Studies in Terpenoid Biosynthesis. Part 35.[1] Biosynthetic Sequences leading to the Diterpenoid Aphidicolin in *Cephalosporium aphidicola*

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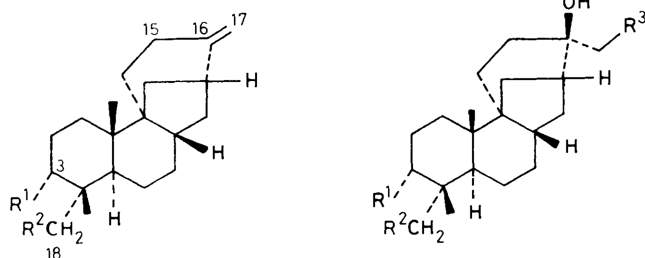
[17-^{14}C]-Labelled samples of 18-hydroxyaphidicol-16-ene, 3α,18-dihydroxyaphidicol-16-ene, 16β,17- and 16β,18-dihydroxyaphidicolane, and 3α, 16β, 18- and 16β,17,18-trihydroxyaphidicolane have been prepared from aphidicolin and shown to be incorporated into aphidicolin by *Cephalosporium aphidicola* to the extent of 0.86, 16.4, 3.5, 20.5, 52.6, and 16.9%, respectively. These results suggest that although the major pathway of aphidicolin biosynthesis involves the 16β-alcohols, the 16-enes may also be utilized whilst a metabolic grid relationship may exist between the variously hydroxylated 16β-alcohols.

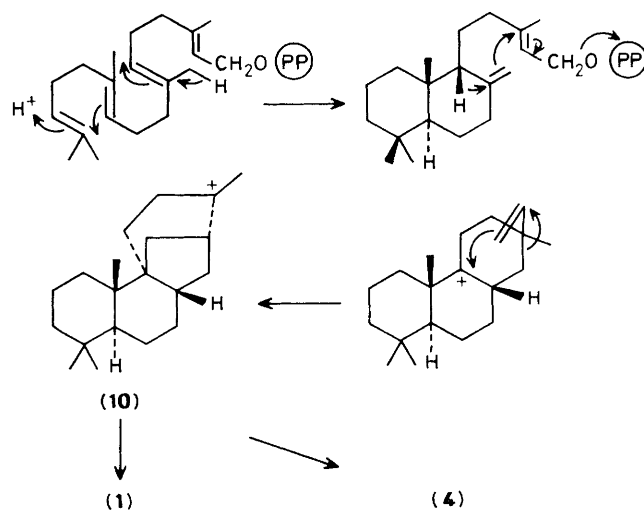
The diterpenoid tetraol, aphidicolin (**9**) is a biologically interesting metabolite of *Cephalosporium aphidicola*.[2] It possesses a unique tetracarbocyclic skeleton. Previous biosynthetic studies utilizing acetate and mevalonate have established [3,4] the position of the constituent isoprene units and have revealed that a hydrogen rearrangement takes place from C-9 to C-8. At the outset of our work, the only other metabolite of this series which had been detected [2] in *C. aphidicola* was the hydrocarbon, aphidicol-16-ene (**1**). By analogy with the relationship of *ent*-kaurene [5] with the hydroxylated kaurenes, the gibberellins and the kaurenolides, we therefore assumed that aphidicol-16-ene (**1**) would play a major role in the biosynthesis of aphidicolin. However, we have subsequently shown that both aphidicol-16-ene and aphidicolan-16β-ol (**4**) are produced [6] by the fungus and are transformed into aphidicolin (0.09 and 7.9% incorporation, respectively). In this paper we present evidence for the biosynthetic sequence from aphidicol-16-ene and aphidicolan-16β-ol in *C. aphidicola* leading to aphidicolin.



