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THE HYDROXYLATION AT C-17 IN THE BIOSYNTHESIS OF THE DITERPENOID APHIDICOLIN

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Key Word Index—*Cephalosporium aphidicola*; biosynthesis; diterpenoid; aphidicolin; C-17 hydroxylation.

Abstract—The hydroxylation at C-17 in aphidicolin biosynthesis is inhibited by a 17-thiol. A metabolite hydroxylated at C-17 and retaining the cyclopropane ring was obtained from 3α,18-dihydroxy-15β,16β-methanoaphidicolane whilst aphidicolin itself was obtained from 3α,18-dihydroxyaphidicolane when these substrates were incubated with the fungus, *Cephalosporium aphidicola*. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

The tetracyclic diterpenoid aphidicolin (**1**) is produced by the fungus *Cephalosporium aphidicola*. It has attracted interest because it is a specific inhibitor of DNA polymerase α and possesses potentially useful tumour-inhibitory and antiviral properties [1]. The biosynthesis of **1** involves the formation of aphidicolan-16β-ol (**2**) [2] and its sequential hydroxylation at C-18, C-3α and C-17 [3]. Whereas the hydroxylation of an isolated methyl group to form a primary alcohol is a common diterpenoid biosynthetic step, the conversion of a methyl carbinol into a 1,2-glycol is much less common. Mechanisms other than simple hydroxylation can be envisaged, involving the participation of the adjacent oxygen at C-16 in the attack at C-17. In this paper we report some further studies on this stage in the biosynthesis.

RESULTS AND DISCUSSION

In a study of the geometry of the active site involved in the hydroxylation of C-17, we examined [4] the hydroxylation of 17-methyl-3α,16β,18-trihydroxyaphidicolane (**3**) and showed that this preferentially generated the 17(*R*)-homologue of aphidicolin. An X-ray crystal structure (Fig. 1) of the 17-methyltriol (**3**) has shown that the ethyl side chain adopts the conformation assumed in our earlier work [4] on the basis of molecular models. If this represents the conformation within the hydroxylase, then it suggests that the predominant hydroxylation is taking place from the (*R*)-face at C-17. There is a minor biosynthetic pathway leading to **1**, which involves the epoxidation of 3α,18-dihydroxyaphidicol-16-ene to form the 16β,17-epoxide (**4**) [5]. The processes leading to hydroxylation of C-17 and epoxidation of the 16-ene are therefore taking place from the same face of the molecule. This stereochemical pattern is well established in other biosyntheses [5].

The inhibitory effect of a 17-thiol on the hydroxylation was then examined. 17-Desoxy-17-thioaphidicolin (**5**) was prepared from **4** [5] by reaction with benzylthiol followed by hydrogenolysis with sodium in liquid ammonia. When this thiol was incubated with an intact culture of *C. aphidicola* over a period of five days at a concentration of 0.25 mM, it reduced the incorporation of 3α,16β,18-trihydroxy[17-^{14}C] aphidicolane (**6**) into **1** by 36%.

The facile opening of the cyclopropane ring in a cyclopropylmethyl radical has been used [6] as a probe for radical intermediates in biosynthesis. Consequently, the biotransformation of the 15β,16β-methylene adduct **9** by *C. aphidicola* was examined. The adduct was prepared by methylenation of 3α,18-dihydroxyaphidicol-15-ene (**7**) [7]. The diol was protected as the acetonide (**8**). Methylenation with zinc–copper–methylene diiodide reagent, followed by acid hydrolysis to remove the acetonide, gave 3α,18-dihydroxy-15β,16β-methanoaphidicolane (**9**). The stereochemistry of the cyclopropane ring was assigned by analogy with the known pattern of 'exo' addition to the 15,16 double bond [7] and from the X-ray crystal structure of the biotransformation product.

