

Article

The Incubation of 13 α ,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 13 α ,17-dihydroxystemodane (**3**) with the fungus *Cephalosporium aphidicola* afforded 13 α ,17,18-trihydroxystemodane (**4**), 3 β ,13 α ,17-trihydroxystemodane (**5**), 13 α ,17-dihydroxy-stemodan-18-oic acid (**6**), 3 β ,11 β ,13 α ,17-tetrahydroxystemodane (**7**), 11 β ,13 α ,17,18-tetrahydroxystemodane (**8**) and 3 β ,13 α ,17,18-tetrahydroxystemodane (**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

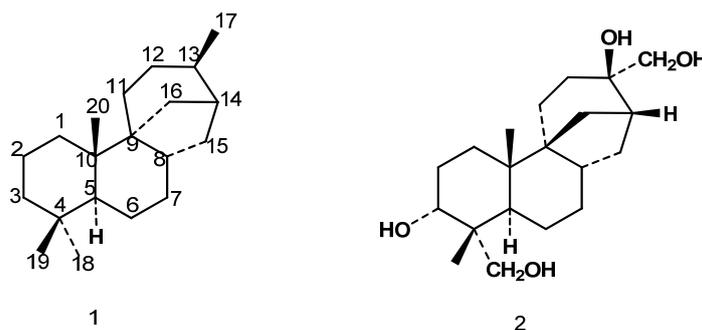
1. Introduction

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 a 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 13 α ,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 13 α ,17-dihydroxystemodane (**3**) with the fungus *C. aphidicola* afforded 13 α ,17,18-trihydroxystemodane (**4**), 3 β ,13 α ,17-trihydroxystemodane (**5**), 13 α ,17-dihydroxystemodan-18-oic acid (**6**), 3 β ,11 β ,13 α ,17-tetrahydroxystemodane (**7**), 11 β ,13 α ,17,18-tetrahydroxystemodane (**8**) and 3 β ,13 α ,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 13 α ,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

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 13 α ,17-dihydroxystemodan-18-oic acid (**6**) to the original metabolite formed in the biotransformation.

Table 1. ^{13}C -NMR data of compounds **3**, **4**, **6a**, **6am**, **8a** and **9a** (CDCl_3).

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

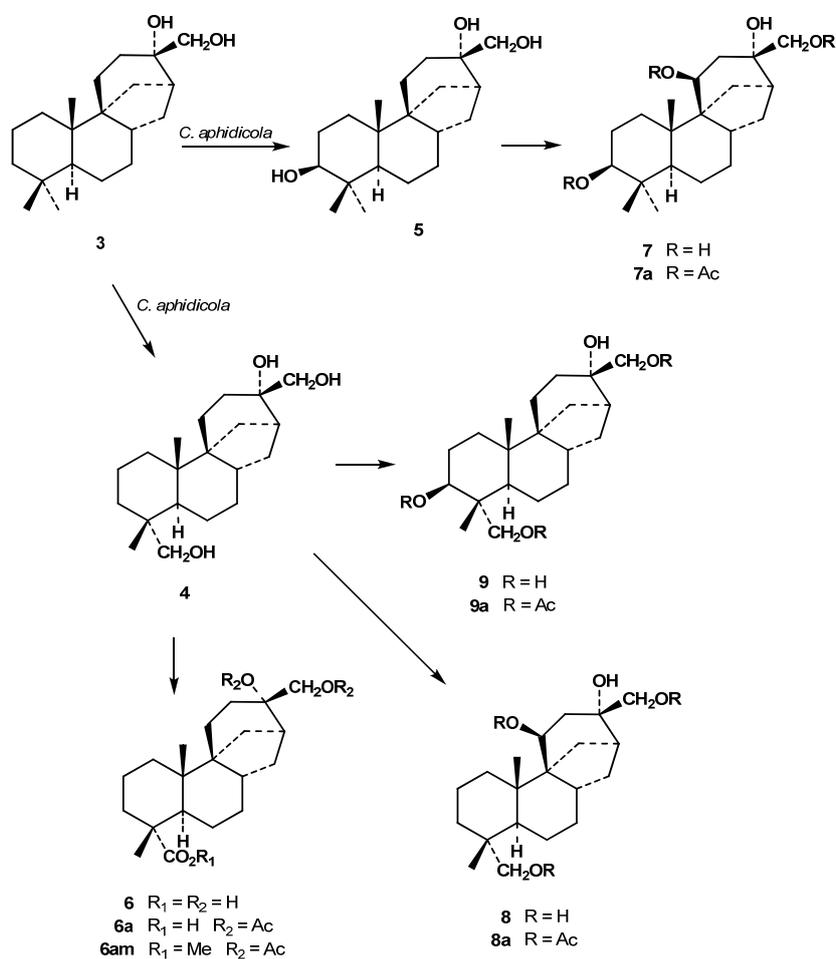
Compound **8** was obtained as its triacetate **8a**, the mass spectrum of which showed a peak at m/z 446.2687 formed from the molecular ion by loss of water. Thus, its molecular formula was determined as $\text{C}_{26}\text{H}_{40}\text{O}_7$. Its NMR spectra showed two $-\text{CH}_2\text{OAc}$ groups, 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 δ_{H} 3.61 and 3.98 (each 1H, d, $J = 10.8$ Hz) and at δ_{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 δ_{H} 5.36 (t, $J = 7.6$ Hz) and δ_{C} 71.7(d). These chemical shifts and couplings were analogous to those of an 11 β -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 11 β ,13 α ,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_2OAc , in the ^1H -NMR spectrum of the triacetate **8a** another acetoxymethylene group was detected at δ_{H} 3.67 and 3.88 (each 1H, d, $J = 11.6$ Hz) and δ_{C}