(**1**) R′ = R² = H
(**2**) R′ = H, R² = OH
(**3**) R′ = R² = OH

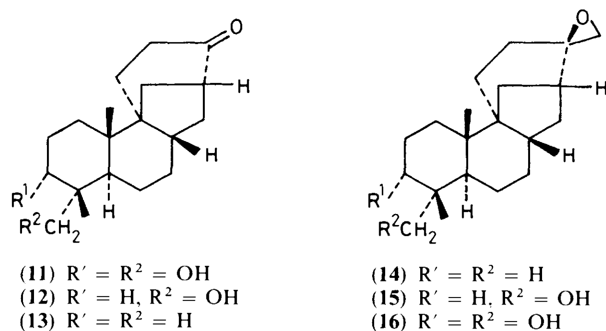
(**4**) R′ = R² = R³ = H
(**5**) R′ = R² = H, R³ = OH
(**6**) R′ = R³ = H, R² = OH
(**7**) R′ = R² = OH, R³ = H
(**8**) R′ = H, R² = R³ = OH
(**9**) R′ = R² = R³ = OH

Simple chromatography of both the mycelial and broth extracts of *C. aphidicola* has failed to yield putative intermediates. However 3-deoxyaphidicolin (**8**) has been isolated [7] together with aphidicolin (**9**) from *Phoma betae*. Both aphidicol-16-ene (**1**) and aphidicolan-16β-ol (**4**) are inter-related through the cation (**10**) which itself represents a likely step in the biosynthesis (see the Scheme). Hence we considered two possibilities: (*a*) that there were two pathways, a major pathway involving the 16-alcohols and a minor pathway involving the 16-enes, or (*ii*) that aphidicol-16-ene (**1**) is converted into aphidicolan-16β-ol (**4**) and then the latter is transformed into aphidicolin (**9**) without the intervention of further alkenic intermediates. The isolation of 3-deoxyaphidicolin (**8**) from *Phoma betae* [7] led us to consider the possibility that oxidation at C-3 was a late hydroxylation and that it was preceded by either hydroxylation at C-17 or C-18. Hence the following substrates were selected for study: 18-hydroxyaphidicol-16-ene (**2**), 3α,18-dihydroxyaphidicol-16-ene (**3**), 16β,17-dihydroxyaphidicolane (**5**), 16β,18-dihydroxyaphidicolane (**6**), 3α,16β,18-trihydroxyaphidicolane (**7**) and 16β,17,18-trihydroxyaphidicolane (**8**). Aphidicolin (**9**) is readily oxidized [2] with periodic acid to afford the 17-nor-16-one (**11**) and C-17 may be recovered as the formaldehyde dimethone derivative. Thus in order to demonstrate specificity of labelling, the substrates were labelled at C-17. They were prepared from aphidicolin as follows.



Scheme.

Aphidicolin (**9**) was cleaved to the 17-nor-16-one (**11**)[2] and the 3-oxygen function was removed by dehydration of the 1,3-glycol with dimethylformamide dimethyl acetal [6,8] and hydrogenation to afford 18-hydroxy-17-noraphidicolan-16-one (**12**).[6] The C-18 oxygen function was removed from the latter by protecting the C-16 ketone group as an ethylenedioxy derivative, chlorination at C-18 using triphenylphosphine–carbon tetrachloride, and hydrogenolysis with tributyltin



(**11**) R′ = R² = OH
(**12**) R′ = H, R² = OH
(**13**) R′ = R² = H

(**14**) R′ = R² = H
(**15**) R′ = H, R² = OH
(**16**) R′ = R² = OH

hydride to afford (**13**).[6] The 16-enes (**2**) and (**3**) were prepared by Wittig methylenation of the 17-nor-16-ones with [^{14}C]methyltriphenylphosphonium iodide. The 16β-alcohols and the 16β,17-glycols were obtained *via* the 16β, 17-epoxides (**14**)—(**16**). Reaction of the nor ketones with [^{14}C]trimethylsulphoxonium iodide affords predominantly the 16β,17-epoxides, *i.e.* with the aphidicolin stereochemistry, although they are accompanied by variable amounts of the 16α-epimer.[9] Although the epimeric epoxides were difficult to separate, the corresponding C-16 alcohols were more easily purified and may be distinguished by their ^{1}H n.m.r. spectra in that the 17-H signal of the C-16β alcohol resonates at higher field than its epimer. The C-16β alcohols (**6**) and (**7**) were prepared by reduction of the epoxides (**15**) and (**16**), respectively, with lithium aluminium hydride. The 16β,17-glycols (**5**) and (**8**) were obtained by base-catalysed hydrolysis (potassium hydroxide, in dimethyl sulphoxide) of the epoxides (**14**) and (**15**). These conditions were chosen to avoid further epimerization or rearrangement at C-16.

