Studies in Terpenoid Biosynthesis. Part 32.[1] The Incorporation of Aphidicol-16-ene and Aphidicolan-16β-ol into the Diterpenoid Aphidicolin by the Fungus *Cephalosporium aphidicola*

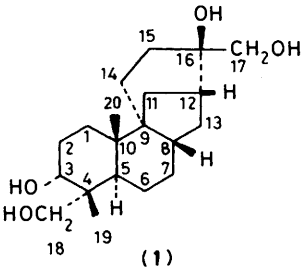
Mark J. Ackland, James R. Hanson,* and Boon Leng Yeoh
School of Molecular Sciences, University of Sussex, Brighton, Sussex, BN1 9QJ
Arnold H. Ratcliffe
I.C.I. Pharmaceuticals Division PLC, Alderley Park, Macclesfield, Cheshire, SK10 4TG

The preparation of [17-^{14}C]aphidicol-16-ene and [17-^{14}C]aphidicolan-16β-ol and their incorporation into aphidicolin by *Cephalosporium aphidicola* to the extent of 0.09 and 7.9% respectively, is described.

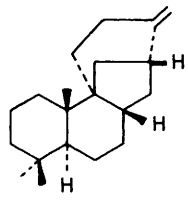
The antiviral tetracyclic diterpenoid aphidicolin (**1**)[2] has attracted interest because of its biological activity as a specific inhibitor of DNA polymerase α.[3] It is a metabolite of several fungi including *Cephalosporium aphidicola*,[2] *Harziella entomophora*,[4] *Nigrospora sphaerica*,[5] and *Onychopora coprophila*.[6] Its structure was established by a combination of chemical and *X*-ray crystallographic studies. Biosynthetic investigations have revealed the diterpenoid origin of the carbon skeleton.[7] The labelling pattern of aphidicolin biosynthesized from [1-^{13}C] and [2-^{13}C] and [1,2-$^{13}C_2$]-acetates defined the constituent isoprene units whilst the generation of a ^{2}H:^{13}C-coupling in the n.m.r. spectrum of material biosynthesized from [4-$^2H_2$,3-^{13}C]-mevalonic acid established that a hydrogen atom had migrated from C-9 to C-8 during the formation of aphidicolin.[8]

In the search for biosynthetic intermediates we examined the mycelial extract by gas chromatography–mass spectrometry. The extract proved to be particularly complex. However we were not only able to confirm the presence of aphidicol-16-ene (**2**)[2] which had been reported previously but also to identify aphidicolan-16β-ol (**3**) by comparison with an authentic sample (*vide infra*). Consequently we have examined the role of these compounds in the biosynthesis of aphidicolin (**1**).

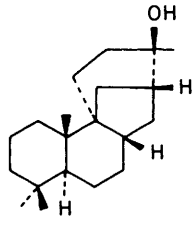
As with other tetracyclic diterpenoids,[9] C-17 is a suitable site for a label since this atom can be introduced *via* the 17-nor-16-ketone whilst specificity in the subsequent biosynthesis may be demonstrated by the periodate cleavage of the 16,17-glycol in the resultant aphidicolin. The substrates were therefore prepared by routes which permitted this labelling. Aphidicol-16-ene (**2**) had previously been prepared[2] during the structural studies on aphidicolin. However the route involved the preparation and desulphurization of a sulphide and hence a pleasanter alternative was chosen. The readily available 3α,18-dihydroxy-17-noraphidicolan-16-one (**4**), obtained by the periodate cleavage of aphidicolin,[2] was treated with freshly distilled dimethylformamide dimethyl acetal.[10] The resulting dimethylformamide acetal was quaternized with methyl iodide and heated to afford 18-formyloxy-17-noraphidicol-2-en-16-one (**5**),† $\nu_{max.}$ 1 740 and 1 720 cm^{-1}; δ 3.88 (2 H, CH_2O), 8.07 (1 H, O–C*H*=O), 5.38 (1 H, dd, *J* 2 and 10 Hz, 3-H), and 5.78 (1 H, ddd, *J* 2, 6, and 10 Hz, 2-H).[11] The multiplicity of the olefinic 3-H proton resonance revealed both vicinal and long-range couplings whilst that of the 2-H signal was consistent with couplings both to 3-H and to the two protons at C-1. Hydrolysis of the formate with aqueous methanolic sodium hydroxide gave the parent alcohol (**6**), $\nu_{max.}$ 3 500 and 1 710 cm^{-1}; δ 3.1 and 3.4 (each 1 H, d, *J* 10 Hz, 18-H_2), 5.25 (1 H, dd, *J* 3 and 10 Hz, 3-H), and 5.85 (1 H, ddd, *J* 3, 5, and 10 Hz, 2-H). The alkene was hydrogenated over 10% palladium on charcoal to afford the saturated alcohol (**7**), $\nu_{max.}$ 3 400 and 1 710 cm^{-1}; δ 3.1 and 3.4 (each 1 H, d, *J* 10 Hz, 18-H_2). The 16-ketone was protected as its ethylene acetal (**8**), $\nu_{max.}$ 3 475 cm^{-1}; δ 3.9 (4 H, m, OCH_2CH_2O) and then the hydroxy group was converted into the chloride (**9**) with triphenylphosphine–carbon tetrachloride. The product (**9**) lacked hydroxy absorption in the i.r. spectrum and possessed the AB doublets (δ 3.1 and 3.53, *J* 12 Hz) characteristic of the primary alkyl chloride, showing that no rearrangement of the



