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THE BIOSYNTHESIS OF THE STEROID, VIRIDIOL, BY *GLIOCLADIUM DELIQUESCENS**

JAMES R. HANSON, MARGARET A. O'LEARY, HARRY J. WADSWORTH and BOON LENG YEOH

School of Molecular Sciences, University of Sussex, Brighton, Sussex, BN1 9QJ, U.K.

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Key Word Index—*Gliocladium deliquescens*; biosynthesis; steroid; viridiol.

Abstract—The incorporation of squalene, lanosterol, dehydroxydemethoxyviridin and demethoxyviridin into viridiol by the fungus, *Gliocladium deliquescens*, is described.

INTRODUCTION

Viridiol (**1**) [2] is an antifungal metabolite of *Gliocladium deliquescens*. It is one of a small group of steroidal antibiotics whose other members include viridin (**2**) [3], demethoxyviridin (**3**) [4], wortmannin (**6**) [5], wortmannolone (**7**) and virone (**8**) [6]. The formation of these fungal androstanes invites comparison with bacterial, plant and mammalian steroid biosynthesis. In previous work we have studied [7] the enrichment and labelling patterns of demethoxyviridin (**3**) biosynthesized by *Nodulisporium hinnuleum* from [1-^{13}C]-, [1,2-$^{13}C_2$]-acetate, [2-^{13}C]-, and [5-^{13}C]-mevalonate and from the corresponding [^{3}H]-mevalonates. This work confirmed prior [2-^{14}C]mevalonate experiments with viridin (**2**) [8] and [1,2-$^{13}C_2$]acetate experiments [9] with wortmannin (**6**) which had suggested the steroidal rather than diterpenoid origin of these compounds. The incorporation of lanosterol (**9**) into viridin (**2**) by *Trichoderma viride* has been reported [10]. We then showed [11] that the cleavage of the sterol side chain in the biosynthesis of these fungal androstanes followed the mammalian rather than the bacterial pattern. A group of C_6 and C_7 alcohols (e.g. **11**) isolated from *N. hinnuleum*, when biosynthesized from [2-^{14}C]-mevalonic acid, had a specific activity which was consistent with a common origin with their co-metabolite, demethoxyviridin (**2**). We have isolated [12] a comparable hydroxy-acid (**12**) from *Gliocladium deliquescens*. Experiments involving the feeding of large sterol precursors to *N. hinnuleum* were unsuccessful and did not give convincing incorporations into demethoxyviridin. The initial mycelial growth of this fungus is white but after about 10 days it becomes black and sporulation occurs. Mevalonate feeding studies showed [7] that demethoxyviridin production occurred after this stage and it is possible that the precursors at the C_{30} level were not transported across the cell wall. Consequently we studied some of the later stages of the biosynthesis with viridiol (**1**) produced by *G. deliquescens*.

RESULTS

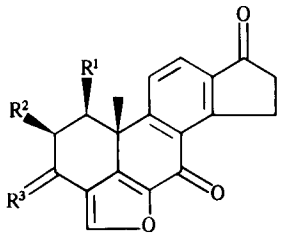
Gliocladium deliquescens (CMI 101523) when grown for eight days on shake culture in a sulphur-limiting medium to restrict gliotoxin production, produced viridiol (**1**) [2] as the major steroidal metabolite. This was further characterized as its diacetate (**4**) [δ 2.2 and 2.37 (each 3H, OAc), 5.37 (H-1 and 6.13 (H-3)]. Preliminary feeding experiments utilizing [^{14}C]acetate established that the optimum time for feeding precursors was on day 2 after inoculation with the culture being harvested on days 6–8.

[11–^{14}C]Squalene (**13**) was fed to *G. deliquescens*. It was incorporated to the extent of 0.41% into viridiol (**1**) [4, 8, 12, 13, 17, 21-^{3}H]Squalene was also fed to *G. deliquescens*. The resultant viridiol had an incorporation of 0.19% whilst the acid (**12**), purified as its *p*-bromophenacyl derivative [12], had an incorporation of 0.17%. In view of the fact that two of the tritiated centres are adjacent to carbonyl groups, a rigorous comparison of the levels of incorporation was not undertaken but nevertheless in the light of our previous work on demethoxyviridin, these figures are consistent with a common origin of the two molecules.