The substrates were fed to *C. aphidicola* approximately 8 days after inoculation. The aphidicolin was isolated after a further 21 days. The results are given in the Table. Specificity in the cases indicated was demonstrated by oxidation of the aphidicolin with periodic acid. The C-17 label was recovered in the formaldehyde dimethone derivative. In all cases that were examined, the nor ketone was inactive.

The following conclusions may be drawn from this work. First, the major route to aphidicolin is *via* the C-16β alcohols rather than *via* the 16-enes although these compounds may also participate in the biosynthesis. Secondly, the relative magnitude of the incorporations of the 16β-alcohols suggests that the major route involves hydroxylation of aphidicolan-16β-ol at C-18, then at C-3, and finally at C-17. However there appears to be relatively little discrimination between the order of hydroxylation at C-3 and C-17, although these studies were carried out over a number of years and it was not possible to eliminate the effects of any variations in the micro-organism. Nevertheless it does suggest that these hydroxylases may not be fully structure-specific and may be able to handle substrate analogues in this biologically interesting series. This is particularly interesting in view of the high incorporations that were obtained and the difficulty of carrying out the transformation $>C(OH)CH_3$ to $>C(OH)CH_2OH$ in a regiospecific chemical manner and without the intervention of skeletal rearrangements.

Experimental

General experimental details have been described previously.[6]

[17-^{14}C]-18-*Hydroxyaphidicol*-16-*ene* (**2**).—Sodium hydride [from an 80% suspension in oil freshly washed with light petroleum (×3) and dried under high vacuum; 78 mg] was added to dimethyl sulphoxide (6 ml) under nitrogen and stirred at 70 °C until it was fully dissolved. [^{14}C]Methyltriphenylphosphonium iodide (1.32 g) in dimethyl sulphoxide (3 ml) was added at room temperature and the mixture was stirred for 15 min. The mixture was then poured through a glass wool plug into a solution of 18-hydroxy-17-noraphidicolan-16-one[6] (200 mg) in tetrahydrofuran (2 ml) and kept at room temperature for 30 min. The mixture was then poured onto ice–water (100 ml) and the products were recovered in chloroform. The extract was washed consecutively with dilute hydrochloric acid and saturated aqueous sodium hydrogen carbonate, dried and the solvent evaporated. The residue was chromatographed on silica. Elution with toluene–ethyl acetate (8:2) gave a gum (134 mg) which crystallized from aqueous methanol to afford [17-^{14}C]-18-*hydroxyaphidicol*-16-*ene* (**2**) as white plates, m.p. 99—100 °C (Found: C, 83.1; H, 11.1. $C_{20}H_{32}O$ requires C, 83.3; H, 11.2%); $\nu_{max.}$ 3 350 and 1 660, 880 cm^{-1}; δ($CDCl_3$) 0.80 (3 H, s, 20-H_3), 1.00 (3 H, s, 19-H_3), 3.00 and 3.38 (each 1 H, d, *J* 11.5 Hz, 18-H_2), and 4.4 (2 H, m, 17-H_2); *m/z* 288 (M^+, 7%), 273 (1), and 257 (100). The *acetate*, prepared with acetic anhydride in pyridine, crystallized from methanol as needles, m.p. 115—117 °C, (Found: C, 80.1; H, 10.6. $C_{22}H_{34}O_2$ requires C, 79.95; H, 10.4%); $\nu_{max.}$ 1 730, 1 660, 1 240, and 880 cm^{-1}; δ($CDCl_3$) 0.86 (3 H, s, 20-H_3), 0.99 (3 H, s, 19-H_3), 2.05 (3 H, s, OAc), 3.62 and 3.85 (each 1 H, d, *J* 11.5 Hz, 18-H_2), and 4.45 (2 H, m, 17-H_2); *m/z* 330 (M^+, 2%), 270 (10), and 257 (43).

