The Incubation of 13α,17-Dihydroxystemodane with Cephalosporium aphidicola

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Received: 30 November 2011; in revised form: 19 January 2012 / Accepted: 3 February 2012 / Published: 9 February 2012

Abstract: The biotransformation of 13α,17-dihydroxystemodane (3) with the fungus Cephalosporium aphidicola afforded 13α,17,18-trihydroxystemodane (4), 3β,13α,17-tri-hydroxystemodane (5), 13α,17-dihydroxy-stemodan-18-oic acid (6), 3β,11β,13α,17-tetra-hydroxystemodane (7), 11β,13α,17,18-tetrahydroxystemodane (8) and 3β,13α,17,18-tetra-hydroxystemodane (9). The hydroxylation at C-18 of the substrate points to a biosynthetically-directed transformation, because aphidicolin (2) is hydroxylated at this carbon. However, the C-3(β) and C-11(β) hydroxylations seem to indicate a xenobiotic biotransformation.

Keywords: Cephalosporium aphidicola; biotransformations; diterpenes; stemodane

1. Introduction

Microbiological transformations can be divided into two groups: xenobiotic biotransformations, in which the substrate is strange to the transforming organism, and biosynthetically-directed...
transformations, also known as “analogue biosynthesis”, in which the substrate possesses a structure analogous to a natural biosynthetic intermediate found in the microorganism [1,2]. We have carried out both types of biotransformations using the fungi *Mucor plumbeus* [3] and *Gibberella fujikuroi* [4] respectively. Now, in this work we have used another fungus, *Cephalosporium aphidicola*, which occupies the borderline between the xenobiotic and biosynthetically-directed biotransformations, because it achieves both [5–8].

Diterpenes with a stemodane skeleton (*i.e.*, 1) have a structural similarity with aphidicolin (2), an antiviral substance and an inhibitor of DNA polymerase, which was isolated from *C. aphidicola* [9], although the C/D ring junctions and the configuration at C-13 in stemodanes and aphidicolanes are different (Scheme 1). Thus, biotransformations of stemodane diterpenes, stemodin and stemodinone, with this fungus, have been carried out [10,11]. Some of us had isolated 13α,17-dihydroxystemodane (3), and analogous compounds of this type, from *Stemonia chilensis*, a plant that grows in the littoral zone of central Chile [12]. This compound had been incubated with *M. plumbeus* [13], a fungus used in xenobiotic biotransformations.

### Scheme 1. Stemodane (1) and aphidicolin (2).

2. Results and Discussion

The microbiological transformation of 13α,17-dihydroxystemodane (3) with the fungus *C. aphidicola* afforded 13α,17,18-trihydroxystemodane (4), 3β,13α,17-trihydroxystemodane (5), 13α,17-dihydroxystemodan-18-oic acid (6), 3β,11β,13α,17-tetrahydroxystemodane (7), 11β,13α,17,18-tetrahydroxystemodane (8) and 3β,13α,17,18-tetrahydroxystemodane (9). Some of these metabolites were obtained as their acetates by acetylation of chromatographic fractions containing them.

The metabolite 4 showed in the HRMS spectrum the ion of higher mass at *m/z* 304.2407, formed from the molecular ion by loss of water, which indicated its molecular formula, C20H34O3. Thus, a new oxygen had been introduced in the molecule during the incubation. In the 1H-NMR spectrum the signal of a new AB system appears at δ 3.11 and 3.35 (1H each, d, *J* = 11 Hz). 13C-NMR spectrum showed a new signal at δ 72.6 (t). This last value is characteristic of an equatorial –CH2OH group at C-4 [14,15], confirmed in the HMBC experiment with correlations of H-18 with C-3, and H-19 with C-3, C-5 and C-18. Therefore, the structure of this compound was determined as 13α,17,18-trihydroxystemodane (4).

