Lanosterol Derivatives as Precursors in the Biosynthesis of Viridin. Part 1

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^{14}C-Labelled lanosterol derived from sodium [1-^{14}C]acetate was efficiently incorporated into the aromatic-C-ring steroid viridin, confirming its derivation *via* a normal steroidal biosynthetic pathway. Studies with [G-^{3}H]lanostene oxidation products were carried out to determine their relative efficiency as precursors of viridin in an attempt to identify the intermediates between lanosterol and viridin. The [G-^{3}H]lanosterol was obtained by catalytic exchange labelling with $^{3}H_2O$ and principal sites of exchange in the sterol were identified by degradation experiments.

As part of an investigation into the synthesis of aromatic-C-ring analogues of the naturally occurring steroid hormones, the possibility is being explored of using *Trichoderma viride*, which is known to produce the aromatic-C-ring steroid viridin,[1] to carry out the aromatisation of other substrates.

Blight *et al.*[2] showed, by using [2-^{14}C]mevalonate that viridin was produced biosynthetically by the normal steroidal route involving the tail-to-tail condensation of two farnesyl pyrophosphate units, squalene 2,3-epoxide and presumably lanosterol. In order to obtain some information on the structural requirements for substrates to be aromatised by the enzyme systems of the micro-organism, a number of derivatives of lanosterol have been fed to cultures of *T. viride*, and the extent of their incorporation into viridin determined.

RESULTS AND DISCUSSION

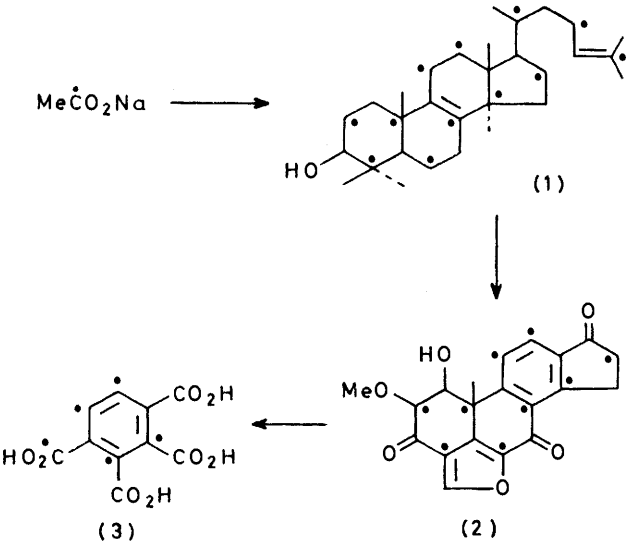
The conclusions drawn by Blight *et al.*[2] concerning the biosynthesis of viridin were confirmed by using [^{14}C]lanosterol (1) (produced biosynthetically by feeding [1-^{14}C]acetate to *Saccharomyces cerevisiae*) as a substrate for the production of viridin by *T. viride*. The viridin (2), isolated from the culture medium, contained 26% of the radioactivity of [^{14}C]lanosterol, after allowance had been made for the number of carbon atoms lost in the biosynthesis, and correction for the recovery of unused [^{14}C]lanosterol.

The benzene-1,2,3,4-tetracarboxylic acid methyl ester (3), obtained by permanganate oxidation (and methylation of the product) of [^{14}C]viridin, contained 4.6 labelled carbon atoms per molecule, which substantiates the proposed biosynthesis of viridin.

The theoretical value is five ^{14}C atoms per molecule, and the variation (8%) is attributed to experimental error rather than to an unusual route for the incorporation of the [1-^{14}C]acetate into lanosterol (Scheme 1).

In order to obtain sufficient radioactively labelled lanosterol and its derivatives for use as substrates, it was necessary to use tritium-labelled material obtained by catalytic exchange with tritiated water. The positions in the [^{3}H]lanosterol where most of the exchange had occurred were identified by performing a series of oxidations, and determining the loss of radioactivity in the products (Scheme 2). Thus, Kuhn–Roth oxidation of the terminal methyl groups produced the equivalent of 6 moles of acetic acid which had less than 1% of the total radioactivity.

