Studies of Terpenoid Biosynthesis. Part 29.[1] The Cleavage of the Sterol Side Chain in the Biosynthesis of Demethoxyviridin

James R. Hanson, Margaret A. O'Leary, and Harry J. Wadsworth
School of Molecular Sciences, University of Sussex, Brighton, Sussex BN1 9QJ

The structures of a group of C_6 and C_7 alcohols obtained from *Nodulisporium hinnuleum* have been elucidated. When biosynthesized from [2-^{14}C]mevalonic acid they have been shown to have a specific activity consistent with a common origin with their co-metabolite, demethoxyviridin. The number of [2-$^2H_2$]mevalonoid hydrogen atoms which were incorporated suggests that they were formed initially at the aldehyde oxidation level.

Microbiological cleavage of the sterol side chain has attracted considerable interest particularly with a view to the development of industrial processes for the production of steroid hormones from naturally occurring sterols.[2] The bacterial cleavage of the sterol side chain differs from the mammalian process. In the latter [3] cleavage of the C(20)–C(22) bond with the formation of isohexanal is followed by cleavage of the C(17)–C(20) bond to give the 17-keto-steroids. On the other hand micro-organisms of the genera, *Nocardia*, *Arthrobacter*, *Mycobacterium*, and *Corynebacterium* commonly shorten the side chain in a stepwise manner by a pathway comparable with that of the oxidation of fatty acids to give acetic and propionic acids.[2,4] Demethoxyviridin (1) which is produced by *Nodulisporium hinnuleum* [5] is a fungal 17-keto-steroid with a modified androstane skeleton. It was of interest to see if the removal of the sterol side chain in the biosynthesis of this fungal metabolite followed the mammalian or bacterial pathway. A preliminary communication describing some of this work has appeared.[6]

Whilst demethoxyviridin (1), ergosterol (2), and the pigment hinnuleaquinone (3) [7] are the major mycelial metabolites of surface cultures of *N. hinnuleum*, a group of alcohols (4)—(9) were obtained from the broth. The mixture, of which the major components were (6) and (7), was separated by preparative g.l.c. on 20% Carbowax 20M at 125 °C. The first fraction obtained from the g.l.c. was identified as 3,4-dimethylpent-1-en-3-ol (4).[8] Its ^{1}H n.m.r. spectrum showed signals with a characteristic multiplicity attributable to a vinylic ABC system (δ 5.0, H_A; 5.13, H_B; and 5.87, H_C; J_{AB} 2 Hz; J_{AC} 10 Hz; J_{BC} 18 Hz), an isopropyl doublet (δ 0.93, *J* 7 Hz) and a relatively low field tertiary methyl group (δ 1.20). The second minor fraction had an n.m.r. spectrum (δ 0.83, 6 H, d, *J* 6 Hz; 0.93, 3 H, d, *J* 7 Hz; 3.42, 2 H, d, *J* 6 Hz) consistent with the structure 2,3-dimethylbutan-1-ol (5). It possessed a low negative optical rotation as does the compound obtained [9] *via* ozonolysis of the side-chain of ergosterol ($[\alpha]_D^{25}$ −2.0°, *cf.* −2.95°) suggesting that it possesses the same chirality at C-2. The third fraction which was a major product, was identified as 3-methylene-4-methylpentan-1-ol (6). Its ^{1}H n.m.r. spectrum contained resonances attributable to an isopropyl group (δ 1.07, 6 H, d, *J* 8 Hz), a hydroxymethyl group (δ 3.70, 2 H, t, *J* 6.5 Hz) which was coupled to an otherwise isolated methylene (δ 2.30). The spectrum also contained the resonances of a vinylic methylene (δ 4.85, 2 H). The ^{13}C n.m.r. spectrum confirmed the presence of these groups leading to the structure (6). The fourth fraction was identified *via* its ^{1}H n.m.r. spectrum as the known [10] 3,4-dimethylpentan-1-ol (7). Its optical rotation ($[\alpha]_D^{25}$ −17.3° lit.,[9] −21.5°) again indicated that it belonged to the same series as the material obtained from ergosterol. The fifth and sixth fractions were identified as the (*Z*)- and (*E*)-3,4-dimethylpent-2-en-1-ols (8) and (9).[11] They were distinguished on the basis of their ^{1}H n.m.r. spectra.[12] The isopropyl ^{1}H n.m.r. resonance of the (*Z*)-isomer was at slightly lower field (δ 1.08) compared to the (*E*)-isomer (δ 1.03) whilst the vinylic methyl group of the (*Z*)-isomer was at slightly higher field (δ 1.62 and δ 1.67) than the (*E*)-isomer. Authentic samples of the (*E*) and (*Z*)-3,4-dimethylpent-2-en-1-ols were prepared from methyl 3-hydroxy-3,4-dimethylpentanoate.[13] Simple acid-catalysed dehydration of the tertiary alcohol in the latter was not regiospecific. Consequently the tertiary alcohol was acetylated with acetyl chloride and *N*,*N*-dimethylaniline in chloroform and the double bond introduced by base-catalysed elimination with sodium methoxide.[14] The unsaturated ester was then reduced with lithium aluminium hydride and aluminium trichloride to afford a mixture of the alcohols (8) and (9) which were separated by preparative g.l.c. A comparable investigation [15] of the broth of *Gliocladium deliquescens* which produces viridiol, revealed the presence of the acid (10) which was isolated as its methyl ester.

