Role of 24- and 28-Hydroxylated Intermediates in the Metabolism of β -Sitosterol in the Insect *Tenebrio molitor*

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(Received 23 April 1979)

1. [28- ^3H]Stigmast-5-ene-3 β ,28-diol and [23,23,25- ^3H]stigmast-5-ene-3 β ,24-diol were synthesized. 2. Each of the samples was mixed with β -[4- ^{14}C]sitosterol and administered to *Tenebrio molitor* larvae. 3. The former compound is not utilized by the insect; the latter, although metabolized to 24(28)-ethylidene sterols and cholesterol, is not a β -sitosterol metabolite. 4. The above results are discussed in relation to the mechanism of formation of the 24(28)-double bond in β -sitosterol metabolism in *T. molitor*.

Phytophagous insects are unable to effect synthesis *de novo* of cholesterol (IV), which they need for the biosynthesis of their steroid moulting hormones. However, they obtain this necessary sterol from phytosterols contained in their diet, through a dealkylation process that removes the C-1 or C-2 alkyl group at C-24 (Thompson *et al.*, 1972).

It is generally accepted that a major route of dealkylation of β -sitosterol (I) proceeds through the formation of fucosterol (II), followed by epoxidation of the 24(28)-double bond, rearrangement to desmosterol (III) and a C₂ unit, and reduction of the 24(25)-double bond (Scheme 1) (Svoboda *et al.*, 1971, 1975;

Allais & Barbier, 1971; Morisaki *et al.*, 1974; Chen *et al.*, 1975).

The first step in Scheme 1 does not appear to be always stereospecific; in fact, we have observed that *Tenebrio molitor* transforms β -sitosterol (I) into both fucosterol (II) and its geometrical isomer isofucosterol (V) (Nicotra *et al.*, 1978).

The mechanism of the formation of the double bond of the above ethylidene compounds is still unknown. Two basic possibilities can be considered for the origin of this double bond: a dehydrogenation mechanism and a hydroxylation–dehydration mechanism. The former mechanism requires the direct

Scheme 1. A major route of dealkylation of β -sitosterol

elimination of two hydrogen atoms, whereas the latter requires that one of the possible hydroxysterols, with a hydroxy group at C-24 or C-28, is an intermediate (Scheme 2).

To see whether the latter route is the correct mechanism, we synthesized labelled stigmast-5-ene-3 β ,28-diol (VI) and stigmast-5-ene-3 β ,24-diol (VII) (both as mixtures of diastereoisomers) and investigated their role as potential intermediates, by administering them to *T. molitor* larvae.

Experimental

Nomenclature

Trivial names are used in the present paper. The systematic names are : cholesterol, cholest-5-en-3 β -ol; desmosterol, cholesta-5,24-dien-3 β -ol; β -sitosterol, (24*R*)-stigmast-5-en-3 β -ol; fucosterol, (24*E*)-stigmasta-5,24(28)-dien-3 β -ol; isofucosterol, (24*Z*)-stigmasta-5,24(28)-dien-3 β -ol.

Reagents

3 β -Hydroxycholest-5-en-24-one was synthesized from 3 β -acetoxy-5-cholenyl chloride (Riegel & Kaye, 1944).

³H₂O (sp. radioactivity 360mCi/mmol) and β -[4-¹⁴C]sitosterol (sp. radioactivity 54mCi/mmol)

were from The Radiochemical Centre, Amersham, Bucks., U.K.

T.l.c.

T.l.c. was carried out on Kieselgel F₂₅₄ plates (0.25mm thickness; Merck) or on Kieselgel G-60 plates impregnated with 20% (w/w) AgNO₃. The compounds were detected by spraying with 50% H₂SO₄ and heating at 110°C for 5min.

G.l.c.

A Carlo Erba Fractovap 2400 V chromatograph was used, fitted with a flame-ionization detector and 4mm-diameter glass columns (2m long). Two stationary phases were used: 1% LAC 796 on Gas Chrom Q and 2.5% SE-30 on Chromosorb W. For preparative g.l.c. a metal splitter was inserted between the detector and the end of the column, so that approx. 1 part in 30 passed to the detector, whereas the remainder was collected in traps cooled at -78°C.

Radioactivity measurements

Radioactivity was measured on a Packard Tri-Carb 3320 scintillation spectrometer. Samples were dissolved into 10ml of a solution consisting of 0.65% (w/v) 2,5-diphenyloxazole and 0.013% (w/v) 1,4-bis-

Scheme 2. The possible hydroxylation-dehydration mechanism for the formation of the 24(28)-double bond

(4-methyl-5-phenyloxazol-2-yl)benzene in toluene/dioxan (1:1, v/v).