Mild oxidation of [G-^{3}H]lanosterol (4) with chromic acid produced [G-^{3}H]lanosta-8,24-dien-3-one (5), which was equilibrated with base to remove enolisable tritium



SCHEME 1 Incorporation of [1-^{14}C]acetate into viridin by *T. viride*; • indicates ^{14}C-atoms

from position 2. This transformation resulted in the loss of 33% of the total radioactivity of the [G-^{3}H]-lanosterol.

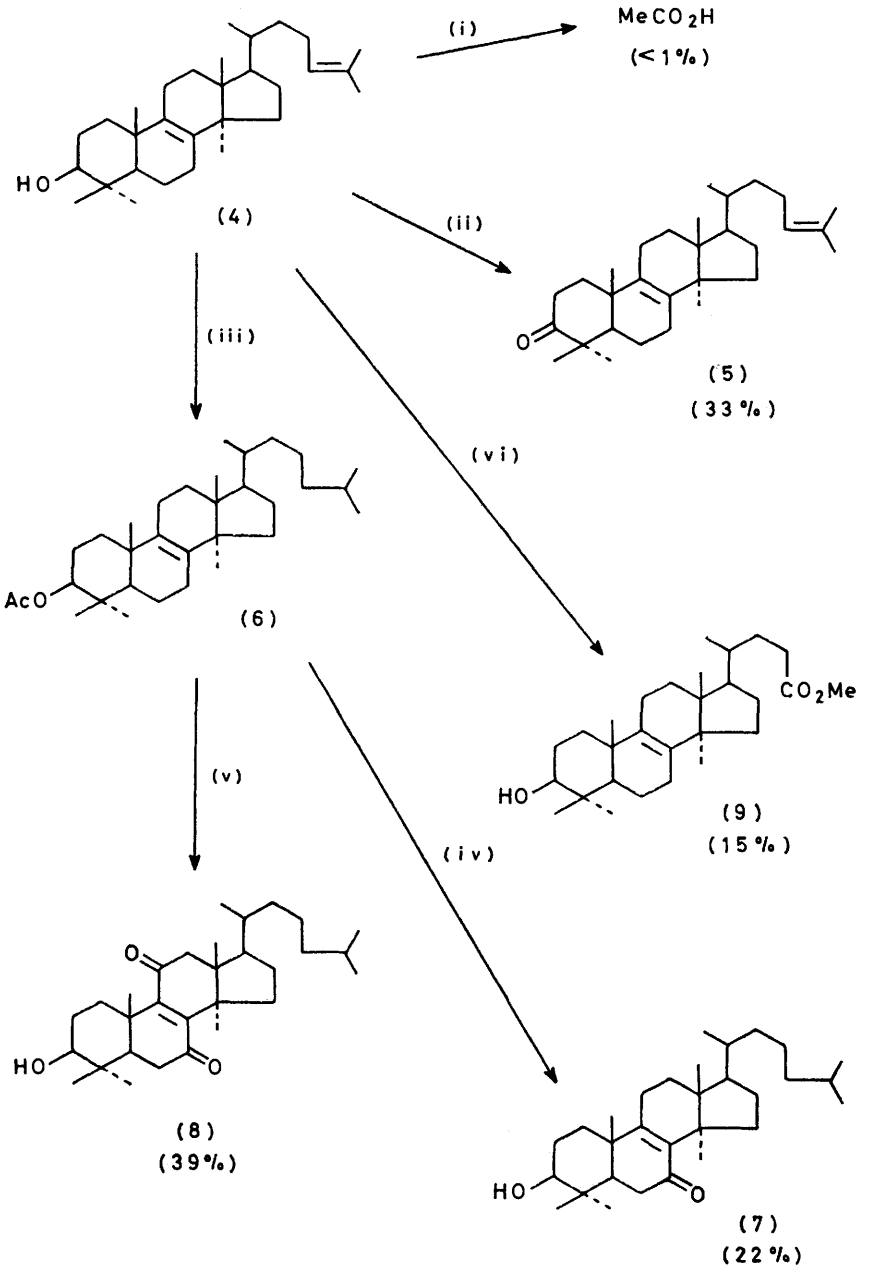
Oxidation of [G-^{3}H]lanostenyl acetate (6) with a mixture of acetic acid, sulphuric acid, and hydrogen peroxide,[3a] followed by alkaline hydrolysis of the acetate, gave 3β-hydroxy[G-^{3}H]lanost-8-en-7-one (7), which had lost 22% of the radioactivity of the [G-^{3}H]lanosterol. Conversion of (6) into the corresponding 7,11-dione by chromium trioxide in acetic acid,[4] followed by saponification of the ester, gave 3β-hydroxy [G-^{3}H]lanost-8-ene-7,11-dione (8), which had 39% less activity than the original compound.