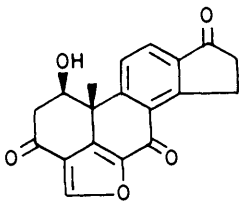
Table. The specific activity of the metabolites of *N. hinnuleum* biosynthesized from [2-^{14}C]-MVA

Compound	Specific activity (d.p.m. mmol^{-1}) Expt. 1	Expt. 2
(1)	119 000 [a]	64 356 [b]
(5)	87 000	44 232 [c]
(7)		38 396
(8)		45 522
(9)		39 900

[a] 2.3% Incorporation of [2-^{14}C]-MVA. [b] 1.17% Incorporation of [2-^{14}C]-MVA. [c] The alcohol (5) was further purified as its crystalline 3,5-dinitrobenzoate which had a specific activity of 43 300 d.p.m. mmol^{-1}.

The structures of these alcohols are reminiscent of sterol, particularly ergosterol, side chains. If these alcohols represent the side chain of a demethoxyviridin precursor, they would be expected to bear two labels from [2-^{14}C]mevalonate whilst the demethoxyviridin should retain three. If they are terminal metabolites the ratio of the specific activities should be approximately 2 : 3. [2-^{14}C]Mevalonic acid was incubated with *Nodulisporium hinnuleum* and the metabolites were isolated. The results are tabulated. The alcohol (6) was further purified as its crystalline 3,5-dinitrobenzoate which retained the radioactivity. The ratio of the specific activities of the alcohols to that of demethoxyviridin was approximately 2 : 3 indicative of a common ancestry.

The distribution of the label from [2-$^2H_2$]mevalonate provided some insight into the possible mechanism of cleavage of the side chain. The isolation of these fragments at the alcohol oxidation level was at first sight surprising. In mammalian steroid biosynthesis (22*R*)-22-hydroxycholesterol



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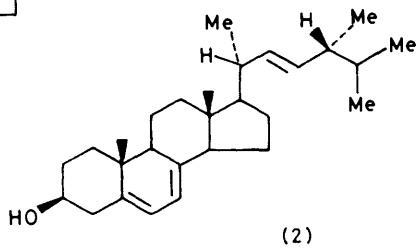

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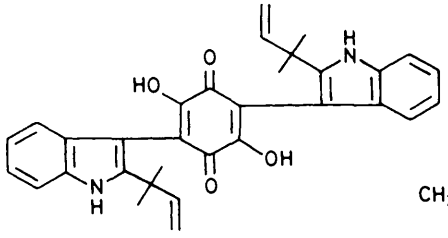