Incubation of **9** with *C. aphidicola* for 14 days gave one major new metabolite, representing a 10% biotransformation. The metabolite was identified as the cyclopropyltriol **10** since the ^{1}H NMR spectrum

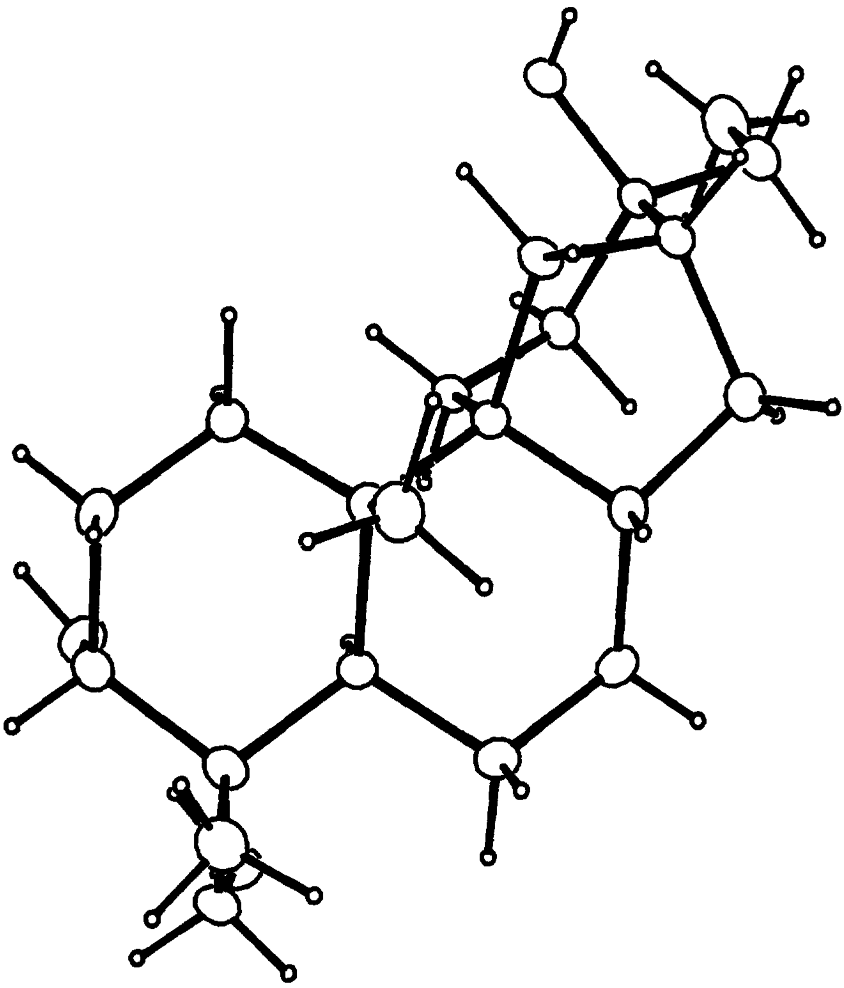


Fig. 1. Crystal structure of 17-methyl-3α,16β,18-trihydroxyaphidicolane (**3**).

contained two doublets (δ 3.17 and 4.14) assigned to each proton of the 17-hydroxymethyl group and retained the highfield resonance (δ 0.62) assigned to the cyclopropane ring. The structure was confirmed by X-ray crystallography (Fig. 2).

Finally, 3α,18-dihydroxy [17-^{14}C] aphidicolane (**11**) was synthesized by hydrogenation of the labelled 15-ene [7]. Incubation with *C. aphidicola* gave **1** with an incorporation of 1.2%. The radioactivity was retained in the bisacetonide. A substantial amount of the substrate was recovered unchanged.

Although the retention of the cyclopropane ring in the biological hydroxylation of **9** was suprising, there are now a number of other instances in which a cyclopropane ring remains intact [8, 9]. The result could be accomodated within the oxygen rebound mechanism for biohydroxylation [10]. The inhibitory effect of a 17-thiol suggests a similarity to steroid methyl group hydroxylations [10]. However, the hydroxylation at C-17 in **9** does establish that the presence of a hydroxyl group at C-16 is not a prerequisite for this step. The formation of **1** from **11**, albeit in low yield, is interesting, revealing that the fungus can both hydrate a carbocation at C-16 and also hydroxylate this centre [11].