Oxidation of [G-^{3}H]lanosteryl acetate with permanganate–periodate,[5] followed by saponification of the

acetate and methylation of the carboxy-group gave methyl 3β-hydroxy[G-^{3}H]trisnorlanost-8-en-24-oate (9), which had lost 15% of the tritium of [G-^{3}H]lanosterol.

These results are summarised in Table 1. The biosynthetic process. Any label at position 17 would also be lost during oxidation to the ketone.

Thus, if [G-^{3}H]lanosterol is a specific precursor of viridin, its incorporation would result in the loss of *ca.*



SCHEME 2 Reactions used to locate tritium in [G-^{3}H]lanosterol; figures in brackets give the percentage of radioactivity lost as a result of the reactions shown.

Reagents: (i) Kuhn–Roth; (ii) Jones oxidation; (iii) Ac_2O–py, room temp.; H_2–Pt, HOAc; (iv) HOAc, H_2SO_4, H_2O_2; methanolic KOH, reflux; (v) CrO_3–HOAc, methanolic KOH, reflux; (vi) Ac_2O–py, room temp.; $KMnO_4$–KIO_4; methanolic KOH, reflux; CH_2N_2

synthetic oxidation of [G-^{3}H]lanosterol to viridin involves the loss of tritium from positions 2, 3, 6, and 7 due to oxidations at these carbon atoms, and probably half of the label at positions 11 and 12 due to aromatisation of ring C. Also tritium atoms at positions 18 and 20—27 are lost since these side-chains are removed in the 79% of the initial radioactivity through the loss of tritium from these sites.

The percentage of the tritium label of [G-^{3}H] lanosterol which was incorporated into viridin by the organism was 21 ± 10.9%, the mean value being very close to that predicted from the foregoing experiments. This value

is also in good agreement with that found for the incorporation of [^{14}C]lanosterol.

Table 2 gives the values, corrected for the loss of tritium, for the extent of incorporation of a number of *[G-^{3}H]lanostene* derivatives. From the data it is

TABLE 1

Relative molar activity of derivatives of [G-^{3}H]lanosterol

Compound	dpm mmol^{-1} ($\times 10^{-6}$)	%	Specific loss
Lanosterol (4)	583	100	
Lanosteryl acetate	580	100	
Lanosta-8,24-dien-3-one (5)	389	67	33% from positions 2 and 3
Methyl 3β-hydroxytrisnorlanost-8-en-24-oate (9)	493	85	15% from positions 23, 24, 25, 26, and 27
Sodium acetate	4.9	0.8	*ca.* 1% from the terminal methyl groups
Lanostenyl acetate (6)	593	100	
3β-hydroxylanost-8-en-7-one (7)	463	78	22% from positions 6 and 7
3β-hydroxylanost-8-ene-7,11-dione (8)	361	61	17% from positions 11 and 12

apparent that none of these substrates is used as efficiently as lanosterol by *T. viride*, but the variation in the results makes it difficult to differentiate between these compounds with respect to their relative efficiency as precursors of viridin. However, it appears that the ester (9) is a more effective substrate for the enzyme