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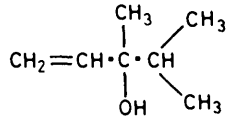

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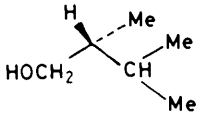

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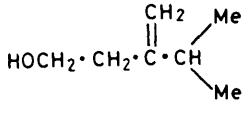

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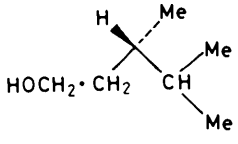

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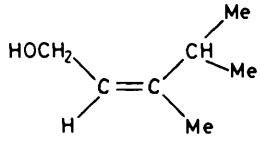

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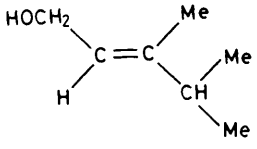

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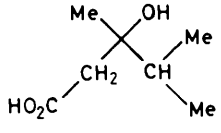

(10)

and (20*R*,22*R*)-20,22-dihydroxycholesterol have been identified [16] as key intermediates whilst the side chain fragment was obtained as the aldehyde. The sterol side chain is labelled from the 2-position of mevalonate at C-22 and C-26 and consequently two centres in the alcohols (6) and (7) should be labelled. [2-$^2H_2$]Mevalonic acid was fed to *Nodulisporium hinnuleum*. Examination of the ^2H n.m.r. spectra of the resultant alcohols showed that signals at δ 3.7 and 1.02 in (6) and at δ 3.6 and 0.83 in (7) were labelled in the ratio 0.6 : 2 and 0.7 : 2 respectively. This suggests that a label is lost from C-1 [= C(22)] during the formation of the alcohols. Since [2-$^2H_2$]mevalonate was used as a substrate, the C(22) of a sterol precursor will bear either two protons or two deuterons. Hence any isotope effect in the cleavage sequence will be reflected in a reduction in the amount of deuterium label retained in the ' inert ' position at C(1) of the alcohols.

The following conclusions may be drawn from this work. The structures of the alcohols (4)—(9) and their specific activity when biosynthesized from [2-^{14}C]mevalonate compared with that of their co-metabolite strongly suggests that the biosynthesis of this fungal 17-keto-steroid follows a mammalian rather than a bacterial side chain cleavage sequence. However the presence of an additional methyl group and its chirality suggest that the common intermediate may have the ergostane side chain rather than a cholestane side chain. The number of [2-$^2H_2$]mevalonoid hydrogen atoms that were incorporated is compatible with an initial cleavage leading to the formation of aldehydes which were subsequently reduced to alcohols. It would not of course exclude other ways, for example the introduction of side chain unsaturation, in which a label could be lost.

Experimental

General experimental details have been described previously.[17]

Isolation of the Alcohols (4)—(9).—*Nodulisporium hinnuleum* (5 l) was grown on surface culture for 20 d as described previously.[1] The mycelium was filtered off, dried, and extracted with chloroform to afford after further purification, demethoxyviridin. The broth was saturated with sodium chloride and extracted with ethyl acetate. The extracts were dried and the solvent evaporated to afford a dark oil which was taken up in ether and filtered through alumina (Woelm grade III, 36 g). The resultant orange coloured oil (1.4 g) was distilled (55—85 °C, 4 mmHg) to give a colourless oil (900 mg) which was separated by preparative g.l.c. on a 15 ft × ⅜ in 20% Carbowax 20M on 80—100 mesh Chromosorb P column at 125 °C, 75 ml min^{-1} nitrogen flow rate to afford the following six major fractions; the collecting efficiency was *ca.* 30—40%.

Fraction no.	R_t	Relative amount
1	9	17.6
2	16	3.9
3	28	32.9
4	32.5	30.6
5	37	1.5
6	40.8	14.1

Fraction 1 was 3,4-dimethylpent-1-en-3-ol (4);[8] δ 0.93 (6 H, d, *J* 7 Hz), 1.20 (3 H, s), 1.6 (1 H, m), 5.0 (1 H, dd, *J* 2 and 10 Hz), 5.13 (1 H, dd, *J* 2 and 18 Hz), 5.87 (1 H, dd, *J* 10 and 18 Hz); *m/z* 97 (M^+ − OH) (5%), 83 (32), 81 (48), 71 (100), 55 (80), 43 (90), and 41 (80).

