 7. The metabolite 5 was obtained as its triacetate 6, while 7 was isolated as the triacetate 8 and the tetraacetate 9, by acetylation of the fractions containing them. The least polar compound 3 was isolated as colorless needles in 7.3% yield. Its MS was in accordance with the formula C\(_{20}\)H\(_{32}\)O\(_3\), indicating that a new oxygen was introduced in the molecule of 1. This must form a part of a secondary hydroxyl group, because a new geminal proton was observed in the \(^1\)H NMR spectrum resonating at \(\delta 3.68\) (dd, J = 8.9, 7.7 Hz). The form of resonance was typical of a \(\beta\)-axial hydrogen at C-1, C-3, or C-12, but the effect produced by the new alcohol in the resonance of the hydroxymethylene group (\(\delta 3.35\) and 3.59, each 1H, d) with respect to the substrate (\(\delta 2.94\) and 3.46) permitted it to be located at C-3(\(\alpha\)). This location was confirmed by assignments of its \(^13\)C NMR spectrum, and that of its acetate 4, using 2D NMR data. Thus, the structure of this metabolite was determined as 3\(\alpha\),7\(\beta\),18-trihydroxy-ent-kaur-16-ene. This compound has been isolated previously as a natural product from Sideritis linearifolia and named foliol (3).20

The second compound obtained in this biotransformation with a 0.93% yield was 5, identified as its triacetate 6 by acetylation of the fraction containing it. Spectroscopic data of 6 were the same as those described in the literature for the triacetate of sideritiol.21 We have now assigned the \(^1\)H and \(^13\)C NMR spectra of this compound utilizing two-dimensional NMR data. The corresponding alcohol sideritriol (5) had been isolated from the corollas of Sideritis sicula.21,22
The incubation of candicandiol (2) with M. plumbeus for 6 days afforded the hydroxylation products 10–12. This last compound was obtained as the triacetate 13 and the tetraacetate 14 by acetylation of the fractions containing it.

The least polar metabolite 10 was isolated as colorless crystals in 1.3% yield. The molecular formula was found to be C$_{20}$H$_{30}$O$_{8}$ on the basis of an ion peak at m/z 302.2194 [M – H$_{2}$O]$^+$ and other spectroscopic data. The 1H and 13C NMR spectra of 10 were similar to those of 2 except for resonances attributed to rings B and C. The 13C NMR spectrum revealed the presence of an additional quaternary carbonyl signal at $\delta$ 78.5. This hydroxyl group was determined as 7$\beta$-orientation. Thus, the structure of this triacetate confirmed this assignment. The two H-17 appear now as a pair of doublets at $\delta$ 4.43 and 4.86 (J = 12.5 Hz); C-16 resonates at 89.9 and C-17 at $\delta$ 63.1. These carbon chemical shifts are in accordance with a 16$\alpha$-OH stereochemistry. Therefore, the original metabolite obtained in the feeding was the corresponding alcohol 7$\beta$,16$\alpha$-hydroxy-ent-kaurane (7).

The least polar metabolite 10 was isolated as colorless crystals in 1.3% yield. The molecular formula was found to be C$_{20}$H$_{30}$O$_{8}$ on the basis of an ion peak at m/z 302.2194 [M – H$_{2}$O]$^+$ and other spectroscopic data. The 1H and 13C NMR spectra of 10 were similar to those of 2 except for resonances attributed to rings B and C. The 13C NMR spectrum revealed the presence of an additional quaternary carbonyl signal at $\delta$ 78.5. This hydroxyl group was determined from the HMBC spectrum to be located at C-9 due to the presence of correlations between this carbon (78.5) and the methyl group protons at H-20 ($\delta$ 1.17), the methylene protons at H-11 ($\delta$ 1.73, m), H-14 (1.26, m and 1.98, dd), and H-15 ($\delta$ 2.43, dt, and 2.46, d), and the methine proton of H-5 ($\delta$ 1.89, dd). This was further supported by the expected downfield shifts for C-8 ($\delta$ 4.9 ppm), C-10 ($\delta$ 4.6 ppm), and C-11 ($\delta$ 13.0 ppm).
and the upfield shift, due to γ-gauche effects, for C-1 (δ = 7.7 ppm), C-5 (δ = 7.0 ppm), C-7 (δ = 4.2 ppm), and C-15 (δ = 7.8 ppm) relative to those of 2. Moreover, shift displacements in the carbon resonances of C-8, C-9, C-10, and C-11 in comparison with those of the diterpene 7α,9α,16β,17-tetrahydroxy-ent-kaurane are in accordance with this assignment. Therefore the structure of 10 was established as 7α,9β,18-trihydroxy-ent-kaur-16-ene.

The second metabolite obtained in this feeding was identified as 7α,15α,18-trihydroxy-ent-kaur-16-ene (canditriol) (11). This triol had been described previously as a natural product from Sideritis infernalis, and comparison of spectroscopic data confirmed its identity. We have now assigned the 1H and 13C NMR spectra utilizing 2D NMR data (COSY, HSQC, and HMBC).