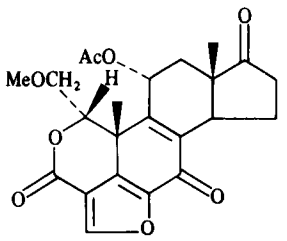
When [2-$^2H_3$]acetate was incorporated [7] into demethoxyviridin (**3**) by *N. hinnuleum*, C-19 retained three deuterium labels thus precluding a cycloartenol type of precursor characteristic of higher plant steroid biosynthesis. [2-$^3H_2$]Lanosterol (**9**) was prepared by an exchange experiment with lanosta-8,24-dien-3-one (**10**) [13] in dry dioxan containing ^3H_2O in the presence of potassium hydroxide and 18-crown-6. Subsequent reduction of the lanostadienone with sodium borohydride gave the [2-$^3H_2$]lanosterol. This was incorporated into viridiol (**1**) to the extent of 0.14% (=0.28% since one label is replaced by a methoxyl group) thus confirming the results obtained with viridin [10] which appeared after our experiments were completed (HJW D.Phil thesis, University of Sussex, 1979).

A number of stages can be distinguished in the biosynthesis of viridiol from lanosterol. These include the formation of the furan ring, the removal of the side chain, the loss of C-18 and the aromatization of ring C and the oxidative modification of ring A. Inspection of the structures of the viridin series suggests that the latter is a late stage in the sequence of events. The 1,2-vicinal oxygen

*Part 36 in the series 'Studies in Terpenoid Biosynthesis', for part 35 see ref. [1].



1 R^1 = OH, R^2 = OMe, R^3 = α-H,β-OH
2 R^1 = OH, R^2 = OMe, R^3 = O
3 R^1 = OH, R^2 = H, R^3 = O
4 R^1 = OAc, R^2 = OMe, R^3 = α-H,β-OAc
5 R^1 = R^2 = H, R^3 = O



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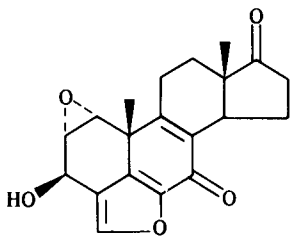

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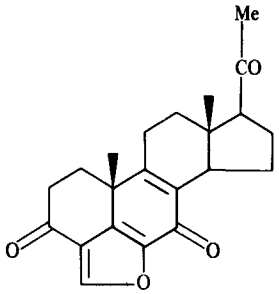

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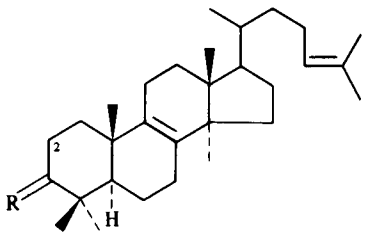
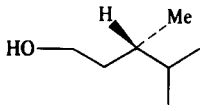


9 R = α-H,β-OH
10 R = O



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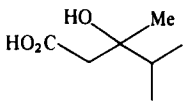

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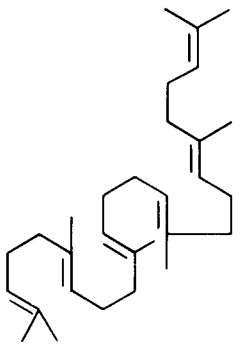

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functions may be introduced in a single or in separate steps (cf. the presence of a 1,2-epoxide in wortmannolone (**7**)).

[^{14}C]Demethoxyviridin (**3**) was readily prepared by incubating [1-^{14}C]acetate with *N. hinnuleum*. Dehydration via the 1-methanesulphonate and catalytic reduction of the resultant 1-ene afforded [^{14}C]-deshydroxydemethoxyviridin (**5**) [14]. Incubation of the latter with *G. deliquescens* gave viridiol (**1**) (1.2 and 2.3% incorporations in separate experiments). A trapping experiment with a fermentation fed with [^{14}C]acetate has established the formation of (**5**) in *N. hinnuleum* [14]. Incubation of [^{14}C]demethoxyviridin (**2**) with *G. deliquescens* gave viridiol (**1**) (4.1 and 6.6% incorporations in separate experiments). Labelled viridin was not obtained. Apart from the selective reduction of the 3-carbonyl group, this showed that the introduction of the vicinal oxygen functions at C-1 and C-2 of viridiol are independent steps involving hydroxylation rather than the addition of oxygen to some unsaturated intermediate.