Fraction 2 was (*S*)-2,3-dimethylbutan-1-ol (5);[9] δ 0.83 (6 H, d, *J* 6 Hz), 0.93 (3 H, d, *J* 7 Hz), 3.42 (2 H, d, *J* 6 Hz); *m/z* 102 (5), 84 (40), 71 (100), 69 (40), 59 (40), and 43 (80); $[\alpha]_D^{25}$ −2° (*c* 2.5 in EtOH) (lit.,[9] $[\alpha]_D^{25}$ −2.95°).

Fraction 3 was 3-*methylene*-4-*methylpentan*-1-*ol* (6). On redistillation it had b.p. 70 °C/2 mmHg (Found: C, 73.5; H, 12.3. $C_7H_{14}O$ requires C, 73.6; H, 12.4%); δ 1.07 (6 H, d, *J* 8 Hz), 1.83 (s, OH), 2.3 (2 H, t, *J* 6.5 Hz), 3.70 (2 H, t, *J* 6.5 Hz), and 4.85 (2 H, d, *J* 6 Hz); ^{13}C δ 21.7 (q, C-4′ and C-5), 33.7 (d, C-4), 37.4 (t, C-2), 60.85 (t, C-1), 108.7 (t, C-3′), and 152.2 (s, C-3); $\nu_{max.}$ 3 320, 1 640, and 890 cm^{-1}; *m/z* 114 (10), 96 (15), 83 (100), 81 (90), 69 (80), 55 (100), 43 (48), and 41 (90). The 3,5-*dinitrobenzoate* had m.p. 40—41 °C (Found: C, 54.75; H, 5.39; N, 9.11. $C_{14}H_{16}N_2O_6$ requires C, 54.54; H, 5.23; N, 9.09%).

Fraction 4 was 3,4-dimethylpentan-1-ol (7),[10] δ 0.83 (6 H, d, *J* 7 Hz), 0.98 (3 H, d, *J* 7 Hz), 3.67 (2 H, t, *J* 6 Hz); *m/z* 98 (5), 83 (50), 70 (90), 56 (100), 55 (90), 43 (80), and 41 (80). The 3,5-dinitrobenzoate had m.p. 50 °C (lit.,[10] 51—52 °C).

The alcohol had $[\alpha]_D^{25}$ −17.3 (*c* 0.2 in $CHCl_3$) (lit.,[9] $[\alpha]_D^{25}$ −21.5°).

Fraction 5 was (*Z*)-3,4-dimethylpent-2-en-1-ol (8) [11,12] δ 1.08 (6 H, d, *J* 7 Hz), 1.62br (3 H, s), 2.8 (1 H, m), 4.01 (2 H, d, *J* 6 Hz), 5.27 (1 H, t, *J* 6 Hz); *m/z* 114 (10), 96 (10), 83 (30), 81 (20), 71 (100), 70 (50), 55 (100), 43 (90), and 41 (90).

Fraction 6 was (*E*)-3,4-dimethylpent-2-en-1-ol (9) [11,12] δ 1.03 (6 H, d, *J* 7 Hz), 1.67br (3 H, s), 2.25 (1 H, m), 4.08 (2 H, d, *J* 6 Hz), 5.37 (1 H, t, *J* 6 Hz); *m/z* 114 (2), 96 (20), 83 (30), 81 (30), 71 (90), 70 (50), 55 (100), 43 (80), and 41 (90).

