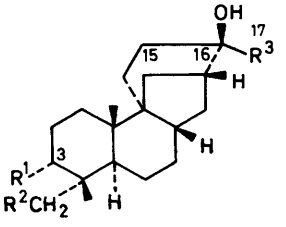
Studies in Terpenoid Biosynthesis. Part 38.[1] The Role of an 16β,17-Epoxyaphidicolane in the Minor Biosynthetic Pathway Leading to Aphidicolin

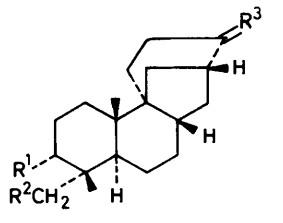
Mark J. Ackland, John F. Gordon, and James R. Hanson
School of Molecular Sciences, University of Sussex, Brighton, Sussex, BN1 9QJ
Arnold H. Ratcliffe
ICI Pharmaceuticals Division plc, Alderley Park, Macclesfield, Cheshire, SK10 4TG

The hydroxylation of [15-^{2}H, 17-$^2H_3$]aphidicolane-3α,16β,18-triol at C-17 in the biosynthesis of aphidicolin is shown to involve an isotope effect whilst there is no effect in the incorporation of [15-^{2}H, 17-$^2H_2$]aphidicol-16-ene-3α,18-diol suggesting that the transformation of the 16-ene involves epoxidation and hydrolysis rather than hydration and hydroxylation. Feeding experiments suggest that the 16β,17-epoxyaphidicolane-3α,18-diol is involved in this transformation.

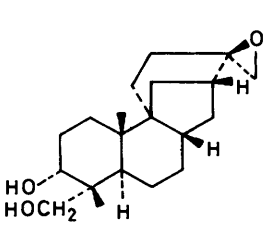
We have presented evidence[2] to show that the biosynthesis of the tetracyclic diterpenoid aphidicolin (**1**) by the fungus, *Cephalosporium aphidicola* involves a major pathway *via* the aphidicolan-16β-ols (**2**) and a minor pathway *via* the aphidicol-16-enes (**4**). In this paper we consider the minor route by which the 16-ene is converted into the 16,17-glycol of aphidicolin (**1**). There are a number of plausible routes for this bio-transformation. Firstly the 16-ene may be hydrated to give a 16β-ol which is then hydroxylated at C-17 by the enzyme systems responsible for the major pathway leading to aphidicolin. Hydration of a 16-ene is a common diterpenoid bio-transformation for example, in *Gibberella fujikuroi*.[3] Secondly the 16-ene may be epoxidized, the oxirane reductively cleaved and then C-17 hydroxylated. Thirdly, the 16-ene may be epoxidized and the oxirane may then be hydrolysed. In some steroid transformations the hydroxylation of a saturated centre is paralleled by epoxidation of the corresponding unsaturated centre.[4] However apart from the steroids,[5] there are relatively few situations in which this has been examined. The 7β-hydroxylation of *ent*-kaurenoic acid and the 6β,7β-epoxidation of some *ent*-kaur-6,16-dienes may be a diterpenoid example.[6] The first two routes involve hydroxylation of a 16α-methyl group whereas the latter does not. Hence if the micro-organism utilizes for this minor pathway, a route which generates a C-17 methyl group which is then hydroxylated, there should be a loss of deuterium from C-17 of a [17-$^2H_2$]aphidicol-16-ene with a corresponding isotope effect. In contrast, a pathway involving epoxidation and hydrolysis would involve no loss of deuterium.



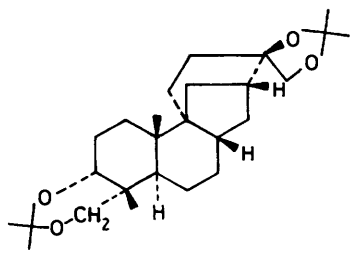
(**1**) $R^1 = R^2 = OH$, $R^3 = CH_2OH$
(**2**) $R^1 = R^2 = H$, $R^3 = Me$
(**3**) $R^1 = OH$, $R^2 = OH$, $R^3 = Me$



(**4**) $R^1 = R^2 = H$, $R^3 = CH_2$
(**5**) $R^1 = R^2 = OH$, $R^3 = CH_2$
(**6**) $R^1 = R^2 = OH$, $R^3 = O$



(**7**)



(**8**)

Aphidicol-16-ene-3α,18-diol (**5**) was the best olefinic precursor of aphidicolin (16.4% incorporation) and hence the bio-transformation was examined with this compound as the substrate. To test the validity of this distinction and to assess the magnitude of any isotope effect, the hydroxylation was also examined with the intermediate [17-$^2H_3$]aphidicolane-3α,16β,18-triol (**3**).