Synthesis of (24RS,28RS)-[28-³H]stigmast-5-ene-3 β ,28-diol (VI)

(24RS,28RS)-6 β -Methoxy-3 α ,5-cyclo[28-³H]stigmastan-28-ol (Busca *et al.*, 1979) (6.60×10^8 d.p.m.) was dissolved in 1 ml of dioxan and stirred for 6 h at 75–80°C with 1 ml of water and 2 mg of *p*-toluenesulphonic acid. Removal of the solvent *in vacuo* and usual work-up afforded a crude product, which was purified by preparative t.l.c. with benzene/ethyl acetate (7:3, v/v) as developing solvent, yielding pure (analysis by g.l.c., 2.5% SE-30, T_c 260°C) (24RS,28RS)-[28-³H]stigmast-5-ene-3 β ,28-diol (6.14×10^8 d.p.m., 3.3×10^8 d.p.m./mg).

Synthesis of (24RS)-[23,23,25-³H]stigmast-5-ene-3 β ,24-diol (VII)

3 β -Hydroxycholest-5-en-24-one (3.5 mg) was dissolved into dioxan (10 ml) containing 10 μ l of ³H₂O (200 mCi) and 5 mg of NaOH, and refluxed under N₂ for 120 h. The solution was freeze-dried and the solid residue dissolved into water/diethyl ether; the ether layer was washed with water, dried over Na₂SO₄ and evaporated *in vacuo*. The above process was repeated twice, by using the recovered ³H₂O/dioxan solution and 3.5 mg of 3 β -hydroxycholest-5-en-24-one each time. The collected solid residues, chromatographed on silica gel/Celite (1:1, w/w) with benzene/ethyl acetate (4:1, v/v) as eluent, afforded 8 mg of pure 3 β -hydroxy[23,23,25-³H]cholest-5-en-24-one (5.9×10^9 d.p.m.). A solution of this ³H-labelled ketone in 1 ml of dry diethyl ether was added dropwise under N₂ to ethyl magnesium bromide (23 mg of magnesium, 0.3 ml of ethyl bromide and 0.8 ml of dry diethyl ether) and the mixture was refluxed with stirring for 3 h (Hayazu, 1957). The excess of Grignard reagent was destroyed with aq. NH₄Cl and the resulting aqueous solution was extracted with diethyl ether. The obtained extracts were washed with water, dried over Na₂SO₄ and evaporated *in vacuo* to yield a residue. Chromatography of the residue (10 mg) on silica gel/Celite (1:1, w/w) with benzene/ethyl acetate (4:1, v/v) as eluent afforded, after crystallization from methanol, 3 mg of pure (24RS)-[23,23,25-³H]stigmast-5-ene-3 β ,24-diol (1.48×10^9 d.p.m./ 4.95×10^8 d.p.m./mg).

Administration of (24RS,28RS)-[28-³H]stigmast-5-ene-3 β ,28-diol (VI) and isolation of fucosteryl acetate and isofucosteryl acetate (Expt. 1)

A mixture of (24RS,28RS)-[28-³H]stigmast-5-ene-3 β ,28-diol (1.03×10^8 d.p.m. of ³H), β -[4-¹⁴C]sito-

sterol (1.28×10^7 d.p.m. of ¹⁴C; ³H/¹⁴C ratio 8.05), fucosterol (2 mg) and isofucosterol (2 mg) was deposited on 130 mg of finely ground oatmeal and fed to 50 young larvae (1.3 g) of *T. molitor* (yellow mealworm), 1–1.5 cm long, which had been starved for 48 h. After 3.5 days the insects were frozen and macerated in ethanol (15 ml); the homogenate was subjected to alkaline hydrolysis (5% ethanolic KOH) and the non-saponifiable fraction was extracted. The crude material (17 mg) was acetylated and, after analysis by g.l.c. (1% LAC 796, T_c 200°C), separated by preparative t.l.c. on AgNO₃/silica gel developed with hexane/benzene (3:2, v/v; two elutions). The fraction corresponding to the acetylated 24(28)-ethylidene sterols (2 mg) was diluted with unlabelled β -sitosteryl acetate (5 mg), fucosteryl acetate (15 mg), isofucosteryl acetate (15 mg) and 3 β ,28-diacetoxystigmast-5-ene (5 mg) and subjected to column chromatography on 20% (w/w) AgNO₃/silica gel eluted with hexane/benzene (9.5:0.5, v/v). Fucosteryl acetate and isofucosteryl acetate were obtained pure, as shown by t.l.c. on AgNO₃ and by g.l.c. (1% LAC 796, T_c 200°C). These two compounds were each diluted with the corresponding carrier material to 30 mg and repeatedly crystallized to constant specific radioactivity from chloroform/methanol. The total radioactivities and the ³H/¹⁴C ratios of the two compounds are shown in Table 1.