Preparation of (E)- *and* (Z)-3,4-*Dimethylpent*-2-*en*-1-*ol*.—Methyl 3-hydroxy-3,4-dimethylpentanoate (300 mg) [13] and 40 drops each of acetyl chloride and *N*,*N*-dimethylaniline in chloroform (5 ml) was heated under reflux for 12 h. The resultant deep blue solution was washed with water, dilute hydrochloric acid, and aqueous sodium hydrogen carbonate, and then dried and filtered through silica. Evaporation of the solvent gave the crude acetate, δ 0.91 (6 H, d, *J* 7 Hz), 1.39 (3 H, s), 2.00 (3 H, s), 2.28 (1 H, m), 2.88 (2 H, d), and 3.6 (3 H, s). The crude acetate (320 mg) in methanol (3 ml) was added to a solution of sodium (50 mg) in methanol (2 ml). After 1 h the solution was poured into water and the product recovered in ether and purified by preparative t.l.c. to afford methyl 3,4-dimethylpent-2-enoate (127 mg), δ 1.02 (d, *J* 7 Hz, minor isomer), 1.1 (d, *J* 7 Hz, major isomer), 1.78br (s, minor isomer), 2.1br (s, major isomer), 3.6 (s, OMe), and 5.4—5.55 (m, =CH); $\nu_{max.}$ 1 720 and 1 642 cm^{-1}.

Aluminium trichloride (50 mg) was added to a suspension of lithium aluminium hydride (45 mg) in ether (15 ml) at 0 °C. The mixture was stirred for 5 min at 0 °C and then for 10 min at room temperature. The crude esters (100 mg) in ether (1 ml) were then added and the mixture was stirred for 40 min. Water was added and the product recovered in ether to afford a pale yellow oil (70 mg) which was separated by preparative g.l.c. as above to afford the alcohols (8) and (9) identified by their n.m.r. spectra.

Incorporation of Labelled Mevalonic Acids.—(a) [2-^{14}C]-Mevalonic acid (25.1×10^6 d.p.m.) in ethanol (10 ml) was evenly distributed between 20 10-day old surface cultures of *Nodulisporium hinnuleum*. Growth was allowed to continue for a further 10 days and the metabolites were isolated as above to afford demethoxyviridin (1.57 g), m.p. 145—150 °C (370 d.p.m. mg^{-1}, 2.3% incorporation). The broth was saturated with sodium chloride and extracted with ethyl acetate. The extract was dried over sodium sulphate and the solvent evaporated to afford a dark gum (2 g). The gum was filtered through alumina to afford a pale-yellow oil which was further chromatographed on alumina–10% silver nitrate (50 g). Elution with 25% ether–light petroleum gave 3-methylene-4-methylpentan-1-ol which then distilled at 70 °C/2 mmHg to give an oil (140 mg; 765 d.p.m. mg^{-1}, 0.43% incorporation).

(b) In a repeat of the above experiment [2-^{14}C]mevalonic acid (1.21×10^7 d.p.m.) was distributed over 5 l of *Nodulisporium hinnuleum* after 10 days growth. After a further 10 days incubation the metabolites were isolated. The demethoxyviridin (710 mg) had 200 d.p.m. mg^{-1}, 1.17% incorporation). The alcohols were separated by preparative g.l.c. 3-Methylene-4-methylpentanol had 388 d.p.m. mg^{-1}, its 3,5-dinitrobenzoate had 140 d.p.m. mg^{-1}, 3,4-dimethylpentanol had 331 d.p.m. mg^{-1}, (*Z*)-3,4-dimethylpent-2-en-1-ol had 373 d.p.m. mg^{-1}, (*E*)-3,4-dimethylpent-2-en-1-ol had 350 d.p.m. mg^{-1}. The specific activities in d.p.m. $mmol^{-1}$ are given in the Table.

(c) [2-$^2H_2$]Mevalonic acid lactone (710 mg) was distributed between 49 10-day old surface cultures of *Nodulisporium hinnuleum*. The metabolites were isolated after a further 10 days and the major alcohols (6) and (7) were separated by preparative g.l.c. to afford 50 mg samples. The ^{2}H n.m.r. spectra were determined on these samples in $CHCl_3$ on a Bruker WH 360 operating at 55.25 MHz by Dr. I. H. Sadler (University of Edinburgh).

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