[17-^{14}C]-3α,18-*Dihydroxyaphidicol*-16-*ene* (**3**).—Sodium hydride (purified as above; 78 mg) in dimethyl sulphoxide (6 ml) was stirred at 70 °C under nitrogen until it was fully dissolved. [^{14}C]Methyltriphenylphosphonium iodide (1.32 g) in dimethyl sulphoxide (3 ml) was added at room temperature and the mixture was filtered through glass wool into a solution of 3α,18-dihydroxyaphidicolan-16-one[2] (300 mg) in tetrahydrofuran (2 ml) and the filtrate was left at room temperature for 30 min. The mixture was poured onto ice–water (100 ml) and extracted with chloroform. The extract was washed with dilute hydrochloric acid and saturated aqueous sodium hydrogen carbonate, and

Table. Incorporation of precursors into aphidicolin by *C. aphidicola*

Substrate	Amount fed (mg)	Amount fed (KBq)	Volume (l)	Amount aphidicolin isolated (mg)	Amount aphidicolin isolated (KBq)	% Incorporated
18-Hydroxyaphidicol-16-ene (**2**)	20	16.5	2.25	490	0.142	0.86
3α,18-Dihydroxyaphidicol-16-ene (**3**)	23.6	14.69	2.25	438	2.41	16.4
16β,17-Dihydroxyaphidicolane (**5**)	24.6	0.9	3	108	0.032	3.5
16β,18-Dihydroxyaphidicolane (**6**)	40	14.6	7.5	500	3.00	20.5
3α,16β,18-Trihydroxyaphidicolane (**7**)	76.8	27	6.75	446	14.22	52.6
16β,17,18-Trihydroxyaphidicolane (**8**)[a]	52	1.83	9	350	0.383	16.9

[a] Fed as a 3:1 mixture of β:α epimers; the results were calculated on the assumption that only the β-epimer is utilized.

dried. The solvent was evaporated and the residue was chromatographed on silica. Elution with toluene–ethyl acetate (7:3) gave [17-^{14}C]-3α,18-dihydroxyaphidicol-16-ene (**3**) (200 mg) which crystallized from ether–light petroleum as needles, m.p. 146—149 °C (lit.,[2] 147—148 °C) (Found: C, 78.9; H, 10.6. Calc. for $C_{20}H_{32}O_2$: C, 78.9; H, 10.6%); $\nu_{max.}$ 3 250, 1 650, and 880 cm^{-1}; δ($CDCl_3$) 0.79 (3 H, s, 20-H_3), 0.83 (3 H, s, 19-H_3), 3.34 and 3.43 (each 1 H, d, *J* 11.5 Hz, 18-H_2), 3.66 (1 H, m, 3-H), and 4.43 (2 H, m, 17-H_2).

[17-^{14}C]-16β,17-*Epoxy-18-hydroxyaphidicolane* (**15**).—Dimethyl sulphoxide (9 ml) and [^{14}C]trimethylsulphoxonium iodide (1.3 g) were added under nitrogen to sodium hydride (200 mg) (freshly purified as above). The mixture was stirred until the evolution of hydrogen had ceased. 18-Hydroxy-17-noraphidicolan-16-one[6] (700 mg) in tetrahydrofuran (2.5 ml) was added. The mixture was stirred for 2 h and then poured into ice–water. The products were recovered in ethyl acetate and washed with water. The solvent was evaporated and the residue chromatographed on silica. Elution with toluene–ethyl acetate (1:1) gave a solid (700 mg) which comprised a mixture (3:1 by n.m.r. spectroscopy). of [17-^{14}C]-16β,17-epoxy-18-hydroxyaphidicolane (**15**) and its 16α-epimer. δ(360 MHz; $CDCl_3$) 0.79 (3 H. s. 20-H_3). 1.01 (3 H, s, 19-H_3), 2.61 and 2.62 (1.5 H, 2 × d, *J* 4.5 Hz. 17-H, 16β-epimer), 2.65 and 2.68 (0.5 H, 2 × d, *J* 5 Hz, 17-H, 16α-epimer), and 3.05 and 3.39 (each 1 H, d, *J* 11 Hz, 18-H). *m/z* (−ve FAB) 303 (MH^+).