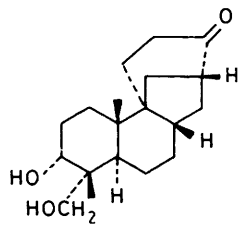
(1)



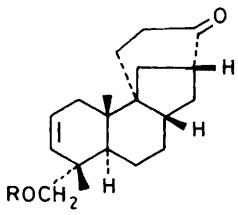
(2)



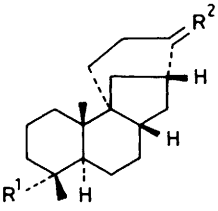
(3)



(4)



(5) R = CHO
(6) R = H



(7) R^1 = CH_2OH, R^2 = O
(8) R^1 = CH_2OH, R^2 = $-OCH_2CH_2O-$
(9) R^1 = CH_2OH, R^2 = $-OCH_2CH_2O-$
(10) R^1 = Me, R^2 = $-OCH_2CH_2O-$
(11) R^1 = Me, R^2 = O
(12) R^1 = Me, R^2 = 

† The scope of this reaction is the subject of further investigations with model compounds.

neopentyl system had occurred. Hydrogenolysis of the chloride with tributyltin hydride gave the acetal (**10**), δ 0.85 (6 H, s,), 0.95 (3 H, s,) (3 × C-Me), and 3.9 (4 H, m, OCH_2CH_2O). The protecting group was removed by mild acid hydrolysis to afford the known 17-noraphidicolan-16-one (**11**).[2] Wittig methylenation with [^{14}C]methyltriphenylphosphonium iodide then gave [17-^{14}C]aphidicol-16-ene (**2**).[2] Reaction of the norketone (**11**) with [^{14}C]trimethylsulphoxonium iodide and sodium hydride in dimethyl sulphoxide gave the 16,17-epoxide (**12**), δ 2.67 (2 H, s, $>C\overset{O}{\diagdown\!\diagup}CH_2$). This reaction is known[2] to regenerate the aphidicolin stereochemistry at C-16. Reduction of the epoxide with lithium aluminium hydride then gave [17-^{14}C]aphidicolan-16β-ol (**3**), $\nu_{max.}$ 3 350 cm^{-1}; δ 0.86 (6 H, s,), 0.97 (3 H, s,), and 1.13 (3 H, s, 17-H_3).