The labelled aphidicol-16-ene-3α,18-diol (**5**) was prepared from the readily accessible 3α,18-dihydroxy-17-noraphidicolan-16-one (**6**).[7] Since there is the possibility of exchange at C-15 and the loss of deuterium from the alkene during the Wittig olefination,[8] this reaction was examined under a number of conditions. When the ylide from [$^2H_3$]methyltriphenylphosphonium bromide was generated with sodium hydride in [$^2H_6$]dimethyl sulphoxide, the olefinic signals (δ 4.38 and 4.45) in the product had an integral which was 7% that of a 1 H multiplet at δ 3.60 (3-H). For mass spectral analysis, the negative ion fast atom bombardment spectrum provided a useful M − H peak. This mass spectrum, with the presence of peaks at 306 and 307 a.m.u. ($^2H_3$ and $^2H_4$), showed that extensive exchange had also occurred, presumably at C-15. The use of alkyl-lithiums as bases in the Wittig reaction has been reported[9,10] to give [^{2}H]-labelled material without scrambling. However initial experiments with 3α,18-dihydroxy-17-noraphidicolan-16-one (**6**) and the [$^2H_3$]phosphonium ylide generated with butyl-lithium in tetrahydrofuran–ether gave an olefin containing only 60% $^2H_2$ species. Prior exchange of the hydroxy protons using methan[$^2H_2$]ol led to an improvement in the deuterium content at C-17 although there was still some exchange at C-15. Indeed because the overall biosynthetic incorporation of aphidicol-16-ene-3α,18-diol (**5**) into aphidicolin (**1**) was relatively low, the extent of deuteriation at C-17 in the resultant aphidicolin was determined relative to that at C-15 and hence the exchange due to enolization in the Wittig olefination was turned to advantage.

[17-$^2H_3$]Aphidicolane-3α,16β,18-triol (**3**) was prepared from 3α,18-dihydroxy-17-noraphidicolan-16-one (**6**) using [$^2H_9$]trimethylsulphoxonium iodide and butyl-lithium in tetrahydrofuran.[11] In trial experiments with unlabelled material this gave a 1:1 mixture of the 16α- and the required 16β-epoxide (**7**). However when the reaction was carried out in dimethyl

sulphoxide, the ratio improved to 1:3. The tendency of the sulphoxonium ylide to give the thermodynamically more stable product may be due to the reversibility of the intermediate betaine formation.[12] This would be favoured by the addition of an aprotic dipolar co-solvent. 1,3-Dimethyl-4,5-dihydroimidazolone (DMI) is a non-toxic alternative to HMPA which has been used in the Wittig olefination.[13] Its use in this case favoured the formation of the thermodynamically more stable 16β-epoxide to the extent of 1:4 (α:β) and hence these conditions were used for the labelling work. However substantial exchange also occurred under these conditions at C-15. The ^2H n.m.r. spectrum of the product exhibited peaks at δ 2.1 and 1.1 (15-^2H) and 2.6 (17-^2H) with a relative integral of 1:1:2, whilst the mass spectrum (negative ion f.a.b.) contained peaks corresponding to $^2H_4$ (60%), $^2H_3$ (30%), and $^2H_2$ (10%) species. Reduction of this epoxide with lithium aluminium deuteride gave [15-^2H, 17-$^2H_3$]aphidicolane-3α,16β,18-triol which from mass spectral analysis (negative ion f.a.b.), contained $^2H_5$ (60%), $^2H_4$ (31%), and $^2H_3$ (8%) species. The corresponding [17-^{14}C]aphidicol-16-ene-3α,18-diol and [17-^{14}C]aphidicolane-3α,16β,18-triol were prepared using [^{14}C]-methyltriphenylphosphonium bromide and [^{14}C]trimethylsulphoxonium iodide.