Administration of (24RS)-[23,23,25-³H]stigmast-5-ene-3 β ,24-diol (VII) and isolation of fucosteryl acetate, isofucosteryl acetate and cholesteryl acetate (Expt. 2)

A mixture of (24RS)-[23,23,25-³H]stigmast-5-ene-3 β ,24-diol (2.05×10^8 d.p.m. of ³H), β -[4-¹⁴C]sitosterol (2.02×10^7 d.p.m. of ¹⁴C; ³H/¹⁴C ratio 10.12), fucosterol (4 mg) and isofucosterol (4 mg) was deposited on 250 mg of finely ground oatmeal and fed to 110 young larvae (2.5 g) of *T. molitor*, which had been starved for 48 h.

After 3.5 days the crude non-saponifiable fraction (190 mg), recovered as described for Expt. 1, was submitted to column chromatography on silica gel/celite (1:1, w/w). Elution with benzene afforded the sterol fraction (16 mg), which was acetylated and submitted to preparative t.l.c. on AgNO₃/silica gel developed with hexane/benzene (3:2, v/v, two elutions). Two bands were separated, the first of which (7 mg) corresponded to the acetates of β -sitosterol plus cholesterol (monitored by g.l.c., 1% LAC 796, T_c 200°C), whereas the second (6 mg) corresponded to the acetates of fucosterol and isofucosterol (monitored by g.l.c., 1% LAC 796, T_c 200°C).

The acetates of β -sitosterol and cholesterol contained in the first band were separated by pre-

parative g.l.c. (2.5% SE-30, T_c 220°C). The obtained pure cholesteryl acetate was diluted to 50mg with carrier material and crystallized to constant specific radioactivity from diethyl ether/methanol (see Table 1). The second band, diluted with unlabelled β -sitosteryl acetate (5mg), fucosteryl acetate (20mg), isofucosteryl acetate (20mg) and 3 β -acetoxy-5-ene-24-ol (5mg) was chromatographed on 20% (w/w) AgNO₃/silica gel. Hexane/benzene (9.5:0.5, v/v) eluted first fucosteryl acetate and then isofucosteryl acetate (the complete separation was measured by g.l.c., 1% LAC 796, T_c 200°C). Both compounds were diluted to 50mg with the corresponding carrier material and crystallized to constant specific radioactivity from chloroform/methanol. The total radioactivities and the $^3\text{H}/^{14}\text{C}$ ratios are shown in Table 1.

Administration of β -[4- ^{14}C]sitosterol and attempted isolation of labelled (24RS)-stigmast-5-ene-3 β ,24-diol (Expt. 3)

A mixture of β -[4- ^{14}C]sitosterol (1.5×10^7 d.p.m.) and unlabelled (24RS)-stigmast-5-ene-3 β ,24-diol (8mg) was deposited on 240mg of finely ground oatmeal and fed to 100 young larvae (2.4g) of *T. molitor*, which had been starved for 48h. After 3.5 days the crude non-saponifiable fraction, recovered as in Expt. 1, was diluted with unlabelled β -sitosterol (5mg) and (24RS)-stigmast-5-ene-3 β ,24-diol (5mg) and submitted to column chromatography on silica gel/Celite. Benzene/ethyl acetate (9.5:0.5, v/v) eluted 4mg of (24RS)-stigmast-5-ene-3 β ,24-diol, which was further purified by acetylation and preparative t.l.c. on silica gel developed with benzene/ethyl acetate (9:1, v/v). The product was diluted to 40mg with carrier material and repeatedly crystallized from diethyl ether. The specific radioactivity decreased rapidly, reaching after the fifth crystallization a value (16d.p.m./mg) comparable with the background value of the scintillation spectrometer.

Results and Discussion

Preliminary experiments showed that the extent of conversion of β -sitosterol into the ethylidene sterols by *T. molitor* larvae varied remarkably depending on several parameters, such as the length of the larvae, the duration of the experiment etc.