EXPERIMENTAL

General fermentation conditions. Gliocladium deliquescens (CMI 101525) was maintained on potato dextrose agar slopes. The fermentation medium contained (g/l), sucrose (15), ammonium tartrate (1.5), KH_2PO_4 (0.5), $MgSO_4$ (0.4), $(NH_4)_2SO_4$ (0.1), peptone (0.02) and $FeCl_3$ (0.01). Shake flasks (250 ml) containing 100 ml of the above medium were grown for 8 days at 25°. In a typical fermentation 25 shake flasks were harvested after 8 days of growth. The broth was filtered and extracted with $CHCl_3$. The solvent was evapd and the residue taken up in MeOH (25 ml) and allowed to slowly evaporate to 10 ml when gliotoxin (150 mg), identified by its IR and NMR spectra, crystallized. The mother liquors were subjected to prep. TLC on silica gel in EtOAc to afford viridiol (**1**) (140 mg) which crystallized from Me_2CO as needles, mp 199–200° (lit. [2] 198–201°), IR $\nu_{max}cm^{-1}$: 3460, 1695, 1680, 1585; ^1H NMR (360 MHz, acetone-d_6): δ1.76 (3H, *s*, H-19), 2.66 (2H, *m*, H-16), 3.63 (2H, *m*, H-15), 3.73 (3H, *s*, OMe), 3.84 (1H, *t*, J = 5 Hz, H-2), 4.399 (1H, *d*, J = 5-Hz, H-1) 5.05 (1H, *d*, J = 5 Hz, H-3), 7.83 (1H, *d*, J = 8 Hz, H-12), 7.93 (1H, *s*, furan-H), 8.50 (1H, *d*, J = 8 Hz, H-11). Further quantities of viridiol were obtained by extraction of the mycelium with $CHCl_3$. The diacetate, prepared with Ac_2O in C_5H_5N, crystallized from Et_2O as cubes, mp 195–196°, (Found: C, H, 65.0; H, 5.2. $C_{24}H_{22}O_8$ requires C, 65.7; H, 5.1%). IR $\nu_{max}cm^{-1}$: 1740, 1705, 1675, 1620, 1580, and 1550; ^1H NMR (90 MHz, $CDCl_3$): δ1.8 (3H, *s* H-19), 2.2 and 2.37 (each 3H, *s* OAc), 2.7 (2H, *m*, H-16), 3.53 (3H, *s* OMe), 3.8 (3H, *m*, H-2 and H-15), 5.37 (1H, *d*, J = 4 Hz, H-1), 6.13 (1H, *d*, J = 4 Hz, H-3), 7.70 (1H, *d*, J = 8 Hz, H-11), 7.83 (1H, *s*, furan-H), 7.97 (1H, *d*, J = 8 Hz, H-12).

Incubation of [11-^{14}C]*squalene with* G. deliquescens. [11-^{14}C]Squalene (NEN Radiochemicals) (13.7×10^6 dpm) in EtOH (1 ml) was evenly distributed between 50 shake cultures (100 ml) of *G. deliquescens* 2 days after inoculation. After a further 6 days the metabolites were isolated as above to afford viridiol (55 mg) which was crystallized to constant sp. act (1029 dpm/mg, 0.41% incorporation).

The above experiment was repeated with [4,8,12,13,17, 21-^{3}H] squalene (NEN Radiochemicals) (4.75×10^7 dpm). The broth extract was separated into acidic and neutral fractions. Purification of the acid fraction by prep. TLC (silica gel, EtOAc–petrol 1:1) gave the acid 12 (210 mg) (identified by its NMR spectrum) (385 dpm/mg, 56 200 dpm/mmol) which was converted to its *p*-bromophenacyl derivative, mp 44–45° (lit. [12] 44–45°) (168 dpm/mg, 57 624 dpm/mmol) (0.17% incorporation). The viridiol (**1**) (83 mg) had 1104 dpm/mg (0.19% incorporation).