[15-^2H, 17-$^2H_3$]- and [17-^{14}C]-Aphidicolane-3α,16β,18-triols (**3**) were co-fed to an 8-day old culture of *Cephalosporium aphidicola*. The fermentation was harvested after a further 21 days. The resultant aphidicolin showed a 22.7% incorporation of the [^{14}C]-labelled triol and, from its mass spectrum, a 17.9% incorporation of the [^2H]-triol. The same result was obtained from the ^1H n.m.r. spectrum. The relative integrals of the 17-H and 18-H AB quartets were 11:18, *i.e.* 35.9% of the isolated aphidicolin was [17-$^2H_2$]-material corresponding to an incorporation of 17.9% of the [^2H]-triol.

The ratio of the incorporation of the [^{14}C]-labelled triol to that of the [^2H]-triol was 1.28:1 suggesting an isotope effect of comparable magnitude to those for steroid hydroxylations.[5]

Since the incorporation of the aphidicol-16-ene-3α,18-diol (**5**) into aphidicolin (**3**) was considerably lower than that of the triol, the ^2H n.m.r. spectrum was used for the analysis with the 15-^2H signals acting as an internal standard. The ^2H n.m.r. spectrum of the [15-^2H, 17-$^2H_2$]aphidicol-16-ene-3α,18-diol showed signals at δ 4.4 (17-^2H), 2.3 and 2.09 (15-^2H) in the ratio of 2.33:1. The resultant aphidicolin (**1**), examined as its bisacetonide (**8**)[7] possessed signals at δ 3.5 and 3.7 (17-^2H) and δ 1.6 and 1.4 (15-^2H) in the ratio of 2.34:1 and thus no deuterium had been lost from C-17. The incorporation of [17-^{14}C]-aphidicol-16-ene-3α,18-diol into aphidicolin in this experiment was 3.4%. Since the hydroxylation of a methyl group at C-17 shows an isotope effect and yet there was no variation in the amount of deuterium at C-17 in the transformation of a 16-ene, the biosynthesis utilizing the latter must involve an epoxidation and hydrolysis rather than hydration and hydroxylation.

The intervention of the epoxide was tested in the following ways. Firstly to eliminate hydration, [17-^{14}C]aphidicol-16-ene-3α,18-diol (**5**) was incubated with *C. aphidicola* and aphidicolane-3α,16β,18-triol (**3**) was added as a trap. Although on this occasion there was a 14.5% incorporation of the 16-ene into aphidicolin, there was no incorporation into the trap. Secondly [17-^{14}C]-16β,17-epoxyaphidicolin-3α,18-diol (**7**) and [2-^3H]-mevalonic acid were co-fed to *C. aphidicola*. The fermentations were harvested at different times (see Table). The epoxide was converted into aphidicolin rapidly reaching a peak at the same time as the incorporation of the mevalonate. Since the epoxide is likely to be unstable under the slightly acidic (pH 4—4.4) conditions of the fermentation, a parallel control experiment was carried out in which the epoxide was stirred with the fermentation medium in the absence of the fungus. The aphidicolin produced after 28 days represented a conversion of 30%. Bearing in mind the more rapid conversion into aphidicolin in the presence of the fungus, we conclude that some microbiological hydrolysis of the epoxide had taken place although its extent is difficult to quantify.

In conclusion we propose that the minor pathway which utilizes the aphidicol-16-enes as substrates, involves an epoxidation and hydrolysis rather than a hydration and hydroxylation, in order to generate the 16,17-glycol of aphidicolin.