EXPERIMENTAL

General experimental details. ^{1}H NMR: 360 MHz; IR: nujol mulls; Merck 9385 silica gel; column chromatography; extracts were dried over Na_2SO_4.

Culture of fungus. Cephalosporium aphidicola (IMI 68689) was grown on surface culture in Thompson bottles on a medium (750 ml per bottle) which contained (l^{-1}): glucose (50 g), KH_2PO_4 (5 g), $MgSO_4 \cdot 7H_2O$ (2 g), KCl (1 g), glycine (2 g) and trace elements sol (2 ml). The last contained (l^{-1}), $Co(NO_3)_2 \cdot 6H_2O$ (0.1 g), $FeSO_4 \cdot 7H_2O$ (1 g), $CuSO_4 \cdot 5H_2O$ (0.15 g), $ZnSO_4 \cdot 7H_2O$ (1.61 g), $MnSO_4 \cdot 4H_2O$ (0.1 g) and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (1 g).

Preparation of 17-thio-3α,16β,18-trihydroxyaphidicolane (**5**). Benzylthiol (3.66 ml) was added dropwise to a stirred suspension of NaH (375 mg) in THF (50 ml) under Ar. Compound **4** [5] (1 g) in THF

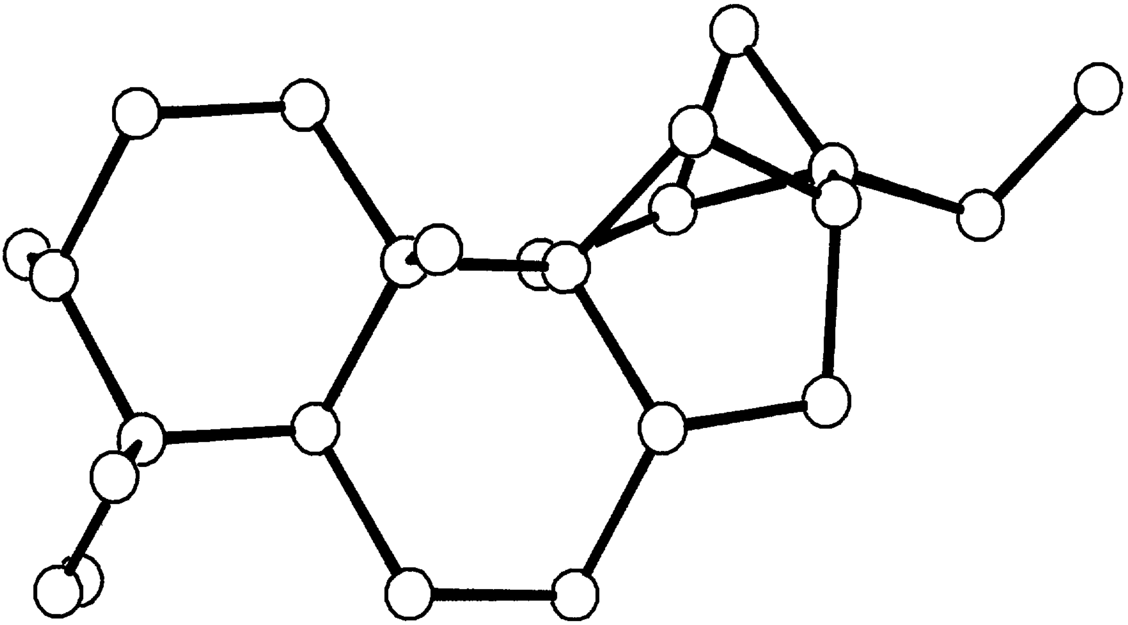


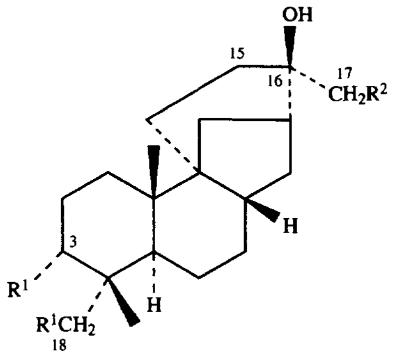
Fig. 2. Crystal structure of 3α,17,18-trihydroxy-15β,16β-methanoaphidicolane (**10**).

