



## THE INCUBATION OF 3 $\alpha$ ,16 $\beta$ -DIHYDROXYAPHIDICOLANE WITH *CEPHALOSPORIUM APHIDICOLA*

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**Abstract**—The preparation of 3 $\alpha$ ,16 $\beta$ -dihydroxyaphidicolane and its [17,18- $^2\text{H}_2$ ]-derivative from aphidicolin is described. This substrate was hydroxylated at C-6 and C-7 by *Cephalosporium aphidicola*. No [ $^2\text{H}$ ]-label was detected in the aphidicolin produced by the fermentation, indicating that 18-hydroxylation cannot occur in the presence of a 3 $\alpha$ -hydroxyl group.

### INTRODUCTION

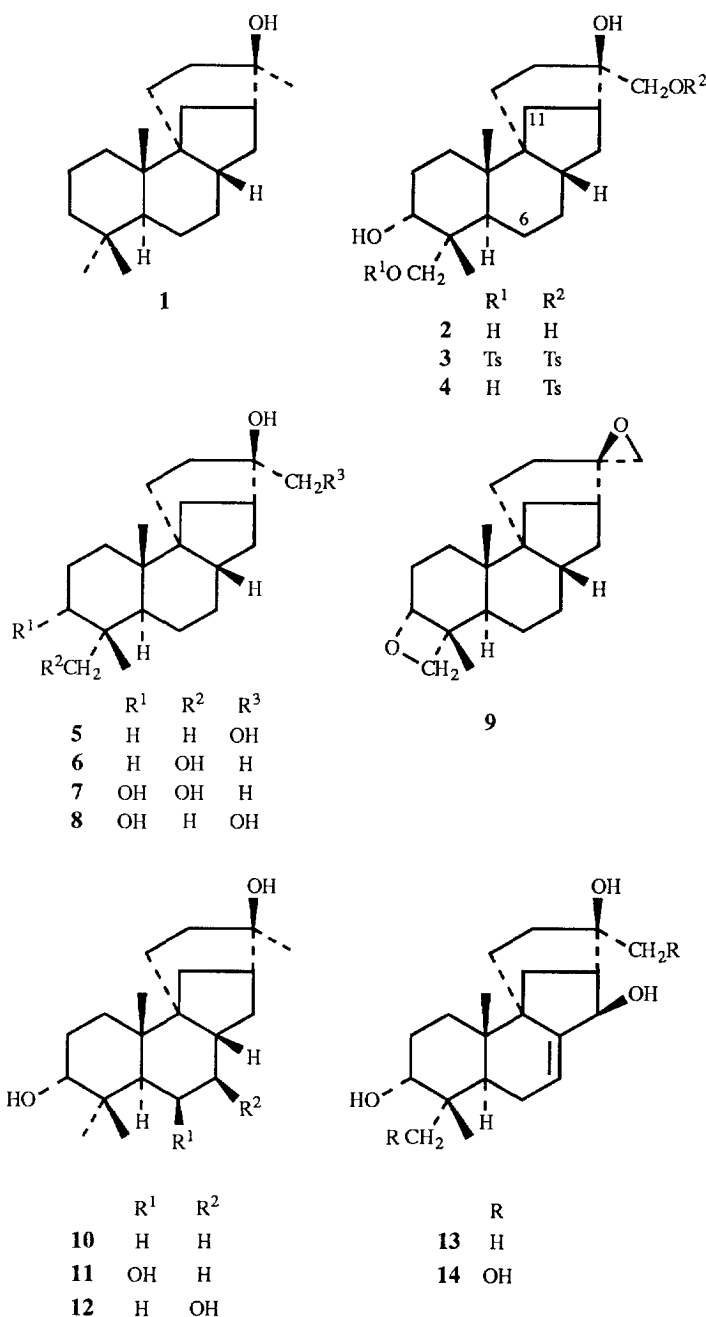
The biosynthesis of the diterpenoid fungal metabolite aphidicolin (**2**) by *Cephalosporium aphidicola* involves the sequential hydroxylation of aphidicolan-16 $\beta$ -ol (**1**) at C-18, C-3 and C-17 [1]. Further hydroxylation then takes place at C-6 $\beta$  and C-11 $\beta$  [2]. We have been interested to know to what extent a metabolic grid relationship might exist between the variously hydroxylated aphidicolan-16 $\beta$ -alcohols [1]. In our previous work we have demonstrated the incorporation of both 16 $\beta$ ,17-dihydroxy aphidicolane (**5**) (3.5%) and 16 $\beta$ , 18-dihydroxyaphidicolane (**6**) (20.5%) into aphidicolin (**2**). In this report we describe the preparation of 3 $\alpha$ ,16 $\beta$ -dihydroxyaphidicolane (**10**) and its biotransformation by *C. aphidicola*. This compound, whilst possessing a 3 $\alpha$ -hydroxyl group, lacks the 18-hydroxyl group which would have been inserted earlier in aphidicolin biosynthesis. There are several possibilities for its biotransformation. Firstly the fungus might 'correct' the mistake by inserting the 18-hydroxyl group and then produce aphidicolin as normal. Secondly the fungus might 'ignore' the mistake and produce the 3 $\alpha$ ,16 $\beta$ ,17-triol (**8**) which is an 18-desoxy analogue of aphidicolin. Thirdly the fungus might reject the substrate or hydroxylate it using a different pathway.