65.9(t), which was assigned to C-4 with an α -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 δ_C 74.6 and δ_H 4.78 (dd, $J = 11.7$ and 4.1 Hz). The coupling constant of this geminal proton to this acetoxy group indicated a β -equatorial configuration for this oxygenated function. Consequently, the structure of the original alcohol formed in the feeding was determined as 3 β ,13 α ,17,18-tetrahydroxystemodane (**9**).

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

Scheme 2. Biotransformation of **1** by *Cephalosporium aphidicola*.



3. Experimental

3.1. General Procedures

^1H - 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 \times 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), NH_4NO_3 (0.48 g), KH_2PO_4 (5 g), MgSO_4 (1 g), and trace elements solution (2 mL). The trace elements solution contained (per 100 mL) $\text{Co}(\text{NO}_3)_2$ (0.01 g), CuSO_4 (0.015 g), ZnSO_4 (0.16 g), MnSO_4 (0.01 g), $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ (0.01 g). 13 α ,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 Na_2SO_4 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), 13 α ,17,18-trihydroxystemodane (**4**, 4 mg), 3 β ,13 α ,17-trihydroxystemodane (**5**, 6 mg), 13 α ,17-dihydroxystemodane-18-oic acid (**6**) (1 mg), 3 β ,11 β ,13 α ,17-tetrahydroxystemodane (**7**, 2 mg), 11 β , 13 α ,17,18-tetrahydroxystemodane (**8**, 1.5 mg) and 3 β ,13 α ,17,18-tetrahydroxystemodane (**9**, 3 mg).

13 α ,17,18-Trihydroxystemodane (**4**). $^1\text{H-NMR}$ (CDCl_3): 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-7 β), 2.15 (1H, br s, $W_{1/2} = 16$ 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 [$\text{M}-\text{H}_2\text{O}$] $^+$ (2), 291 (66), 286 (17), 273 (100), 255 (54), 230 (8), 215 (12), 206 (18), 203 (40), 173 (29), 159 (22). Found [$\text{M}-\text{H}_2\text{O}$] $^+$ at m/z 304.2407. $\text{C}_{20}\text{H}_{32}\text{O}_2$ requires 304.2402.

13 α ,17-Dihydroxystemodane-18-oic acid (**6**). Obtained as its diacetate **5a** by acetylation and chromatography of the fractions containing it, $^1\text{H-NMR}$ (CDCl_3): 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 [$\text{M}-\text{C}_2\text{H}_4\text{O}_2$] $^+$ (5), 318 (33), 305 (16), 300 (24), 285 (12), 277 (19), 255 (12), 239 (7), 220 (22), 204 (17), 184 (35), 159 (20). Found [$\text{M}-\text{C}_2\text{H}_4\text{O}_2$] $^+$ at m/z 360.2291. $\text{C}_{22}\text{H}_{32}\text{O}_4$ requires 360.2301. Acetate methyl ester (**5am**). $^1\text{H-NMR}$ (CDCl_3): 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 [$\text{M}-\text{C}_2\text{H}_4\text{O}_2$] $^+$ (9), 332 (82), 314 (52), 299 (24), 277 (38), 255 (75), 239 (27), 234 (44), 220 (17), 199 (15), 185 (27). Found [$\text{M}-\text{C}_2\text{H}_4\text{O}_2$] $^+$ at m/z 374.2456. $\text{C}_{23}\text{H}_{34}\text{O}_4$ requires 374.2457.

11 α ,13 α ,17,18-Tetrahydroxystemodane (**8**). Obtained as its triacetate **8a** from the fractions containing it, ¹H-NMR (CDCl₃): δ 0.89 (3H, s, H-19), 1.02 (3 H, s, H-20), 1.72 (2H, m, H-1 and H-15), 2.00, 2.06 and 2.08 (each 3H, s), 2.10 (1 H, 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 1 H, d, $J = 11.2$ Hz, H-17), 5.36 (1H, t, $J = 7.6$ Hz, H-11). EIMS m/z (rel. int.): 446 [M-H₂O]⁺ (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-H₂O]⁺ at m/z 446.2687. C₂₆H₃₈O₆ requires 446.2668.

3 β ,13 α ,17,18-Tetrahydroxystemodane (**9**). Obtained as its triacetate **9a** from the fractions containing it, ¹H-NMR (CDCl₃): δ 0.90 (3 H, 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-C₂H₄O₂]⁺ (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-C₂H₄O₂]⁺ at m/z 404.2544. C₂₃H₃₅O₅ requires 404.2563.

4. Conclusions

Several conclusions can be deduced from the microbiological transformation of 13 α ,17-dihydroxystemodane (**3**) with *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 *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 *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 *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 3 α -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.