TABLE 2

Incorporation of [G-^{3}H]lanostene derivatives into viridin

[G-^{3}H]-Compound	% ^{3}H-Incorporation into viridin [a]
[^{3}H]Lanosterol (4)	21.0 ± 10.9
[^{3}H]Lanosta-8,24-dien-3-one (5)	5.6 ± 2.6
3β-Hydroxy[^{3}H]lanost-8-en-7-one (7)	7.9 ± 3.7
3β-Hydroxy[^{3}H]lanost-8-ene-7,11-dione (8)	5.7 ± 1.9
Methyl 3β-hydroxy[^{3}H]trisnorlanost-8-en-24-oate (9)	9.2 ± 4.2

[a] Means of duplicate experiments and the range.

systems than the ketones, which would indicate that this modification of the side-chain does not affect the extent of substrate incorporation into viridin as much as oxidation of the polycyclic nucleus.

Further work to identify natural precursors of viridin is in progress.

EXPERIMENTAL

Catalytic Exchange Labelling of Lanosterol with Tritium.—[G-^{3}H]Lanosterol was prepared by New England Nuclear Corporation, Boston, Massachusetts, as follows. Lanosterol (50 mg, recrystallised three times from Me_2CO–MeOH) was dissolved in EtOAc containing ^3H_2O (10 Ci) and rhodium–alumina catalyst (5%; 50 mg). The mixture was heated at 80 °C overnight with stirring and the ' labile ' tritium removed with Me_2CO–MeOH (10 ml) twice by distillation *in vacuo*. The radioactively labelled lanosterol was supplied dissolved in MeOH (20 ml).

All radioactive samples were counted using a Packard Tri-Carb model 3004 liquid scintillation spectrometer, equipped for external standardisation, and were dissolved in a phosphor solvent containing methanol (10% v/v), 2,5-diphenyloxazole (0.5% w/v), and 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene (0.005% w/v) in scintillation grade toluene. Chromatoplate scanning was performed using a Chromoscan J552 double beam recording and integrating densitometer (Joyce-Loebl and Co. Ltd., Princesway, Gateshead-on-Tyne, England) using reflectance photometry (red filter). Melting points were determined on a heated stage (Kofler block) apparatus and are corrected.

Purification of the Tritium-exchanged Lanosterol.—[G-^{3}H]Lanosterol, prepared by the catalytic exchange procedure above, was diluted with non-radioactive lanosterol and purified [6] *via* the 24,25-dibromide. The final product (4) had m.p. 138—139° (from Me_2CO–MeOH) (lit.,[6] 139—140°) and specific activity 5.83 × 10^8 dpm mmol^{-1}.

Location of Tritium Label in [G-^{3}H]*Lanosterol.*—*Conversion of* (4) *into* [G-^{3}H]*lanosta-8,24-dien-3-one* (5). Compound (85 mg) was dissolved in Me_2CO and titrated with Jones reagent [7] until oxidation of the secondary hydroxy-group was complete. After the usual work-up, the product was recrystallised (Me_2CO–MeOH) to constant specific activity, 3.89 × 10^8 dpm mmol^{-1}, m.p. 87—89° (lit.,[6] 87—89°).

Conversion of [G-^{3}H]-3β-*acetoxylanost-8-ene* (6) *into* 3β-*hydroxy*[G-^{3}H]*lanost-8-en-7-one* (7). This conversion was carried out according to the method of Pinhey *et al.*[3a] using as oxidant H_2O_2 in HOAc–H_2SO_4 and with stirring at room temperature for 8 h. The deacetylated recrystallised product (MeOH) had m.p. 135—136° (lit.,[3b] 135°) and constant specific activity 4.63 × 10^8 dpm mmol^{-1}.