### RESULTS AND DISCUSSION

3 $\alpha$ ,16 $\beta$ -Dihydroxyaphidicolin (**10**) was prepared from aphidicolin (**2**). Treatment of the 17,18-ditoluene-*p*-sulphonate (**3**) [3] of aphidicolin with  $\text{NaHCO}_3$ : DMSO affords 16 $\beta$ ,17-epoxy-3 $\alpha$ ,18-oxidoaphidicolane (**9**). Vigorous reduction of this with LAH gave 3 $\alpha$ ,16 $\beta$ -dihydroxyaphidicolane (**10**). The diol was also obtained by direct reduction of the ditoluene-*p*-sulphonate with LAH although this reaction was rather more capricious. The diol **10** possessed  $^1\text{H}$  NMR signals  $\delta_{\text{H}}$  3.39 (1H), 0.86 (3H), 0.94 (6H), and 1.12 (3H) and  $^{13}\text{C}$  NMR signals

(Table 1) assigned to a secondary alcohol and four C-methyl groups. Repetition of the reduction with lithium aluminium deuteride gave [17,18- $^2\text{H}$ ]-3 $\alpha$ ,16 $\beta$ -dihydroxyaphidicolane. The reaction with toluene-*p*-sulphonyl chloride also gave some of the 17-monotoluene-*p*-sulphonate (**4**). Treatment of this with base gave the 16 $\beta$ , 17-epoxide [3] thus confirming the location of the toluene-*p*-sulphonoxy group at C-17. On reduction with LAH this monotoluene-*p*-sulphonate (**4**) afforded, 3 $\alpha$ ,16 $\beta$ ,18-trihydroxyaphidicolane (**7**).

3 $\alpha$ ,16 $\beta$ -Dihydroxyaphidicolane (**10**) was incubated with *C. aphidicola* in the presence of chlorocholine chloride (CCC) [4]. The latter is a diterpenoid cyclization inhibitor and reduces the formation of the natural diterpenoid metabolites thus facilitating the identification of the metabolites of the exogenous substrates. The metabolites were separated by chromatography. The  $^1\text{H}$  NMR spectrum of the first metabolite (**11**) to be eluted still contained the four C-methyl  $^1\text{H}$  NMR signals of the starting material but those assigned to the 19-H and 20-H had moved downfield. There was a new CH(OH) signal at  $\delta_{\text{H}}$  4.22. The location of this proton at C-6 $\alpha$  followed from changes in the  $^{13}\text{C}$  NMR spectrum (Table 1) and from an NOE experiment. Irradiation of the signal at  $\delta_{\text{H}}$  0.99 (18-H) gave a 4% enhancement at  $\delta_{\text{H}}$  3.28 (3-H) and a 12% enhancement at  $\delta_{\text{H}}$  4.22 (6-H). There was a 6% enhancement of a doublet ( $J = 2.6$  Hz) at  $\delta_{\text{H}}$  1.77 which was assigned to the 5-H. The 5-H signal, which in the presence of an 18-hydroxyl group appears at lower field ( $\delta_{\text{H}}$  2.2–2.33) is normally a doublet ( $J = 3.0$  and 12.5 Hz). In this metabolite it lacks the large coupling and thus the new hydroxyl group was at C-6 $\beta$ . A further metabolite (**12**) possessed a new CH(OH)  $^1\text{H}$  NMR signal as a triplet ( $J = 10$  Hz) of doublets ( $J = 4$  Hz). The  $^{13}\text{C}$  NMR spectrum (Table 1) was in accord with the location of the hydroxyl group at C-7 and hence this metabolite was 3 $\alpha$ ,7 $\beta$ ,16 $\beta$ -trihydroxy aphidicolane (**12**).