The most polar compound, 12, of this biocconversion was obtained as its triacetate 13 and its tetraacetate 14 by acetylation and chromatography of the fractions containing it. Comparison of the spectroscopic data of 13 with those of 8 indicated that those compounds were very similar. Thus, the absence in their 1H NMR spectrum of vinylic proton signals similar to those observed for the diterpene 7α,16α,17,18-tetrahydroxy-ent-kaurane (12).

Several conclusions can be obtained from these microbiological transformations:

1. A spatial change in the orientation of the hydroxyl group at C-17 from axial in candidicandiol (1) to equatorial in candidicandiol (2) affected the way in which these kauranes bind to the oxidative enzymes affording a different hydroxylation pattern in the A and B rings. Thus, the main difference in both feedings was the formation of a 3α-hydroxylated derivative of 1 in the first and a 9β-hydroxylated one of 2 in the second.

2. Incubations of both candidicandiol 1 and 2 with M. plumbeus afforded mainly 7 and 12 in 15.0% and 22.7% yield, respectively. These 16,17-dihydroxylated compounds can be formed by enzymatic epoxidation of the exocyclic double bond to give the corresponding epoxides, followed by opening of these epoxides in the medium. In this context, we have reported that 16α,17-dihydroxy derivatives were formed when 16α,17-epoxy-ent-kauranes were added without the fungus to a medium prepared for growing Gibberella fujikuroi.

3. The formation of sideritriol (5) in the incubation of 1 and of canditriol (11) in that of 2 should be due to the same enzyme. Thus, 5 can be formed by enzymatic abstraction of a hydrogen at C-15 in 1 with formation of a carbonium ion, migration of the double bond to the 15,16-position, and neutralization of the cation at C-17 by a −OH group, primarily originating from water. The presence of the 7β-OH in the substrate can anachronistically assist this process. Sideritriol (5) can also be formed by enzymatic opening of the epoxide 15 and concomitant formation of the 15,16-double bond. We think that this is not the mechanism of formation in our case, because analogously in the biotransformation of candidicandiol (2) this process must also imply the formation of the 7α-epimer of the diterpene sideritriol (5), which was not isolated therefrom.

4. The hydroxylations produced in these incubations of the substrates 1 and 2 with M. plumbeus resemble those observed in the Sideritis genus, where these compounds were transformed into the metabolites 3 and 11, respectively, and from which compound 5 was also obtained.

**Experimental Section**

**General Experimental Procedures.** Melting points were determined with a Reichert Thermovar apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 1600 FT. 1H NMR spectra were recorded in CDC13 solutions at 500.13 MHz with a Bruker AMX250 spectrometer. 13C NMR spectra were run in CDC13 at 125.15 MHz with a Bruker AC-200 or a Bruker AMX250 spectrometer. Chemical shifts are given in ppm (δ). Mass spectra and HRMs were taken at 70 eV in a Micromass Autospec spectrometer. HPLC was performed using a Beckman System Gold 125P. Purification by HPLC was achieved using a Si gel column (Ultrasphere Si 5 µm, 10 × 250 mm) with a gradient of hexane–EtOAc.

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(18), 197 (23), 183 (25), 169 (23), 157 (26), 145 (41); HREIMS m/z 446.2726 [M – AcOH]⁺ (calcd for C26H38O6, 446.26683).

Biotransformation of Candicandiol (2). Candicandiol (7α,18-dihydroxy-ent-kaur-16-ene) (2) (230.8 mg) dissolved in EtOH (8 ml) and 3 drops of Tween-80 was distributed among 40 conical flasks and incubated for a further 6 days. The metabolites were isolated as above and chromatographed on Si gel. Elution with n-hexane–EtOAc (3:2) gave starting material (128.4 mg). Further elution with n-hexane–EtOAc (1:1) gave 7α,9β,18-trihydroxy-ent-kaur-16-ene (10) (2.5 mg).

Fractions eluted with n-hexane–EtOAc (1:4) were purified by HPLC using EtOAc as isocratic eluent to give canditriol (11) (3.4 mg). Acetylation of fractions eluted with EtOAc–MeOH (1:1) and further chromatography on a Si gel column eluted with n-hexane–EtOAc mixtures gave the tetraacetate (14) (9.1 mg) and the triacetate (13.5 mg).

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1 developed starting material 1 (1.5 mg). Fraction 2 was purified by column chromatography on Si gel, using a n-hexane–EtOAc gradient. Fractions eluted with n-hexane–EtOAc (9:1) were purified by HPLC using a gradient of 35% EtOAc to 55% in n-hexane, giving foliol (3) (4.8 mg). Fractions eluted with n-hexane–EtOAc (85:15) were acetylated and purified by HPLC using EtOAc as isocratic eluent to give foliol triacetate (4) (2.3 mg). Chromatographic separation of the acetylated fraction 3 afforded (4) (3.1 mg) and sidertriol triacetate (13) (5.3 mg).

Fraciation 4 was acetylated and purified by chromatography on a Si gel column with a gradient of ethyl acetate–hexane (4:1 to 1.1, v/v) and then by HPLC with a gradient of 30% EtOAc to 50% in n-hexane to give the tetraacetate (9) (1 mg) and the triacetate (18) (2.9 mg).

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References and Notes