Conversion of [G-^{3}H]-3β-*acetoxylanost-8-ene* (6) *into* 3β-*hydroxy*[G-^{3}H]*lanost-8-ene-7,11-dione* (8). The conversion was performed according to the method of Ruzicka *et al.*[4] using CrO_3–HOAc at 40 °C for several hours. The neutral product was extracted with ether and, after successive washings with dilute NaOH and water, drying ($MgSO_4$), and evaporation of solvent, was dissolved in hexane and absorbed on a column of alumina (Activity II). Hexane–benzene (1 : 2 v/v) eluted a yellow product which, after saponification and recrystallisation (× 3) from MeOH, had m.p. 113—115° (lit.,[4] 113—115°) and constant specific activity 3.61 × 10^8 dpm mmol^{-1}.

Conversion of 3β-*acetoxy*[G-^{3}H]*lanosta-8,24-diene to methyl* 3β-*hydroxy*[G-^{3}H]*trisnorlanost-8-en-24-oate* (9). This conversion was performed by a modification of the method of Mallory *et al.*[5] The sterol acetate was dissolved in t-butyl alcohol and oxidised with $KMnO_4$–KIO_4 in K_2CO_3 solution overnight at 37 °C. After deacetylation, the product was treated with diazomethane–MeOH and chromatographed on alumina. Hexane–benzene (1 : 1 v/v) eluted a pale yellow compound which after two recrystallisations from MeOH to constant specific activity (4.93 × 10^8 dpm mmol^{-1}) had m.p. 153—155° (lit.,[4] 152.5—154.5°).

Kuhn–Roth Oxidation of the Terminal Methyl Groups of [G-^{3}H]*Lanosterol.*—The apparatus employed was a modification of that of Grant.[8] The NaOAc formed after neutralisation of the steam-distillate was treated with *S*-benzylisothiuronium chloride [9] and recrystallised three times from water–ethanol 50 : 1 v/v). The salt had m.p. 135—136° and constant specific activity 4.88 × 10^6 dpm mmol^{-1}. Correction was made for ' quenching ' of the phosphor by the derivative.

Incorporation of [G-^{3}H]*Lanosterol in Viridin using* Trichoderma viride CMI 24039.—*Growth conditions.* Sterile Weindling medium (pH 3.5; 3 l) [10] was distributed into thirty 2-l conical flasks (100 ml per flask). Each flask was inoculated with a 1 × 3 cm square of a Weindling-agar

plate culture of *T. viride* and [G-^{3}H]lanosterol (5.83×10^8 dpm mmol^{-1} 0.047 mmol) in EtOH (20 ml) aseptically added to each of twenty flasks (1 ml per flask). These flasks served as ' test ' cultures, the other ten (inoculated with micro-organism alone) were used as ' controls ' to assess antibiotic production in the absence of added sterol. All flasks were incubated at 28 °C for 8 days in still culture.

Isolation and assay procedures. All cultures were harvested, the mycelium washed with water, dried at room temperature and then over P_2O_5, powdered, and extracted continuously with light petroleum (b.p. 40—60°) for 1 h followed by $CHCl_3$ for 7 h. Culture filtrates were extracted by intermittent shaking with solvents in separatory funnels ($2 \times \frac{1}{5}$ vol) sequentially. The combined $CHCl_3$ extracts of the mycelium and culture filtrate were washed with water, dried over $MgSO_4$, and evaporated *in vacuo* to dryness. The yellow-brown oils were made up to known volumes with $CHCl_3$ and the components separated on preparative silica gel GF_{254} plates (0.5 mm) using $CHCl_3$–EtOH (98 : 3 v/v) as solvent. The steroid bands, detected using u.v. light, were removed from the plates, eluted with $CHCl_3$–EtOH (75 : 25 v/v), and extensively purified by t.l.c. using (C_6H_6–Me_2CO, 70 : 30 v/v). The amount of steroid present in each solvent sample was estimated by use of a chromatoplate scanner; the radioactivity present was assayed by liquid scintillation counting.