The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of the third metabolite (**13**) revealed the presence of a trisubstituted double bond ( $\delta_{\text{H}}$  6.18;  $\delta_{\text{C}}$  148.7, 121.3 ppm) and an allylic alcohol ( $\delta_{\text{H}}$  4.63;  $\delta_{\text{C}}$  74.8 ppm). The alkene proton resonance was a narrow triplet ( $J = 3.1$  Hz) whilst the  $\text{CH}(\text{OH})$  resonance was a singlet. The metabolite was assigned the structure **13** by analogy with the metabolite **14** which had previously been obtained from *C. aphidicola* [2]. The structure of that metabolite had been established by X-ray crystallography of the acetone.

The fermentation was repeated with  $3\alpha,16\beta$ -dihydroxy-[17,18- $^2\text{H}$ ] aphidicolane. Whereas deuterium was detected by  $^2\text{H}$  NMR in samples of the metabolites **11**–**13**, no

deuterium was detected in the aphidicolin (**2**) produced by the fermentation.  $3\alpha,16\beta,18$ -Trihydroxy aphidicolane was not detected by TLC.

The interesting feature of these biotransformations is that the hydroxylations at C-18 and the C-17 which occur in aphidicolin biosynthesis did not take place. This suggests that the hydroxylation at C-18 plays a role in directing the biosynthesis. The effect of an oxygen function at C-3 $\alpha$  in blocking hydroxylation of the equivalent  $\alpha$ -orientated methyl group (C-19) has been noted in gibberellin biosynthesis [5]. The hydroxylation pattern of the metabolites that were formed corresponds to that of the minor metabolites of aphidicolin.

Table 1.  $^{13}\text{C}$  NMR data for the metabolites of 3 $\alpha$ ,16 $\beta$ -dihydroxyaphidicolane determined in  $\text{CDCl}_3$  at 125 MHz

C	10	11	12	13*
1	26.6	28.3	26.4	26.9
2	26.0	25.8	26.0	26.5
3	76.5	77.8	76.1	75.2
4	37.6	38.4	37.7	37.3
5	29.7	42.6	39.5	37.2
6	23.3	69.5	28.8	30.2
7	27.2	38.6	71.7	121.3
8	40.0	33.5	47.3	148.7
9	48.3	48.3	49.9	52.1
10	39.9	40.7	39.2	37.8
11	33.0	33.2	33.4	35.7
12	46.7	46.8	47.8	56.9
13	32.9	33.2	33.0	74.8
14	25.2	24.9	25.9	24.8
15	32.1	31.6	33.4	31.6
16	72.8	72.8	72.5	69.7
17	27.5	27.7	27.7	28.2
18	22.1	28.9	29.1	29.3
19	28.8	24.8	21.9	22.8
20	14.6	17.8	14.6	16.9

\*Determined in pyridine- $d_5$ .

## EXPERIMENTAL

**General.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were determined at 500 and 125 MHz, respectively. IR spectra were determined as Nujol mulls. Silica gel for chromatography was Merck 9385. Petrol refers to light petroleum bp 60–80°. Extracts were dried over anhydrous  $\text{Na}_2\text{SO}_4$ .

**Reduction of 3 $\alpha$ ,16 $\beta$ -dihydroxy-17,18-ditoluene-*p*-sulphonyaphidicolane.** The 17,18-ditoluene-*p*-sulphonate (**3**) [3] (700 mg) and LAH (400 mg) in dry THF (50  $\text{cm}^3$ ) were heated under reflux for 1 hr. The mixt. was cooled to 0° and the excess reagent destroyed by the careful addition of EtOAc, followed by  $\text{H}_2\text{O}$  and dil. HCl. The THF was removed *in vacuo* and the residue extracted with EtOAc. The extract was washed with aq.  $\text{NaHCO}_3$ , brine and dried. The solvent was evapd to give 3 $\alpha$ ,16 $\beta$ -dihydroxyaphidicolane (**10**) (360 mg) which crystallized from MeOH as rods, mp. 83–85°. (Found: C, 74.9; 74.7; H, 11.3, 10.9,  $\text{C}_{20}\text{H}_{34}\text{O}_2 \cdot \text{MeOH}$  requires C, 74.6; H, 11.2%.  $\nu_{\text{max}}$   $\text{cm}^{-1}$ : 3330 *m*, 3255, 1035,  $\delta_{\text{H}}$  ( $\text{CDCl}_3$ ) 0.86 (H, *s*, 19-H), 0.94 (6H, *s*, 18 and 20-H), 1.12 (3H, *s*, 17-H), 3.39 (1H, *br s*, 3-H).

**Reduction of 3 $\alpha$ ,18;16 $\beta$ ,17-diepoxyaphidicolane (**9**).** The diepoxide **9** [3] (120 mg) was heated with LAH (100 mg) in dry THF (15  $\text{cm}^3$ ) under reflux for 30 min. The product was recovered as above to give 3 $\alpha$ ,16 $\beta$ -dihydroxyaphidicolane (**10**) (80 mg) identified by TLC, mp and  $^1\text{H}$  NMR.