[2-$^3H_2$]*Lanosterol* (**9**). Lanosterol (purified via its dibromide) (2 g) in Me_2CO (100 ml) was treated with the 8N CrO_3 reagent (3 ml) at room temp. for 1 hr. Excess aq. Na_2SO_3 was added and the Me_2CO was removed *in vacuo*. The lanosterone (1.6 g) was recovered in EtOAc and crystallized as plates, mp 93–94° (lit. [13] 80–81°). It was identified by its IR and NMR spectra. Lanosterone (100 mg) in dry dioxan (2 ml) was treated with KOH (10 mg), 18-crown-6 (10 mg) and ^3H_2O (10 μl, 3×10^9 dpm) under reflux for 1 hr. HOAc (0.1 ml) was added and the soln was diluted with EtOAc. The organic phase was washed with excess aq. $NaHCO_3$ and dried over Na_2SO_4. The solvent was evapd to give [2-$^3H_2$]lanosterone (60 mg) which crystallized from MeOH as plates, mp 91–93° (lit. [13] 80–81°) (4.2×10^6 dpm/mg). The lanosterone (50 mg) in MeOH (5 ml) was treated with $NaBH_4$ (50 mg) for 30 min. at room temp. Water (5 ml) was added dropwise and the lanosterol was collected. It was recrystallized from EtOAc as needles, mp 136–138° (lit. [13] 140°) (4.2×10^6 dpm/mg).

Incubation of [2-$^3H_2$]*lanosterol* (**9**) *with* G. deliquescens. [2-$^3H_2$]Lanosterol (**9**) (10 mg, 4.2×10^6 dpm/mg) was added to 15 shake flasks (100 ml broth each) of *G. deliquescens* after 2 days of growth. After a further 6 days, the broth was filtered and extracted with $CHCl_3$. The solvent was evapd and the residue was taken up in MeOH (25 ml) and the gliotoxin (85 mg) allowed to crystallize. The mother liquors were separated by prep. TLC on silica gel in EtOAc to afford viridiol (58 mg) which crystallized from Me_2CO as needles, mp 202° (1000 dpm/mg, 0.14% incorporation).

Incubation of dehydroxydemethoxyviridin (**5**) *with* G. deliquescens. [^{14}C]Demethoxyviridin was prepared biosynthetically from [1-^{14}C]acetate by incubation with *N. hinnuleum*. It was converted as described previously [14] to dehydroxydemethoxyviridin (**5**). [^{14}C]Dehydroxydemethoxyviridin (**5**) (22.5 mg, 5.4×10^4 dpm) in DMSO (15 ml) was added to 24 shake flasks of *G. deliquescens*. After a further 7 days growth, the fermentation was harvested as above to give an oil (2.4 g). This was chromatographed on silica and then the relevant fractions were further purified by prep. TLC on silica gel in EtOAc to afford viridiol (290 mg) (1160 dpm, 2.3% incorporation). Repetition of the experiment with dehydroxydemethoxyviridin (22 mg, 2.3×10^5 dpm, from another preparation) and using only 10 flasks gave viridiol (20 mg, 2.78×10^3 dpm, 1.2% incorporation).

Incubation of [^{14}C]*demethoxyviridin with* G. deliquescens. [^{14}C]Demethoxyviridin (**3**) (prepared biosynthetically from[1-^{14}C]acetate) (23 mg 2.2×10^5 dpm) in DMSO (10 ml) was distributed between 10 flasks of *G. deliquescens*. After a further 7 days of growth, the broth and the mycelium were extracted and the combined extracts were chromatographed on silica and then the relevant fractions subjected to prep. TLC on silica in EtOAc to afford viridiol (100 mg), mp 200° (lit. [2] 198–201°). (14972 dpm, 6.6% incorporation). Repetition of the experiment with demethoxyviridin (52 mg, 4.9×10^5 dpm) gave viridiol (30 mg, mp. 196–199°, 2×10^4 dpm, 4.08% incorporation).

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