[17-^{14}C]Aphidicol-16-ene (**2**) was fed to *C. aphidicola* on day 7 after inoculation. The fermentation was harvested 21 days later. The resultant aphidicolin (**1**) showed an incorporation of 0.09%. It was cleaved to afford 3α, 18-dihydroxy-17-noraphidicolan-16-one (**4**) which was inactive whilst C-17 was isolated as the formaldehyde bisdimethone derivative possessing 96% of the activity of the parent aphidicolin. The [17-^{14}C]aphidicolan-16β-ol (**3**) was also fed to *C. aphidicola* for the same period of time. The resultant aphidicolin showed an incorporation of 7.9%. Degradation to 3α,18-dihydroxy-17-noraphidicolan-16-one (**4**) showed that the radioactivity was located at C-17.

Aphidicolan-16β-ol (**3**) may be formed by hydration of aphidicol-16-ene (**2**) or, more probably, directly by hydration of a carbocation which is formed during the cyclization stage of the biosynthesis. Our [2-^{13}C]-acetate results[8] confirmed that a C(13)–C(16) bond migrates to C-12 during the cyclization to form rings C and D. The hydroxy group in aphidicolan-16β-ol is *trans* to this bond. This stereochemistry is consistent with hydration of the incipient carbocation which would arise during this rearrangement. The relative magnitude of the biosynthetic incorporations suggest that although the fungus has the ability to transform exogenous aphidicol-16-ene (**2**) into aphidicolin (**1**), the major biosynthetic pathway may lie *via* the tertiary alcohol, aphidicolan-16β-ol (**3**).

Experimental

General experimental conditions have been described previously.[8] ^{1}H N.m.r. spectra were determined at 360 MHz for solutions in deuteriochloroform. Light petroleum refers to that fraction of b.p. 60—80 °C. *C. aphidicola* (CMI 68689) was grown as described previously.[8]

18-*Formyloxy*-17-*noraphidicol*-2-*en*-16-*one* (**5**).—3α,18-Dihydroxyaphidicolan-16-one[2] (1 g) in freshly distilled dimethylformamide dimethyl acetal (10 ml) was heated under reflux until t.l.c. showed the complete disappearance of the starting material. The solvent was evaporated to afford a yellow oil which was treated with toluene (40 ml) and methyl iodide (10 ml) under reflux for 2 h. The solvent was evaporated to give a brown oil which was taken up in chloroform, washed with dilute hydrochloric acid and water, and dried. The solvent was evaporated and the residue chromatographed on silica to give the *title compound* (**5**) (500 mg) which crystallized from methanol as plates, m.p. 120—123 °C (Found: C, 75.8; H, 8.7. $C_{20}H_{28}O_3$ requires C, 75.9; H, 8.9%); $\nu_{max.}$ 1 740 and 1 720 cm^{-1}; δ 0.93 (3 H, s, 10-Me), 1.07 (3 H, s, 4-Me), 3.88 (2 H, s, 18-H_2), 5.38 (1 H, dd, *J* 2 and 10 Hz, 3-H), 5.78 (1 H, ddd, *J* 2, 6, and 10 Hz, 2-H), and 8.07 (1 H, s, OC*H*O).

18-*Hydroxy*-17-*noraphidicol*-2-*en*-16-*one* (**6**).—The above ester (400 mg) in methanol (10 ml) was treated with 10% sodium hydroxide (2 ml) under reflux for 5 min. The product was recovered in ethyl acetate, washed with dilute hydrochloric acid and water, and dried. The solvent was evaporated to give the *title compound* (**6**) (290 mg) which crystallized from ethyl acetate–light petroleum as small prisms, m.p. 120—122 °C (Found: C, 78.85; H, 9.3. $C_{19}H_{28}O_2$ requires C, 79.1; H, 9.8%); $\nu_{max.}$ 3 500 and 1 710 cm^{-1}; δ 0.9 (3 H, s, 10-Me), 1.12 (3 H, s, 4-Me), 3.1 and 3.4 (2 H, AB q, *J* 10 Hz, 18-CH_2), 5.25 (1 H, dd, *J* 3 and 10 Hz, 3-H), and 5.85 (1 H, ddd, *J* 3, 5, and 10 Hz, 2-H).