Compound 6 was obtained as its diacetate 6a by acetylation of the fractions containing it. The molecular formula of 6a was determined as C24H38O6 considering HRMS data. Therefore, the substrate had gained two oxygens and lost two hydrogens during the fermentation. The two oxygens must form a part of an acid, because in the 13C-NMR spectrum a new signal was detected at δ 182.0, typical of
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this group, while the disappearance of a methyl signal was noted. The presence of this new group was confirmed because 6a formed a methyl ester (compound 6am) by treatment with diazomethane. We considered that the C-18 acid must be formed by oxidation of the corresponding alcohol in 4 (Scheme 2), and consequently assigned the structure of 13α,17-dihydroxystemodan-18-oic acid (6) to the original metabolite formed in the biotransformation.

Table 1. 13C-NMR data of compounds 3, 4, 6a, 6am, 8a and 9a (CDCl3).

| Position | 3   | 4   | 6a  | 6am | 8a   | 9a   |
|----------|-----|-----|-----|-----|------|------|
| 1        | 36.2| 35.8| 35.7| 35.9| 36.0^a| 33.5 |
| 2        | 18.8| 18.1| 18.1| 18.7| 17.6 | 23.2 |
| 3        | 41.8| 35.2| 36.8| 36.8| 36.3^b| 74.6 |
| 4        | 33.2| 37.6^a| 47.2| 47.7| 36.5 | 40.8 |
| 5        | 47.2| 40.4| 41.6| 41.9| 41.1 | 39.5 |
| 6        | 22.2| 22.0| 24.6| 24.6| 22.1 | 21.6 |
| 7        | 36.6| 36.2| 36.7| 36.7| 34.6 | 36.1 |
| 8        | 37.3| 37.2| 37.7| 37.7| 33.6 | 37.2 |
| 9        | 50.7| 50.8| 50.6| 50.6| 55.0 | 50.9 |
| 10       | 38.5| 38.3^a| 37.9| 38.0| 39.1 | 38.1 |
| 11       | 27.2| 27.3| 26.0| 26.1| 71.7 | 27.1 |
| 12       | 28.1| 28.2| 27.0| 27.1| 36.6^b| 28.1 |
| 13       | 74.3| 74.2| 84.4| 84.5| 74.0 | 74.6 |
| 14       | 40.4| 40.5| 39.1| 39.1| 40.3 | 41.0 |
| 15       | 37.4| 37.4| 35.2| 35.2| 35.5^a| 37.2 |
| 16       | 29.7| 29.8| 29.8| 29.9| 28.4 | 29.4 |
| 17       | 68.0| 68.1| 64.7| 64.7| 69.9 | 69.8 |
| 18       | 34.5| 72.6| 182.0| 179.6| 73.3 | 65.9 |
| 19       | 22.8| 18.6| 17.7| 17.8| 18.5 | 13.9 |
| 20       | 18.8| 19.6| 19.0| 19.0| 20.6 | 19.4 |

^a,b These values can be interchanged.

Compound 8 was obtained as its triacetate 8a, the mass spectrum of which showed a peak at m/z 446.2687 formed from the molecular ion by loss of water. Thus, its molecular formula was determined as C_{26}H_{40}O_{7}. Its NMR spectra showed two –CH_{2}OAc groups, one corresponding to the acetylated C-17 alcohol of the substrate, and the other, formed by acetylation of an hydroxyl group introduced in the incubation, resonates at \( \delta_H \) 3.61 and 3.98 (each 1H, d, \( J = 10.8 \) Hz) and at \( \delta_C \) 73.3 (t). These signals are characteristic of an equatorial acetoxymethylene group at C-4 [14,15]. Other signals observed in the spectra of 8a were those of an oxymethine group at \( \delta_H \) 5.36 (t, \( J = 7.6 \) Hz) and \( \delta_C \) 71.7 (d). These chemical shifts and couplings were analogous to those of an 11β-acetoxy derivative described in the biotransformation of the substrate 3 by *M. plumbeus* [13]. The HMBC experiment of 8a confirmed these assignments with the following crosspeaks: H-11 with C-8; H-18 with C-3, C-4 and C-5; H-19 with C-3, C-4, C-5 and C-18; H-20 with C-1, C-5, C-9 and C-10. Thus, the structure 11β,13α,17,18-tetrahydroxystemodane was assigned to the metabolite 8 (Scheme 2) obtained in this fermentation.