Therefore, to see whether the above hydroxy-sterols are intermediates, we chose to compare the extent of their utilization with that of β -sitosterol, administering each potential intermediate, ^3H -labelled, together with β -[4- ^{14}C]sitosterol and taking into consideration the $^3\text{H}/^{14}\text{C}$ ratio of the ethylidene sterols obtained.

When a mixture of [28- ^3H]stigmast-5-ene-3 β ,28-diol (VI) and β -[4- ^{14}C]sitosterol ($^3\text{H}/^{14}\text{C}$ ratio 8.05) was fed to *T. molitor* young larvae, together with unlabelled fucosterol and isofucosterol as metabolic traps (Expt. 1), both the recovered 24(28)-ethylidene sterols showed a sharp decrease in the $^3\text{H}/^{14}\text{C}$ ratio (Table 1). This indicates that no significant conversion of compound (VI) into the 24(28)-ethylidene sterols had occurred. Although it was theoretically possible that the 24-hydroxylated sterol (VI) might be an intermediate in the formation of the 24-ethylidene sterols if ^3H -labelled VI was converted into the unlabelled form by an oxo-alcohol reductase system in the body of the insect, this possibility was ruled out by our finding (F. Nicotra, F. Ronchetti, G. Russo & L. Toma, unpublished work) that β -[28- ^3H]sitosterol is converted in *T. molitor* into [28- ^3H]fucosterol and [28- ^3H]isofucosterol.

Our result is in agreement with that of a nutritional experiment effected in *Bombyx mori*, the larvae of which died when reared on a semi-synthetic diet containing stigmast-5-ene-3 β ,28-diol (VI) as a sole sterol source (Morisaki *et al.*, 1974).

On the contrary, [23,23,25- ^3H]stigmast-5-ene-3 β ,24-diol (VII) was found to be converted into compounds (II) and (V) and, even more efficiently, into cholesterol (IV). When a mixture of this compound and β -[4- ^{14}C]sitosterol ($^3\text{H}/^{14}\text{C}$ ratio 10.12) was fed to *T. molitor* young larvae, together with fucosterol

Table 1. $^3\text{H}/^{14}\text{C}$ ratios and total radioactivities of the administered precursors and of the sterols isolated from *T. molitor*

Expt. no.	Administered precursors	$^3\text{H}/^{14}\text{C}$ ratio	Radioactivities in sterol products					
			Fucosteryl acetate isolated		Isofucosteryl acetate isolated		Cholesteryl acetate isolated	
			(d.p.m. of ^{14}C)	($^3\text{H}/^{14}\text{C}$)	(d.p.m. of ^{14}C)	($^3\text{H}/^{14}\text{C}$)	(d.p.m. of ^{14}C)	($^3\text{H}/^{14}\text{C}$)
1	(24RS,28RS)-[28- ^3H]Stigmast-5-ene-3 β ,28-diol (VI) + β -[4- ^{14}C]sitosterol (1.28×10^7 d.p.m. of ^{14}C)	8.05	1.23×10^3	0.6	9.2×10^2	0.9	—	—
2	(24RS)-[23,23,25- ^3H]Stigmast-5-ene-3 β ,24-diol (VII) + β -[4- ^{14}C]sitosterol (2.02×10^7 d.p.m. of ^{14}C)	10.12	5.75×10^4	12.2	3.9×10^4	9.8	2.04×10^6	7.11

and isofucosterol as in Expt. 1 (Expt. 2), the two ethylidene sterols and cholesterol had a $^3\text{H}/^{14}\text{C}$ ratio in agreement with that of the administered mixture (Table 1).

However, when β -[4- ^{14}C]sitosterol was fed together with unlabelled stigmast-5-ene-3 β ,24-diol (VII) as metabolic trap (Expt. 3), no significant amount of radioactive label was found associated with the recovered stigmast-5-ene-3 β ,24-diol. So, stigmast-5-ene-3 β ,24-diol, though transformed into cholesterol, probably through a dehydration process, does not seem to be an intermediate during the process that transforms β -sitosterol into cholesterol.

The results reported in the present paper indicate that, since neither stigmast-5-ene-3 β ,28-diol (VI) nor stigmast-5-ene-3 β ,24-diol (VII) seem to play a role in the formation of the double bond of 24(28)-ethylidene sterols, the hydroxylation-dehydration mechanism can be excluded, the most probable mechanism remaining the direct dehydrogenation of the 24-ethyl group.

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