[17-^{14}C]-16β,17-*Epoxy*-3α,18-*dihydroxyaphidicolane* (**16**).—[^{14}C]Trimethylsulphoxonium iodide (431 mg) in dimethyl sulphoxide (6 ml) was added to sodium hydride (47 mg) (freshly purified as above) and the mixture was stirred until the evolution of hydrogen had ceased. The reaction mixture was filtered through a glass wool plug into a solution of 3α,18-dihydroxy-17-noraphidicolan-16-one[2] (300 mg) in tetrahydrofuran (2 ml). The solution was stirred for 2 h and then at 50 °C for 1 h whereupon it was poured into water and the product was recovered in chloroform. The organic layer was washed with water, dried. and the solvent was evaporated. The residue was chromatographed on silica. Elution with toluene–ethyl acetate (8:2) gave a powder which was recrystallized from ethyl acetate–light petroleum to afford [17-^{14}C]-16β,17-*epoxy*-3α,18-*dihydroxyaphidicolane* (**16**) (180 mg) as fine needles, m.p. 155—158 °C (Found: C, 75.1; H, 10.4. $C_{20}H_{32}O_3$ requires C, 75.0: H, 10.1%); $\nu_{max.}$ 3 400, 3 250, and 910 cm^{-1}; δ($CDCl_3$) 0.65 (3 H, s, 20-H_3), 0.95 (3 H, s, 19-H_3), 2.64 (2 H, s, 17-H_2), 3.35 (brs, 18-H), and 3.64 (1 H. m. 3-H); *m/z* 320 (M^+, 2%), 318 (9), 302 (24), 289 (10), and 272 (47).

[17-^{14}C]-16β,18-*Dihydroxyaphidicolane* (**6**).—[17-^{14}C]-16,17-Epoxy-18-hydroxyaphidicolane (100 mg) in freshly distilled tetrahydrofuran (2 ml) was treated with lithium aluminium hydride (50 mg) in an ice-bath for 30 min. The solution was concentrated, moist ethyl acetate was added, and the extract was washed with water and dried and the solvent evaporated to afford a residue which was separated by preparative layer chromatography to afford [17-^{14}C]-16β,18-dihydroxyaphidicolane (**6**) (60 mg) as a gum, $\nu_{max.}$ 3 350 cm^{-1}; δ($CDCl_3$) 0.76 (3 H, s, 20-H), 0.96 (3 H, s, 19-H_3), 1.18 (3 H, s 17-H), and 3.15 and 3.25 (each 1 H, d, *J* 10 Hz, 18-H). The second minor component (20 mg) was [17-^{14}C]-16α,18-dihydroxyaphidicolane, $\nu_{max.}$ 3 350 cm^{-1}; δ($CDCl_3$) 0.76 (3 H, s, 20-H), 0.96 (3 H, s, 19-H), 1.30 (3 H, s, 17-H), and 3.15 and 3.25 (each 1 H, d, *J* 10.5 Hz, 18-H).

[17-^{14}C]-3α,16β,18-*Trihydroxyaphidicolane* (**7**).—[17-^{14}C]-16β,17-Epoxy-3α,18-dihydroxyaphidicolane (180 mg) was added to a suspension of lithium aluminium hydride (80 mg) in dry tetrahydrofuran (15 ml) and the mixture was heated under reflux for 1 h under nitrogen. The mixture was then poured into dilute hydrochloric acid and the products were recovered in ethyl acetate. The extract was washed consecutively with dilute hydrochloric acid, aqueous sodium hydrogen carbonate, aqueous sodium chloride, dried and the solvent was evaporated. The residue was recrystallized from methanol to give [17-^{14}C]-3α,16β,18-*trihydroxyaphidicolane* (**7**), m.p. 220—222 °C, (Found: C, 74.2; H, 10.7. $C_{20}H_{34}O_3$ requires C, 74.5; H, 10.6%); $\nu_{max.}$ 3 490 and 3 220 cm^{-1}; δ(C_5D_5N) 0.79 (3 H, s, 20-H), 1.02 (3 H, s, 19-H), 1.32 (3 H, s, 17-H_3), 3.64 and 3.80 (each 1 H, d, *J* 11.5 Hz, 18-H), 3.93 (1 H, m, 3-H), δ(CD_3OD), 0.72 (3 H, s, 20-H_3), 0.99 (3 H, s, 19-H_3), 1.06 (3 H, s, 17-H_3), 18-H_3 obscured by solvent, and 3.6 (1 H, m, 3-H).