Acetylation and chromatography of the fractions containing 9 led to the triacetate 9a, which is an isomer of 8a. In addition to the signals of the 17-CH_{2}OAc, in the \(^1H\)-NMR spectrum of the triacetate 8a another acetoxymethylene group was detected at \( \delta_H \) 3.67 and 3.88 (each 1H, d, \( J = 11.6 \) Hz) and \( \delta_C \)
65.9(t), which was assigned to C-4 with an \( \alpha \)-equatorial configuration. Thus, in the HMBC experiment
the main observed correlations were: H-3 with C-1, C-2, C-4, C-18 and C-19; H-18 with C-3, C-5 and
C-19; H-19 with C-3, C-4, C-5 and C-18. These crosspeaks also showed that another acetoxy group
was located at C-3, with resonances of this oxymethine at \( \delta_C \) 74.6 and \( \delta_H \) 4.78 (dd, \( J = 11.7 \) and
4.1 Hz). The coupling constant of this geminal proton to this acetoxy group indicated a \( \beta \)-equatorial
configuration for this oxygenated function. Consequently, the structure of the original alcohol formed
in the feeding was determined as 3\( \beta \),13\( \alpha \),17,18-tetrahydroxystemodane (9).

Compounds 4 and 6–9 are described here for the first time, whilst 3\( \beta \),13\( \alpha \),17-trihydroxystemodane
(5) and 3\( \beta \),11\( \beta \),13\( \alpha \),17-tetrahydroxystemodane (7) were already isolated from the biotransformation of
3 with \textit{M. plumbeus} [13].

\textbf{Scheme 2.} Biotransformation of 1 by \textit{Cephalosporium aphidicola}.

3. Experimental

3.1. General Procedures

\( ^1 \)H- and \( ^{13} \)C-NMR spectra were recorded at 500.13 and 125.03 MHz, respectively, in a Bruker
AMX-500 spectrometer. Mass spectra were taken at 70 eV (probe) in a Micromass Autospec
spectrometer. HPLC was performed using a Beckman System Gold 125P. Purification by HPLC was
achieved using a silica gel column (Ultrasphere Si 5 μm, 10 × 250 mm). Dry column chromatography was carried out on silica gel Merck 0.040–0.063 mm.

3.2. Microorganism

The fungus strain *Cephalosporium aphidicola* IMI 68689 was a gift from Prof. J. R. Hanson, School of Chemistry, University of Sussex, UK.

3.3. Incubation of 3

*C. aphidicola* was grown in shake culture at 25 °C, in 20 conical flasks (250 mL), each containing 100 mL of a sterile medium comprising (per L) glucose (80 g), NH₄NO₃ (0.48 g), KH₂PO₄ (5 g), MgSO₄ (1 g), and trace elements solution (2 mL). The trace elements solution contained (per 100 mL) Co(NO₃)₂ (0.01 g), CuSO₄ (0.015 g), ZnSO₄ (0.16 g), MnSO₄ (0.01 g), (NH₄)₆Mo₇O₂₄ (0.01 g).