**Reduction of 3 $\alpha$ ,16 $\beta$ ,18-trihydroxy-17-toluene-*p*-sulphonyaphidicolane (**4**).** The 17-monotoluene-*p*-sulphonate (**4**) [3] (2 g) was heated with LAH (1.15 g) in dry THF (150  $\text{cm}^3$ ) under reflux for 2 hr. The product was recovered as above to give 3 $\alpha$ ,16 $\beta$ ,18-trihydroxyaphidi-

colane (**8**) (1.47 g), mp 216–220°. (Found: C, 74.3; H, 10.7.  $\text{C}_{20}\text{H}_{34}\text{O}_3$  requires C, 74.5; H, 10.6%).  $\delta_{\text{H}}$  ( $\text{CDCl}_3$ ) 0.70 (3H, *s*, 19-H), 0.98 (3H, *s*, 20-H), 1.14 (3H, *s*, 17-H), 3.38 and 3.48 (each 1H, *d*,  $J = 11.3$  Hz, 18-H), 3.68 (1H, *br s*, 3-H).

**Incubation of 3 $\alpha$ ,16 $\beta$ -dihydroxyaphidicolane with *Cephalosporium aphidicola*.** *Cephalosporium aphidicola* (IMI 68689) was grown as described previously [4], except that shake culture was used. 3 $\alpha$ ,16 $\beta$ -Dihydroxyaphidicolane (**10**) (500 mg) in DMSO (25  $\text{cm}^3$ ) was mixed with CCC (300 mg) in EtOH (5  $\text{cm}^3$ ) and a few drops of Tween 80 and distributed between 25 flasks on day 6 after inoculation. After a further 11 days, the broth was extracted with EtOAc. The metabolites were sep'd by chromatography on silica gel. Elution with EtOAc–petrol (1:1) gave the following compounds: (i) 3 $\alpha$ ,6 $\beta$ ,16 $\beta$ -trihydroxyaphidicolane (**11**) (20 mg) as a gum [MS 304.240  $\text{C}_{20}\text{H}_{32}\text{O}_2$  ( $M - \text{H}_2\text{O}$ ) requires 304.238],  $\nu_{\text{max}}$   $\text{cm}^{-1}$ : 3410,  $\delta_{\text{H}}$  0.99 (3H, *s*, 18-H), 1.07 (3H, *s*, 20-H), 1.19 and 1.21 (each 3H, *s*, 17 and 19-H), 3.28 (1H, *br s*, 3-H), 4.22 (1H, *br s*, 6-H),  $\delta_{2\text{H}}$  1.15 (*br*); (ii) 3 $\alpha$ ,7 $\beta$ ,16 $\beta$ -trihydroxyaphidicolane (**12**) (6 mg) as a gum [ms 304,  $\text{C}_{20}\text{H}_{32}\text{O}_2$  ( $M - \text{H}_2\text{O}$ ) requires 304],  $\nu_{\text{max}}$   $\text{cm}^{-1}$ : 3410 (*br*),  $\delta_{\text{H}}$  ( $\text{CDCl}_3$ ) 0.89 (3H, *s*, 20-H), 0.95 and 0.98 (each 3H, *s*, 18- and 19-H), 1.15 (3H, *s*, 17-H), 3.44 (1H, *br s*, 3-H), 3.65 (1H, *t*,  $J = 10$  Hz, of doublets,  $J = 4$  Hz, 7-H),  $\delta_{2\text{H}}$  1.00 and 1.19; (iii) 3 $\alpha$ ,13 $\beta$ ,16 $\beta$ -trihydroxyaphidicol-7-ene (**13**) (5 mg)  $\delta_{\text{H}}$  pyridine- $d_5$  1.00 (3H, *s*, 20-H), 1.11 (3H, *s*, 19-H), 1.19 (3H, *s*, 18-H), 1.46 (3H, *s*, 17-H), 3.66 (1H, *br s*, 3-H), 4.63 (1H, *br s*, 13-H), 6.18 (1H, *t*,  $J = 3.1$  Hz, 7-H),  $\delta_{2\text{H}}$  1.14 and 1.44. Elution with EtOAc gave aphidicolin (50 mg) identified by its  $^1\text{H}$  NMR spectrum. The material from the  $^2\text{H}$ -labelling experiment showed no significant enrichment of  $^2\text{H}$  by  $^2\text{H}$  NMR. The  $^2\text{H}$  labelling experiment was carried out as above using 3 $\alpha$ ,16 $\beta$ -dihydroxy [17, 18- $^2\text{H}$ ]-aphidicolane (500 mg) as the substrate.

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