Experimental

General experimental details have been described previously.[2]

Wittig Methylenation of 3α,18-*Dihydroxy*-17-*noraphidicolan*-16-*one*.—(*a*) Sodium hydride (80% suspension in oil) was washed with light petroleum (×3) and dried *in vacuo*. [$^2H_6$]-Dimethyl sulphoxide (3 ml) was heated with sodium hydride (50 mg) at 70 °C under nitrogen for 30 min. and then cooled. [$^2H_3$]Methyltriphenylphosphonium bromide (500 mg) in [$^2H_6$]dimethyl sulphoxide (5 ml) was added and stirring was continued for 30 min before 3α,18-dihydroxy-17-noraphidicolan-16-one (120 mg) was added. After a further hour the mixture was poured into water and the products were extracted with ethyl acetate. The extract was washed with dilute hydrochloric acid, aqueous sodium hydrogen carbonate, and brine, and dried. The solvent was evaporated and the residue was chromatographed on silica to afford aphidicol-16-ene-3α,18-diol (100 mg), m.p. 147—149 °C (lit.,[7] m.p. 147—148 °C); δ($CDCl_3$) 0.70 (3 H, s, 20-H), 0.98 (3 H, s, 19-H), 3.37 and 3.46 (each 1 H, d, *J* 10 Hz, 18-H), 3.66 (1 H, m, 3-H), and 4.40 and 4.48 (each 0.075 H, 17-H); *m/z* (negative f.a.b.) *M* − H peak 307 (60, $^2H_4$), 306 (30, $^2H_3$), and 305 (10, $^2H_2$).

(*b*) Butyl-lithium (1.65M solution in ether; 2ml) was added to a suspension of [$^2H_3$]methyltriphenylphosphonium bromide (700 mg) in dry ether (15 ml) under argon for 1 h. 3α,18-Dihydroxy-17-noraphidicolan-16-one (100 mg) in ether (5 ml) was added dropwise and stirring was continued overnight. The mixture was poured into water (100 ml) and the products were recovered in ethyl acetate. The extract was washed with brine, dried, and evaporated to give a gum which was chromatographed on silica. Elution with toluene–ethyl acetate (1:1) gave aphidicol-16-ene-3α,18-diol (75 mg), m.p. 148—150 °C, *m/z* (negative f.a.b.) *M* − H peak, 305 (60, $^2H_2$), and 304 (40, $^2H_1$).

(*c*) 3α,18-Dihydroxy-17-noraphidicolan-16-one (500 mg) was dissolved in methan[^2H]ol (3 ml) under nitrogen and after a few minutes the solvent was removed under reduced pressure. The procedure was repeated twice more to give 3α,18-di[^2H]-hydroxy-17-noraphidicolan-16-one, $\nu_{max.}$ 2 390 cm^{-1} (O–D stretch). The Wittig procedure was repeated as above to afford aphidicol-16-ene-3α,18-diol, m.p. 145—148 °C; δ 4.38 and 4.45 (each singlet integral 6% of 1 H, 17-H); *m/z* (negative f.a.b.) *M* − H peak, 306 (40, $^2H_3$), 305 (100 $^2H_2$), and 304 (18, $^2H_1$).

[17-^{14}C]Aphidicol-16-ene-3α,18-diol was prepared following procedure (*a*) using [^{14}C]methyltriphenylphosphonium bromide.

Preparation of [17-$^2H_2$]-16β,17-*Epoxyaphidicolane*-3α,18-*diol*.—[$^2H_9$]Trimethylsulphoxonium iodide was prepared by heating trimethylsulphoxonium iodide (4 g) in deuterium oxide (20 ml) containing anhydrous potassium carbonate (15 mg) on a steam-bath for 1 h. The solution was cooled in an ice-bath and the precipitate was filtered off. The procedure was repeated twice and the product {*m/z* 145 (100, CD_3I), 84 [22, $(CD_3)_2S{=}O$)], and 66 (36, CD_3SO)} was dried at 40 °C *in vacuo*.

Butyl-lithium (1.3M ethereal solution; 5.8 ml) was added to a stirred suspension of [$^2H_9$]trimethylsulphoxonium iodide (1.78 g) in tetrahydrofuran (10 ml). After 1 h, 1,3-dimethyl-4,5-