13α,17-Dihydroxystemodane (3, 230 mg) dissolved in EtOH (4.5 mL) was evenly distributed in 20 flasks after one day growth. After a further eight days, the fermentation was harvested. The mycelium was filtered and the culture filtrate was extracted with EtOAc. The extract was dried over Na₂SO₄ and the solvent evaporated to yield a residue (740 mg) that was chromatographed on a silica gel column in a petroleum ether-EtOAc gradient, to afford starting material 3 (30 mg), 13α,17,18-trihydroxystemodane (4, 4 mg), 3β,13α,17-trihydroxystemodane (5, 6 mg), 13α,17-dihydroxystemodane-18-oic acid (6, 1 mg), 3β,11β,13α,17-tetrahydroxystemodane (7, 2 mg), 11β, 13α,17,18-tetrahydroxystemodane (8, 1,5 mg) and 3β,13α,17,18-tetrahydroxystemodane (9, 3 mg).

13α,17,18-Trihydroxystemodane (4). ¹H-NMR (CDCl₃): δ 0.83 (3 H, s, H-19), 1.01 (3H, s, H-20), 1.60 (1H, dd, J = 11.4 and 2.4 Hz, H-5), 1.79 (2H, br s, H-16), 1.89 (1 H, ddt, J = 13.2, 7.4 and 3.1 Hz, H-7β), 2.15 (1H, br s, W₁/₂ = 16 Hz, H-14), 3.11 and 3.35 (each 1H, d, J = 11.0 Hz, H-18), 3.38 and 3.45 (each 1H, d, J = 10.9 Hz, H-17). EIMS m/z (rel. int.): 304 [M−H₂O]+ (2), 291 (66), 286 (17), 273 (100), 255 (54), 230 (8), 215 (12), 206 (18), 203 (40), 173 (29), 159 (22). Found [M−H₂O]+ at m/z 304.2407. C₂₀H₃₂O₂ requires 304.2402.

13α,17-Dihydroxystemodane-18-oic acid (6). Obtained as its diacetate 5a by acetylation and chromatography of the fractions containing it, ¹H-NMR (CDCl₃): δ 1.00 (3H, s, H-20), 1.25 (3H, s, H-19), 1.87 (2H, m, H-1 and H-16), 1.97 (1H, dd, J = 13.7 and 5.6 Hz, H-11), 2.05 and 2.06 (each 3H, s), 2.16 (1H, dd, J = 12.1 and 2.0 Hz, H-5), 2.80 (1 H, br t, J = 7.0 Hz, H-14), 4.36 and 4.51 (each 1H, d, J = 12.2 Hz, H-17). EIMS m/z (rel. int.): 360 [M−C₂H₄O₂]+ (5), 318 (33), 305 (16), 300 (24), 285 (12), 277 (19), 255 (12), 239 (7), 220 (22), 204 (17), 184 (35), 159 (20). Found [M−C₂H₄O₂]+ at m/z 360.2291. C₂₂H₃₂O₄ requires 360.2301. Acetate methyl ester (5am). ¹H-NMR (CDCl₃): δ 1.00 (3H, s, H-20), 1.25 (3H, s, H-19), 2.04 and 2.06 (each 3H, s), 2.14 (1H, dd, J = 12.1 and 2.2 Hz, H-5), 2.81 (1 H, br t, J = 7.0 Hz, H-14), 3.65 (3H, s, -OMe), 4.35 and 4.51 (each 1H, d, J = 12.1 Hz, H-17). EIMS m/z (rel. int.): 374 [M−C₂H₄O₂]+ (9), 332 (82), 314 (52), 299 (24), 277 (38), 255 (75), 239 (27), 234 (44), 220 (17), 199 (15), 185 (27). Found [M−C₂H₄O₂]+ at m/z 374.2456. C₂₃H₃₄O₄ requires 374.2457.
11α,13α,17,18-Tetrahydroxystemodane (8). Obtained as its triacetate 8a from the fractions containing it. 