(10 ml) was added and the mixt. allowed to warm to room temp. over 2 hr. Et_2O (150 ml) was added and the mixt. was washed with aq. NaOH, aq. NaCl and dried. The solvent was evap *in vacuo* to afford 17-benzylthio-3α,16β,18-trihydroxyaphidicolane (1.3 g) as needles, mp 75–80° ^1H NMR: δ 0.69 (3H, *s*, 19-H), 1.00 (3H, *s*, 20-H), 2.55 (2H, *s*, 17-H_2), 3.35 and 3.46 (each 1H, *d*, *J* = 11.5 Hz, 18-H), 3.66 (1H, *br s*, 3-H), 3.73 (2H, *s*, CH_2Ph), 7.30 (5H, *br s*, Ph); CI MS (NH_3) *m/z*: 427 $[M-OH]^+$, 409 $[M-OH-H_2O]^+$, 307 $[M-PhCH_2SCH_2]^+$. The benzylthioether (1.05 g) was added to liquid NH_3 (300 ml) at −78°. Na (1 g) was added in portions until the blue colour persisted. The mixt. was stirred for 1 hr and then excess of NH_4Cl was added. The NH_3 was evapd at room temp. under a stream of N_2 leaving a solid which was partitioned between EtOAc and aq. citric acid. The organic extract was washed with H_2O and brine and dried. The solvent was evaporated to give **5** (985 mg), which was crystallized from EtOAc–MeOH as needles, mp 212–213° (found: C, 67.3; H, 9.8. $C_{20}H_{34}O_3S$ requires C, 67.8; H, 9.6%). ^1H NMR pyridine-d_5: δ 0.78 (3H, *s*, 19-H), 1.04 (3H, *s*, 20-H), 2.75 (2H, *s*, 17-H_2), 2.84 (1H, *dd*, *J* = 3, 12 Hz, 5-H), 3.62, 3.80 (each 1H, *d*, *J* = 11 Hz, 18-H), 3.92 (1H, *br s*, 3-H).

Incubation of 17-*thio*-3α,16β-18-*trihydroxyaphidicolane* (**5**) *with* C. aphidicola. A soln of 3α,16β,18-trihydroxy-[17-^{14}C]aphidicolane[5] (**6**); (37 mg, 3.28×10^4 dpm), the 17-thiol (67 mg) and Tween-80 (1 drop) in EtOH (5 ml) was added to a culture (750 ml) of *C. aphidicola* 6 days after inoculation. After a further 5 days, the broth was filtered, extracted with EtOAc and the extract chromatographed on silica gel to give **1** (49 mg, 1.93×10^4 dpm, 58.8% incorporation). In a parallel control experiment, the triol (37 mg, 3.28×10^4 dpm) gave **1** (68 mg, 3.03×10^4 dpm, 92.4% incorporation).

Preparation of 3α,18-*dihydroxy*-15β,16β-*methanoaphidicolane* (**9**). Zn dust (305 mg) and CuCl (465 mg) were heated in Et_2O (20 ml) under reflux for 30 min under N_2. Compound **8** [7] (550 mg) and CH_2I_2 (0.3 ml) were added and the soln heated under reflux for 28 hr. The mixt. was cooled and filtered and the filtrate washed with dilute HCl, aq.$NaHCO_3$ and H_2O and dried. The solvent was evapd to give a gum (505 mg). ^1H NMR: δ_H 0.35 (2H, cyclopropyl H), 0.70, 1.0, 1.05 (each 3H, *s*, Me), 1.45 (6H, *s*, O_2CMe_2), 3.25 (1H, *d*, *J* = 11 Hz, 18-H), 3.65 (2H, *m*, 3-H, 18-H). The gum was dissolved in a mixt. of MeOH (150 ml) and H_2O (20 ml) containing TsOH (10 mg) and stirred at room temp. for 4 days. The solvent was evapd and the product recovered in EtOAc and chromatographed to give **9** (440 mg) as a gum CIMS (NH_3) *m/z*: 336 $[M+NH_4]^+$, 319 $[M+H]^+$, 301 $[M-OH]^+$, 283 $[M-OH, H_2O]^+$: IR ν_{max} cm^{-1}: 3360, 1035; ^1H NMR($CDCl_3$): δ ($CDCl_3$) 0.35 (1H, *m*, cyclopropyl), 0.70 (3H, *s*, 18-H), 1.0, 1.05 (each 3H, *s*, 17-H, 20-H), 3.4 (2H, *br s*, 18-H), 3.7 (1H, *br s*, 3-H).