This incorporation experiment was repeated using the other tritiated lanostene derivatives in Table 2.

Biosynthetic [^{14}C]*Lanosterol* (1).—Compound (1) was obtained by incubation of [1-^{14}C]NaOAc * (1.2 mCi; 19.6 μmol) with a cell-free yeast homogenate for 12 h at 26 °C. Yeast cells (*S. cerevisiae*, culture 156, 10 g) † were suspended in phosphate buffer (0.1M; pH 7.0; 20 ml) and subjected to freezing with solid CO_2 and subsequent disruption at 41.34 MPa in a cell disintegrator. Any remaining intact cells were removed by differential centrifugation, and the supernatant fraction, together with added co-factors,[11] was used for the incubation. After subsequent hydrolysis of the incubation mixture with methanolic KOH the recovered non-saponifiable residue (50 mg; 4.07×10^7 dpm) was extensively purified on chromatoplates (0.5 mm thick plates, Silica gel GF_{254}) to yield lanosterol as well as squalene and a mixture of sterols all radioactively labelled.

Incorporation of Biosynthetic [^{14}C]*Lanosterol* (1) *into* [^{14}C]*Viridin* (2).—The labelled lanosterol (9.38×10^4 dpm μmol^{-1}; 11.83 μmol) in EtOH was added to Weindling liquid culture medium (400 ml) containing a five-day old culture (obtained from Weindling 3% agar slopes) of *T. viride* CMI 24039. After 10 days' growth in surface culture at 26 °C, the culture was harvested, extracted with solvents as before, and the chloroform extracts chromatographed on preparative plates. The viridin obtained (0.02 mmol) was diluted with a known amount of authentic compound (0.57 mmol) ‡ and co-crystallised from acetone to constant specific activity, 1.36×10^5 dpm mmol^{-1}, as needles, m.p. 220—222° (decomp.).

Location of ^{14}C-*Label in* ^{14}C-*Viridin* (2).—Compound (2) (1.36×10^5 dpm mmol^{-1}; 0.57 mmol) was oxidised by slow addition of aqueous $KMnO_4$ (7%; 20 ml) under reflux at 100 °C for 12 h, a modification of the method of Grove *et al.*[2a] CO_2 was continuously bubbled into the mixture during heating. The mixture was cooled and the precipitated MnO_2 filtered off and washed with boiling water. The combined filtrate and washings were made alkaline (pH 10) by the addition of K_2CO_3, and extracted with ether (2×10 ml). The aqueous phase was separated, acidified (pH 2), concentrated to small volume at 40 °C *in vacuo*, and extracted for 24 h with EtOAc. Concentration of the extract afforded a yellow oil (140 mg) which was dissolved in MeOH and treated at 0 °C with a slight excess of diazomethane. The product was fractionally sublimed and an oil (80 mg) obtained at 160°—170° and 1 mmHg) which gave, on repeated recrystallisation from MeOH, colourless needles (m.p. 131—132°) of methyl benzene-1,2,3,4-tetracarboxylate (3), 0.065 mmol; 6.94×10^4 dpm mmol^{-1}, identical with an authentic sample prepared by oxidation of 1,2,3,4-tetramethylbenzene with $KMnO_4$ under the same conditions.

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* [1-^{14}C]NaOAc obtained from the Radiochemical Centre, Amersham, England, catalogue No. CFA 13.

† Yeast obtained from Anheuser–Bush, Inc., St. Louis, Missouri, U.S.A. and grown by the procedure described in ref. 11.

‡ Viridin obtained from Dr. J. S. Moffatt, I.C.I. Pharmaceutical Division, Mereside, Alderley Park, Macclesfield, Cheshire SKIO 4TG, England.

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