dihydroimidazolone (distilled from barium oxide, 40 ml) was added and stirring was continued for a further hour. 3α,18-Di[^{2}H]hydroxy-17-noraphidocolan-16-one (780 mg) in 1,3-dimethyl-4,5-dihydroimidazolone (10 ml) was then added and the reaction mixture stirred overnight. It was poured into water and the products were recovered in ethyl acetate. The extract was washed with brine, dried, and evaporated to give an oil which was chromatographed on silica. Elution with toluene–ethyl acetate (1:1) gave a white gum (650 mg), a small sample of which was recrystallized from ethyl acetate to give [17-$^2H_2$]-16β,17-epoxyaphidicolane-3α,18-diol, m.p. 146—150 °C (lit.,[7] m.p. 148—151 °C); δ(^{1}H) (360 MHz) 0.70 (3 H, s, 20-H), 1.00 (3 H, s, 19-H), 3.28 and 3.77 (each 1 H, dd *J* 10 Hz, 18-H), 3.51 (1 H, m, 3-H); δ(^{2}H) 1.10 and 2.12 (15-^{2}H), and 2.57 (17-^{2}H); *m/z* (negative f.a.b.) 323 (100), 322 (50), and 321 (15). When this reaction was carried out using only ^{1}H material the relative integrals of the 17-H_2 signals (16:16), 2.63 and 2.64; 2.66 and 2.69 were 4:1.

Preparation of [15-^{2}H, 17-$^2H_3$]*Aphidicolane*-3α,16β,18-*triol*.—The crude product (600 mg) from the above, in tetrahydrofuran (10 ml) was added dropwise to a stirred suspension of lithium aluminium deuteride (250 mg) in tetrahydrofuran (20 ml) under argon. After 30 min the reaction was quenched with deuterium oxide and the mixture was then poured into dilute hydrochloric acid and the product recovered in ethyl acetate. The extract was washed with aqueous sodium hydrogen carbonate and brine, dried, and evaporated and the product was repeatedly crystallized from methanol to give [15-^{2}H, 17-$^2H_3$]aphidicolane-3α,16β,18-triol (324 mg), m.p. 218—222 °C (lit.,[7] m.p. 220—222 °C); $\nu_{max.}$ 3 380 cm^{-1};δ(C_5D_5N) 0.77 (3 H, s, 20-H), 1.01 (3 H, s, 19-H), 3.49 and 3.90 (each 1 H, d, *J* 11 Hz, 18-H), and 3.78 (1 H, m, 3-H); δ(^{2}H; C_5H_5N), 1.19 (17-^{2}H), 1.35 and 1.45 (15-^{2}H); m.s. (negative f.a.b.) and 326 (83, 60% $^2H_5$), 325 (42, 31% $^2H_4$), and 324 (12, 8% $^2H_3$). The [17-^{14}C]aphidicolane-3α,16,18-triol was also prepared as above.

Incubation Experiments.—(*a*) A solution of [15-^{2}H, 17-$^2H_3$]-aphidicolane-3α,16β,18-triol (150 mg; from the above material) and [17-^{14}C]aphidicolane-3α,16β,18-triol (31.2 mg, $7.16 \cdot 10^4$ d.p.m.) in dimethyl sulphoxide (2.75 ml) and ethanol (0.25 ml) containing Tween 80 (3 drops) was evenly distributed between 3 Thompson bottles (2.25 l) of an 8-day old culture of *C. aphidicola*.[2] The culture was harvested after a further 21 days incubation. The mycelium was filtered off and the broth was extracted with ethyl acetate. The extract (500 mg) was chromatographed on silica to give aphidicolin (69 mg), m.p. 230—233 °C (lit.,[7] 227—233 °C); *m/z* (negative f.a.b.) 342 (8), 341 (30), 340 (12), 339 (6), and 338 (16); ^{14}C $1.63 \cdot 10^4$ d.p.m.; 22.7% incorp. The aphidicolin (60 mg) was heated under reflux in acetone (25 ml) containing toluene-*p*-sulphonic acid (3 mg) for 30 min. The solvent was removed under reduced pressure and the residue partioned between aqueous sodium hydrogen carbonate and ethyl acetate. The ethyl acetate layer was washed with brine, dried, and evaporated to give 3α,18;16β,17-bisisopropylidenedioxyaphidicolane (55 mg), m.p. 144—145 °C (lit.,[7] 145—146°); δ($CDCl_3$) 0.72 (3 H, s, 20-H), 0.98 (3 H, s, 19-H), 1.26 (3 H, s), 1.34 (3 H, s), and 1.40 (6 H, s) ($OCMe_2O$), 3.23 and 3.63 (each 1 H, d, *J* 12 Hz, 18-H), and 3.54 and 3.75 (total 1.25 H, d, *J* 8 Hz, 17-H); δ(^{2}H) 1.47 and 1.70 (15-^{2}H), and 3.54 and 3.75 (17-^{2}H) (relative integral 15-^{2}H:17-^{2}H, 1:1).