Incubation of 3α,18-*dihydroxy*-15β,16β-*methanoaphidicolane* (**9**) *with* C. aphidicola. The substrate (**9**) (400 mg), chlorocholine chloride (450 mg) and Tween-80 (5 drops) in DMSO (32 ml) and EtOH (10 ml) was evenly distributed between 6 Thompson bottles (4.5 litre) of *C. aphidicola* 11 days after inoculation. After a further 14 days, the mycelium was filtered and the broth was extracted with EtOAc. TLC revealed the presence of one metabolite not present in the control fermentation. The extract was chromatographed on silica gel. Elution with EtOAc–petrol (3:2) gave 3α,17,18-*trihydroxy*-15β,16β-*methanoaphidicolane* (**10**) (43 mg) which was crystallized from EtOAc, mp 220° (decomp) (found: C, 75.2; H, 10.3. $C_{21}H_{34}O_3$ requires C, 75.4; H, 10.3%) IR ν_{max} cm^{-1}: 3380; ^1H NMR (pyridine-d_5): δ 0.62 (1H, *dd*, *J* = 6,



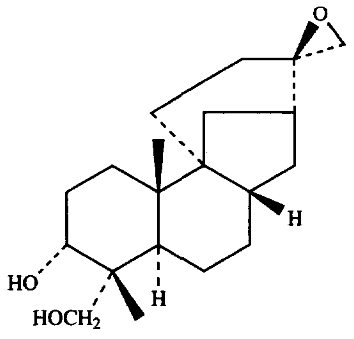
1 $R^1 = R^2 = OH$

2 $R^1 = R^2 = H$

3 $R^1 = OH, R^2 = Me$

5 $R^1 = OH, R^2 = SH$

6 $R^1 = OH, R^2 = H$



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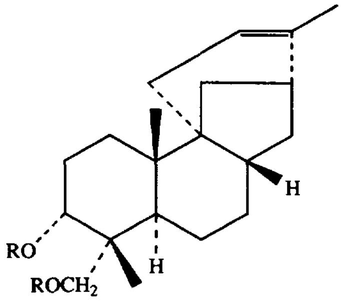
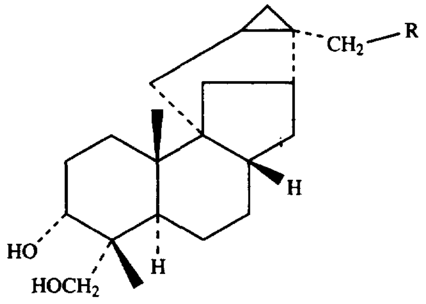


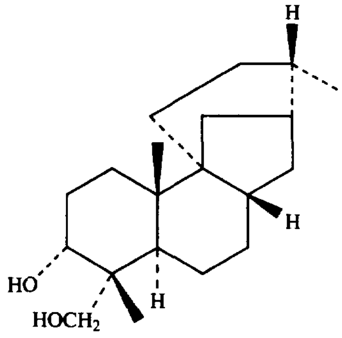
7 R = H

8 R = $\mathrm{Me_2C}<$



9 R = H

10 R = OH



11

10.2 Hz, cyclopropane H), 0.73 (3H, *s*, 19-H), 0.94 (3H, *s*, 20-H), 3.17, 4.14 (each 1H, *d*, $J = 11$ Hz, 18-H), 3.55, 3.73 (each 1H, *d*, $J = 10.8$ Hz, 17-H), 3.87 (1H, *br s*, 3-H).