18-*Hydroxy*-17-*noraphidicolan*-16-*one* (**7**).—The above olefin (250 mg) in ethyl acetate (50 ml) containing 10% palladium on charcoal (50 mg) was shaken in an atmosphere of hydrogen for 45 min. The solution was filtered through a bed of Celite which was then further washed with ethyl acetate. The solvent was evaporated to afford the *title compound* (**7**) (240 mg) which crystallized as plates from methanol, m.p. 153—156 °C (Found: C, 78.3; H, 10.3. $C_{19}H_{30}O_2$ requires C, 78.6; H, 10.4%); $\nu_{max.}$ 3 400 and 1 700 cm^{-1}; δ 0.8 (3 H, s, 10-Me), 1.0 (3 H, s, 4-Me), and 3.1 and 3.4 (2 H, AB q, *J* 10 Hz, 18-H_2).

Preparation of the Ethylene Acetal (**8**).—18-Hydroxy-17-noraphidicolan-16-one (3.45 g) in benzene (160 ml) containing toluene-*p*-sulphonic acid (260 mg) and ethane-1,2-diol (10 ml) was heated under reflux for 1 h under a Dean and Stark water separator. The solvents were evaporated the residue taken up in ethyl acetate, and the solution washed with saturated aqueous sodium hydrogen carbonate and water and dried. Evaporation of the solvent gave the 16-*ethylene acetal* of 18-hydroxy-17-noraphidicolan-16-one (3.3 g) which crystallized from ethyl acetate–light petroleum as prisms, m.p. 153—154 °C (Found: C, 75.4; H, 10.2. $C_{21}H_{34}O_3$ requires, C, 75.4; H, 10.25%); $\nu_{max.}$ 3 475 cm^{-1}; δ 0.85 (3 H, s, 10-Me), 1.0 (3 H, s, 4-Me), 3.1 and 3.4 (2 H, ABq, *J* 10 Hz, 18-H_2), and 3.9 (4 H, m, OCH_2CH_2O).

Chlorination of the 18-*Hydroxy Group*.—The above ethylene acetal (2.5 g) in carbon tetrachloride (20 ml) was heated with pyridine (0.5 ml) and triphenylphosphine (10 g) under nitrogen for 3 h. (t.l.c. control). The solution was diluted with chloroform, washed with dilute hydrochloric acid, saturated aqueous sodium hydrogen carbonate, and water, and dried. The solvent was evaporated and the residue chromatographed on silica to afford the 16-*ethylene acetal* of 18-chloro-17-noraphidicolan-16-one (900 mg) which crystallized from methanol as plates, m.p. 125—127 °C (Found: C, 71.5; H, 9.7. $C_{21}H_{33}ClO_2$ requires C, 71.5; H, 9.45%); $\nu_{max.}$ 1 100 and 720 cm^{-1}; δ 0.9 (3 H, s, 10-Me), 0.93 (3 H, s, 4-Me), 3.3 and 3.53 (2 H, ABq, *J* 12 Hz, 18-H_2), and 3.9 (4 H, m, OCH_2CH_2O).

Hydrogenolysis of the 18-*Chloride* (**9**).—The above chloro compound (800 mg), tributyltin hydride (2 ml), and 2,2-azobisisobutyronitrile (500 mg) in benzene (20 ml) were heated under reflux under nitrogen for 3 h. The products were absorbed directly onto silica and chromatographed in light petroleum and then toluene to afford the 16-*ethylene acetal* of 17-noraphidicolan-16-one (650 mg) which crystallized from methanol as plates, m.p. 105—106 °C (Found: C, 78.9; H, 10.4. $C_{21}H_{34}O_2$ requires C, 79.2; H, 10.7%); $\nu_{max.}$ 1 100 cm^{-1}; δ 0.85 (6 H, s,), 0.95 (3 H, s,) (4- and 10-Me), and 3.9 (4 H, m, OCH_2CH_2O).

17-*Noraphidicolan*-16-*one* (**11**).—The above ethylene acetal (635 mg) in ethanol (10 ml) containing water (0.5 ml) was treated with concentrated sulphuric acid (1 drop) and heated on a steam-bath for 10 min. The solution was concentrated and then diluted with water to afford 17-noraphidicolan-16-one as plates, m.p. 138—140 °C (lit.,[2] 135—139 °C); $\nu_{max.}$ 1 720 cm^{-1}; δ 0.9 (6 H, s) and 1.1 (3 H, s,) (4- and 10-Me).