[17-^{14}C]-16β,17-*Dihydroxyaphidicolane* (**5**).—[17-^{14}C]-16β,-17-Epoxyaphidicolane[6] (260 mg) in dimethyl sulphoxide (15 ml; containing 15% water), was treated with potassium hydroxide (200 mg) at 70 °C overnight. The mixture was poured into water and the products were recovered in ethyl acetate. The extract was washed consecutively with dilute hydrochloric acid, aqueous sodium hydrogen carbonate, and aqueous sodium chloride, and then dried and evaporated. The residue crystallized from ethyl acetate to afford [17-^{14}C]-16β,17-*dihydroxyaphidicolane* (**5**) (170 mg) as needles, m.p. 173—175 °C (Found: C, 78.3; H, 11.2. $C_{20}H_{34}O_2$ requires C, 78.5; H, 11.2%): $\nu_{max.}$ 3 350 and 3 240 cm^{-1}; δ($CDCl_3$) 0.85, 0.86, and 0.95 (each 3 H, s, 18-, 19-, and 20-H_3) and 3.38 and 3.46 (each 1 H, d. *J* 11 Hz, 17-H).

[17-^{14}C]-16β,17,18-*Trihydroxyaphidicolane* (**8**).—16β,17-Epoxy-18-hydroxyaphidicolane [containing the 16α-epimer (3:1) as above; (350 mg)] in dimethyl sulphoxide (15 ml, containing 15% water) was treated with potassium hydroxide (200 mg) at 70 °C overnight. The products were recovered as above to afford [17-^{14}C]-16β,17,18-trihydroxyaphidicolane (310 mg) as an amorphous mixture (3:1) with its 16α-epimer, δ($CDCl_3$) (major component) 0.77 (3 H, s, 20-H_3). 0.99 (3 H, s, 19-H_3), 3.03 and 3.48 (each 1 H, doublets, *J* 11 Hz. 18-H), and 3.35 and 3.42 (each 1 H, d, *J* 11 Hz, 17-H); δ (minor component) 0.76 (3 H, s, 20-H_3), 0.98 (3 H, s, 19-H_3), 3.03 and 3.48 (each 1 H, d, *J* 11 Hz, 18-H), and 3.54 and 3.62 (each 1 H, d, *J* 11 Hz, 17-H). The epimers can be separated as the corresponding acetonides.[9]

Feeding Experiments.—The substrate (see the Table) in ethanol (*ca.* 10 ml) containing Tween 80 (0.75 ml) was distributed evenly between cultures of *Cephalosporium aphidicola* (CMI 68689) growing in Thomson bottles, (750 ml medium each[4]) (total volume see the Table) on day 8 from inoculation. The cultures were harvested on day 28 and the aphidicolin was isolated as described previously.[4] It was purified by chromatography, identified, (t.l.c. and n.m.r. spectroscopy) and crystallized to constant radioactivity. The results are tabulated.

Degradation of Aphidicolin: Typical Procedure.—Aphidicolin (77.5 mg) in pyridine (5 ml) and water (1.6 ml) was treated with periodic acid (50%; 2 ml) at room temperature for 30 min. The solution was acidified with concentrated sulphuric acid and steam distilled. The distillate was collected in a solution of dimedone* (78 mg) in ethanol (6 ml) containing triethylamine (2 drops). The formaldehyde dimedone derivative (26 mg) was recrystallized from methanol as needles, m.p. 189 °C. Repetition of the reaction with periodic acid, acidification with hydrochloric acid and recovery in chloroform gave 3α,18-dihydroxy-17-noraphidicolan-16-one (40 mg) which crystallized from ethyl acetate as needles, m.p. 155 °C (lit.,[2] 155—156 °C).

* 5,5-Dimethylcyclohexa-1.3-dione

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