Incubation of 3α,18-*dihydroxy*[7-^{14}C]*aphidicolane* (**11**) *with* C. aphidicola. The diol **11** [7] (190 mg, 17.9×10^4 dpm) and Tween-80 (1 drop) in DMSO–EtOH (1:1, 24 ml) was evenly distributed between 6

Thompson bottles (4.5 l) of *C. aphidicola* 7 days after inoculation. After a further 20 days the mycelium was filtered and the broth was extracted with EtOAc. The solvent was dried and evapd. The residue was chromatographed on silica. Elution with EtOAc gave **1**, which was recrystallized from EtOAc–MeOH to constant activity (475 mg, 4.547 dpm mg^{-1} total activity, 2.16×10^3 dpm, 1.2% incorporation). A sample was converted into the bisacetonide and recrystallized to constant activity, 4.477 dpm mg^{-1}. The starting diol (95 mg) was also recovered, untransformed, from the fermentation.

Crystallographic data and structural determinations. (i) Compound **3** ($C_{21}H_{36}O_3$), M_r 336.5, monoclinic, space group $P2_1$, $a = 7.731(2)$, $b = 10.399(8)$, $c = 12.080(3)$ Å, $\beta = 96.34(2)^\circ$ $U = 965.3$ Å^3, $Z = 2$, $D_{calc} = 1.16$ g cm^{-3}, $F(000) = 372$, monochromated MoK_α radiation, $\lambda = 0.71069$ Å, $\mu = 0.7$ cm^{-1}. Data were collected using a crystal (*ca* $0.8 \times 0.3 \times 0.1$ mm) on an Enraf-Nonius CAD 4 diffractometer operating in the θ–2θ mode with $\Delta\theta = (0.8 + 0.35 \tan \theta)^\circ$ and a max. scan time of 1 min. A total of 1799 unique reflections were measured for $2 < \theta < 25^\circ$ and $+h+k \pm 1$, and 1337 reflections with $|F^2| > \sigma(F^2)$ were used in the refinement where $\sigma(F^2) = \{\sigma^2(I) + (0.04I)^2\}^{1/2}/L_p$. There was no crystal decay and no absorption correction was applied. The structure was solved by direct methods using MULTAN and the non-H atoms were refined anisotropically by full matrix least squares. H atoms were located on a difference map and their positions refined with isotropic temp. factors fixed at $B = 4.0$Å^2. The weighting scheme was $w = 1/\sigma^2(F)$ and the final residuals were $R = 0.041$; $R' = 0.048$. A final difference map was featureless. The programs from the Enraf-Nonius SDP-Plus package were run on a MicroVax computer.

(ii) Compound **10** ($C_{21}H_{34}O_3$), M_r 334.5, tetragonal, space group $P4_12_12$, $a = b = 11.652(8)$, $c = 26.706(9)$ Å, $U = 3625.7$ Å^3, $Z = 8$, $D_{calc} = 1.22$ g cm^{-3}, $F(000) = 1464$. Monochromated MoK_α radiation, $\lambda = 0.71069$ Å, $\mu = 0.7$ cm^{-1}. Data were collected using a crystal (*ca* $0.5 \times 0.4 \times 0.4$ mm) on an Enraf-Nonius CAD 4 diffractometer operating in the θ–2θ mode with $\Delta \theta = (0.8 + 0.35 \tan \theta)^\circ$ and a max. scan time of 1 min. A total of 3594 reflections were measured for $2 < \theta < 25^\circ$ and $+h$, $+k$, $+1$ and after averaging ($R_{int} = 0.05$), 1248 unique reflections with $|F^2| > \sigma$ (F^2) were used in the refinement where σ $(F^2) = \{\sigma^2(I) + (0.04I)^2\}^{1/2}/L_p$. There was no crystal decay and no absorption corrections were made. The structure was solved by direct methods using SHELXS and the non-H atoms were refined anisotropically by full matrix least squares. H atoms attached to C atoms were placed at calculated positions and held fixed with $B_{iso} = 1.3B_{eq}$ for the atom to which they were bonded. Hydroxyl-H atoms were not located. The weighting scheme was $w = 1/\sigma^2(F)$ and the final residuals were $R = 0.064$; $R' = 0.082$. Tables of crystallographic data have been deposited with the Cambridge Crystallographic Data Centre.

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