Aphidicol-16-ene (**2**).—Sodium hydride (74 mg) in dimethyl sulphoxide (6.4 ml) was stirred under nitrogen at 70 °C until the evolution of hydrogen ceased. The mixture was cooled and methyltriphenylphosphonium iodide (1.25 g) in dimethyl sulphoxide (3.3 ml) was added. The mixture was stirred for 30 min and filtered through a glass wool plug into a solution of 17-noraphidicolan-16-one (85 mg) in THF (3.3 ml). The mixture was stirred for 30 min and then poured into water and the product recovered in chloroform and purified by column and preparative layer chromatography to afford aphidicol-16-ene (50 mg) which crystallized from methanol as needles, m.p. 70—72.5 °C, $[\alpha]^{25}$ −10° (*c* 1.0 in $CHCl_3$) (lit.,[2] 70—72.5 °C, $[\alpha]_D^{23.5}$ −13.2°) (Found: C, 88.1; H, 11.8. Calc. for $C_{20}H_{32}$: C, 88.2; H, 11.8%); $\nu_{max.}$ 3 080, 1 655, and 885 cm^{-1}; δ 0.87 (6 H, s), 0.97 (3 H, s) (4- and 10-Me), and 4.43 (2 H, m, 17-H_2).

16,17-*Epoxyaphidicolane* (**12**).—Sodium hydride (500 mg) was repeatedly washed with light petroleum. A solution of trimethylsulphoxonium iodide (500 mg) in dimethyl sulphoxide (10 ml) was added and the reaction mixture was left at room temperature for 1 h. 17-Noraphidicolan-16-one (400 mg) in dry THF (5 ml) was then added and the resultant reaction mixture was left at room temperature for 3 h and then heated to 60 °C for 1 h. The solution was poured into water and the product recovered in chloroform and chromatographed to afford 16,17-*epoxyaphidicolane* (**12**), m.p. 103—104 °C (Found: C, 83.8; H, 10.8. $C_{20}H_{32}O$ requires C, 83.3; H, 11.2%); δ 0.83 (6 H, s,), 1.0 (3 H, s,) (4- and 10-Me), and 2.63 (2 H, s, 17-H_2).

Reduction of the Epoxide (**12**).—The above epoxide (110 mg) in freshly dried and distilled THF (3 ml) was cooled in an ice-bath and lithium aluminium hydride (180 mg) was then added. The reaction mixture was stirred at room temperature for 1 h. The solvent was partially evaporated and the residue extracted with chloroform. The extract was washed with dilute hydrochloric acid and water, and dried. The solvent was evaporated and the residue chromatographed on silica to afford *aphidicolan-16β-ol* (**3**) which crystallized from methanol as plates, m.p. 134—135 °C, $[\alpha]_D^{26}$ +17.6° (*c* 1.0 in $CHCl_3$) (Found: C, 83.1; H, 11.6. $C_{20}H_{34}O$ requires C, 82.7; H, 11.8%), $\nu_{max.}$ 3 350 cm^{-1}; δ 0.86 (6 H, s,), 0.97 (3 H, s) (4- and 10-Me), and 1.13 (3 H, s, 17-H_3); *m/z* 290, 272, 257, 133, 105, 81, and 69.

Identification of Aphidicol-16-ene and Aphidicolan-16β-ol in Cephalosporium aphidicola.—A sample of the mycelial extract of *C. aphidicola* was slurried with light petroleum, filtered, and the resultant oil chromatographed on activated alumina (Laporte Grade 1, 50 g) in light petroleum. The eluant was examined by g.c.–m.s. (Varian CH 5, 2% OV-17 column, Helium carrier gas) using a temperature programme (90—280 °C, 10 °C $min.^{-1}$ initiated at time of injection). This revealed four minor hydrocarbons, M^+ 272 ($C_{20}H_{32}$) with R_t 13.5, 14.66, 14.84, and 15 min. The latter had a fragmentation pattern and R_t identical with that of aphidicol-16-ene. The experiment was repeated using a Kratos MS 25 and a 3% OV-17 column operating at 250 °C with helium carrier gas (30 ml min^{-1}) and a 2-m column. This revealed a minor component, M^+ 290 ($C_{20}H_{34}O$), R_t 2.35 min with a fragmentation pattern and R_t identical with those of authentic aphidicolan-16β-ol.