(*b*) A solution of [15-^{2}H, 17-$^2H_2$]aphidicol-16-ene-3α,18-diol [210 mg; δ(^{2}H) 2.09 and 2.3 (15-^{2}H): 4.4 (17-^{2}H) 1:2.33] and [17-^{14}C]aphidicol-16-ene-3α,18-diol (13.5 mg, $2.35 \cdot 10^5$ d.p.m.) in dimethyl sulphoxide (3 ml) and ethanol (1 ml) containing Tween 80 (5 drops) was evenly distributed between 4 Thompson bottles (3 l) of a *C. aphidicola* culture 8 days after inoculation. The fermentation was harvested after a further 21 days as described previously to afford aphidicolin (220 mg, $811 \cdot 10^3$ d.p.m., 3.4% incorp.). The aphidicolin (200 mg) was converted into its bisacetonide (180 mg), m.p. 145—146 °C as described previously, δ(^{2}H) 1.6 (15-^{2}H) and 3.5 and 3.7 (17-^{2}H) relative integral 1:2.34.

(*c*) A solution of [17-^{14}C]aphidicol-16-ene-3α,18-diol (10.9 mg, $1.90\ 10^5$ d.p.m.) in dimethyl sulphoxide (1.5 ml) and ethanol (0.5 ml) containing Tween 80 (2 drops) was fed to 2 Thompson bottles (1.5 l) of a *C. aphidicola* culture 8 days after inoculation. Two days later, aphidicolane-3α,16β,18-triol (30 mg) in dimethyl sulphoxide (4 ml) was added. The fermentation was harvested 28 days from inoculation. Extraction of the broth gave an oil (400 mg) to which aphidicolane-3α,16β,18-triol (20 mg) was added. The oil was chromatographed on silica. Elution with toluene–ethyl acetate (1:2) gave aphidicolane-3α,16β,18-triol (23 mg), m.p. 220—222 °C in which, after three recrystallizations from methanol, no radioactivity could be detected. Elution with ethyl acetate gave aphidicolin (148 mg), m.p. 230—239 °C ($2.75 \cdot 10^4$ d.p.m., 14.5% incorporation).

(*d*) A group of 18 Thompson bottles (750 ml each) were inoculated with *C. aphidicola*. A solution of [17-^{14}C]-16β,17-epoxyaphidicolane-3α,18-diol (188 mg; 95.603 MBq mol^{-1}) and [2-^{3}H]mevalonic acid (1.5 ml; 1.06 MBq mol^{-1}) and Tween 80 (6.75 ml) in ethanol (9.75 ml) was fed to groups of three Thompson bottles (1 ml per bottle) at intervals of 4 days over a period of a month. The batches were separately harvested on day 28 and the aphidicolin was purified. The results are tabulated.

Table. Incorporation of [17-^{14}C]-16β,17-epoxyaphidicolane-3α,18-diol and [2-^{3}H]mevalonic acid into aphidicolin

		Radioactivity (Bq mg^{-1})		% Incorporation	
Day of feed	Yield (mg)	^{3}H	^{14}C	^{3}H	^{14}C
4	432	19.19	5.34	3.1	24.7
8	450	37.14	8.58	6.3	41.3
12	429	24.31	8.78	3.9	40.2
16	463	12.53	8.34	2.2	41.3
20	464	9.76	8.49	1.7	42.2
24	465	5.95	6.50	1.1	32.3

The aphidicolin (100 mg) (from the last sample 2.196 MBq mol^{-1}) was cleaved with periodic acid (50%; 2 ml) in pyridine (7 ml) and water (2 ml) at 10 °C for 30 min to afford formaldehyde which was recovered by steam distillation as its dimedone derivative, m.p. 190 °C (22 mg; 2.135 MBq mol^{-1}). Repetition on the aphidicolin from the fourth feed (63.6 mg; 2.82 MBq mol^{-1}) gave 3α,18-dihydroxy-17-noraphidicolan-16-one (55 mg), m.p. 155° which contained no ^{14}C label.

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