\[ ^1H-NMR \ (CDCl_3): \delta \ 0.89 \ (3H, \ s, \ H-19), \ 1.02 \ (3H, \ s, \ H-20), \ 1.72 \ (2H, \ m, \ H-1 \ and \ H-15), \ 2.00, \ 2.06 \]
and 2.08 (each 3H, s), 2.10 (1H, m, H-14), 2.33 (1H, m, H-8) 3.61 and 3.98 (each 1H, d, \( J = 10.8 \) Hz, H-18), 3.96 and 3.99 (each 1H, d, \( J = 11.2 \) Hz, H-17), 5.36 (1H, t, \( J = 7.6 \) Hz, H-11). EIMS m/z (rel. int.): 446 [M−H_2O]^+ (1), 404 (2), 386 (33), 371 (6), 344 (23), 331 (21), 313 (10), 276 (14), 274 (13), 253 (17), 215 (76), 201 (14), 189 (47), 129 (100). Found [M−H_2O]^+ at m/z 446.2687. \( \text{C}_{26}\text{H}_{38}\text{O}_6 \) requires 446.2668.

3β,13α,17,18-Tetrahydroxystemodane (9). Obtained as its triacetate 9a from the fractions containing it. 

\[ ^1H-NMR \ (CDCl_3): \delta \ 0.90 \ (3H, \ s, \ H-19), \ 1.03 \ (3H, \ s, \ H-20), \ 1.30 \ (2H, \ m, \ H-6), \ 1.74 \ (1H, \ m, \ H-8), \ 1.91 \ (2H, \ m, \ H-7 \ and \ H-16), \ 2.02, \ 2.07 \]
and 2.09 (each 3H, s), 2.13 (1H, br t, \( J = 7.0 \) Hz, H-14), 3.67 and 3.88 (each 1H, d, \( J = 11.6 \) Hz, H-18), 3.91 and 4.00 (each 1H, d, \( J = 11.3 \) Hz, H-17), 4.78 (1H, dd, \( J = 11.7 \) and 4.1 Hz, H-3). EIMS m/z (rel. int.): 404 [M−C_2H_4O_2]^+ (6), 391 (24), 331 (9), 326 (29), 311 (14), 284 (16), 271 (14), 269 (12), 266 (49), 251 (39), 223 (17), 197 (14), 186 (100). Found [M−C_2H_4O_2]^+ at m/z 404.2544. \( \text{C}_{23}\text{H}_{35}\text{O}_5 \) requires 404.2563.

4. Conclusions

Several conclusions can be deduced from the microbiological transformation of 13α,17-dihydroxystemodane (3) with \( \text{C. aphidicola} \):

1. The hydroxylations produced in the substrate 3 by this fungus occurred at C-3(β), C-11(β) and C-18.
2. The hydroxylation at C-18 points to a biosynthetically-directed transformation, since aphidicolin (2) is also hydroxylated at this carbon. This position was also functionalized in the biotransformation of stemodine and stemodinone with \( \text{C. aphidicola} \) [10,11]. However, the C-3(β) and C-11(β) hydroxylations, also observed in the incubation of 3, seem to indicate a xenobiotic biotransformation. These hydroxylations were also observed in the feeding of 3 with \( \text{M. plumbeus} \) [13], a fungus used in the latter type.
3. The oxidation of C-18 to acid level, as occurs in the formation of 6 from 4, has now been observed for the first time in a biotransformation with \( \text{C. aphidicola} \).
4. It is probable that the formation of 9 only occurs from 4, and not from 5. Thus the hydroxylation of the C-18 methyl in 5 to form 9 could be inhibited by the presence of the equatorial β-hydroxyl group at C-3 (Scheme 2). In aphidicolin biosynthesis has been noted that an axial α-hydroxyl at C-3 blocks the hydroxylation of C-18 [8], whilst in gibberellin biosynthesis has been observed that an equatorial 3α-OH inhibits hydroxylation of C-19 [16].

Acknowledgements

This work has been supported by grant CTQ2009-14629-C02-01, Ministerio de Ciencia e Innovación (MICINN), Spain.
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Sample Availability: Samples of the compounds 3 and 5 are available from the authors.