Incorporation Experiments.—(a) [17-^{14}C]Aphidicol-16-ene (38 mg, 2.10×10^6 d.p.m.) in ethanol (10 ml) and Tween 80 (0.75 ml) was distributed evenly between three Thompson bottles (2.25 l) of *C. aphidicola* 7 days after inoculation. The cultures were harvested after a further 21 days. The aphidicolin (415 mg) was purified by chromatography and had 2.05×10^3 d.p.m. (0.09% incorporation). The specificity was demonstrated as follows. The aphidicolin (77.5 mg) in pyridine (5.4 ml) and water (1.6 ml) was treated with 50% periodic acid (2 ml) at 10 °C for 30 min. The pH was adjusted to 1 with concentrated sulphuric acid and the solution steam distilled into dimedone (78 mg) in ethanol (6.2 ml) and triethylamine (2 drops). The resultant formaldehyde dimedone derivative, m.p. 189 °C had a specific activity 26.29 kBq mol^{-1} from the aphidicolin 27.5 kBq mol^{-1}. A further sample of the aphidicolin (100 mg) in pyridine (7 ml) and water (2 ml) was treated with 50% periodic acid (2 ml) at 10 °C for 30 min. The mixture was poured into dilute hydrochloric acid and the product recovered in chloroform. The extract was washed with aqueous sodium hydrogen carbonate, dried, and the solvent evaporated to give 3α,18-dihydroxy-17-noraphidicolan-16-one (40 mg), m.p. 155 °C (lit.,[2] 155—156 °C) which contained no radioactivity.

(b) [17-^{14}C]Aphidicolan-16β-ol (20 mg, 5.0×10^5 d.p.m.) in ethanol (5 ml) was incubated with *C. aphidicola* (7.5 l) as above to afford aphidicolin (600 mg) (3.9×10^4 d.p.m., 7.9% incorporation). Degradation as above afforded the 3α,18-dihydroxy-17-noraphidicolan-16-one which was inactive.

Acknowledgements

We thank Dr. Carolyn Perring for help with growing the fermentations and I.C.I. Pharmaceuticals Division for generous financial help during the tenure of a CASE studentship by M. J. A.

References

1 Previous part, J. R. Hanson, M. A. O'Leary, H. J. Wadsworth, and B. L. Yeoh, *J. Chem. Soc., Perkin Trans 1*, 1985, 1311.
2 W. Dalziel, B. Heap, K. M. Stevenson, and J. A. J. Jarvis, *J. Chem. Soc., Perkin Trans. 1*, 1973, 2841.
3 For a review see J. A. Huberman, *Cell*, 1981, **23**, 647.
4 K. Kawada, Y. Kimura, K. Katagiri, A. Suzuki, and S. Tamura, *Agric. Biol. Chem. (Jpn)*, 1978, **42**, 1611.
5 A. N. Starratt and S. R. Loshiavo, *Can. J. Microbiol.*, 1974, **20**, 174.
6 P. J. Fisher, A. N. Anson, J. Webster, H. M. Noble, and J. R. Evans, *Trans. Br. Mycol. Soc.*, 1984, **83**, 149.
7 M. R. Adams and J. R. Bu'Lock, *J. Chem. Soc., Chem. Commun.*, 1975, 389.
8 M. J. Ackland, J. R. Hanson, and A. H. Ratcliffe, *J. Chem. Soc., Perkin Trans. 1*, 1984, 2751.
9 B. E. Cross, R. H. B. Galt, and J. R. Hanson, *J. Chem. Soc.*, 1964, 295.
10 S. Hanessian, A. Bargiotti, and M. La Rue, *Tetrahedron Lett.*, 1978, 737.

Received 22nd *April*, 1985; *Paper* 5/652