Synthesis of (24*R*)- and (24*S*)-24,28-Epoxyergost-5-en-3β-ols. Substrate Stereospecificity in their Metabolism in the Insect *Tenebrio molitor*

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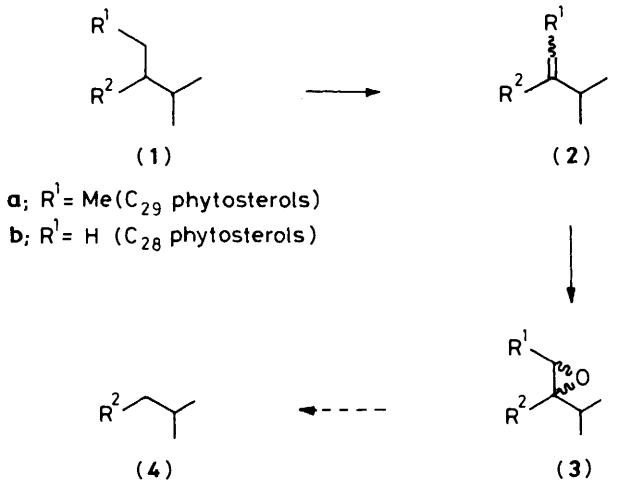
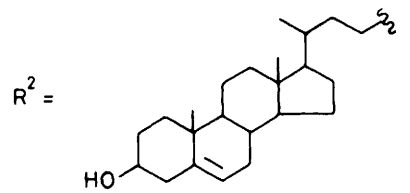
(24*R*)- and (24*S*)-24,28-Epoxyergost-5-en-3β-ols have been synthesized and their configuration at C-24 assigned. When these 24,28-epoxides were tritium labelled and fed in separate experiments to *Tenebrio molitor* larvae, only the (24*R*) stereoisomer was effectively converted into cholesterol, indicating that in this step of phytosterol metabolism the insect exhibits a high degree of substrate stereospecificity.

The sequence of metabolic events through which C_{29} phytosterols (Scheme 1, R^1 = Me) are transformed into cholesterol (**4**) by most phytophagous insects is now well known.[1] The metabolism of C_{28} phytosterols (Scheme 1, R^1 = H) has been less widely studied, though the intermediates involved seem to be analogous to those in the C_{29} phytosterol metabolism; for example, we have observed[2] that 24-methylenecholesterol (**2b**) and the corresponding 24,28-epoxides (**3b**), as a diastereoisomeric mixture, are converted into cholesterol (**4**) by *Tenebrio molitor* larvae.

The utilization of 24,28-epoxides of the C_{29} series by *Tenebrio molitor* shows a low degree of substrate stereospecificity, as three out of the four diastereoisomeric 24,28-epoxides are converted into cholesterol to about the same extent.[3] We now wish to report our results on the substrate stereospecificity shown by the same insect in the utilization of the two diastereoisomeric 24,28-epoxyergost-5-en-3β-ols (**3b**).





Scheme 1.

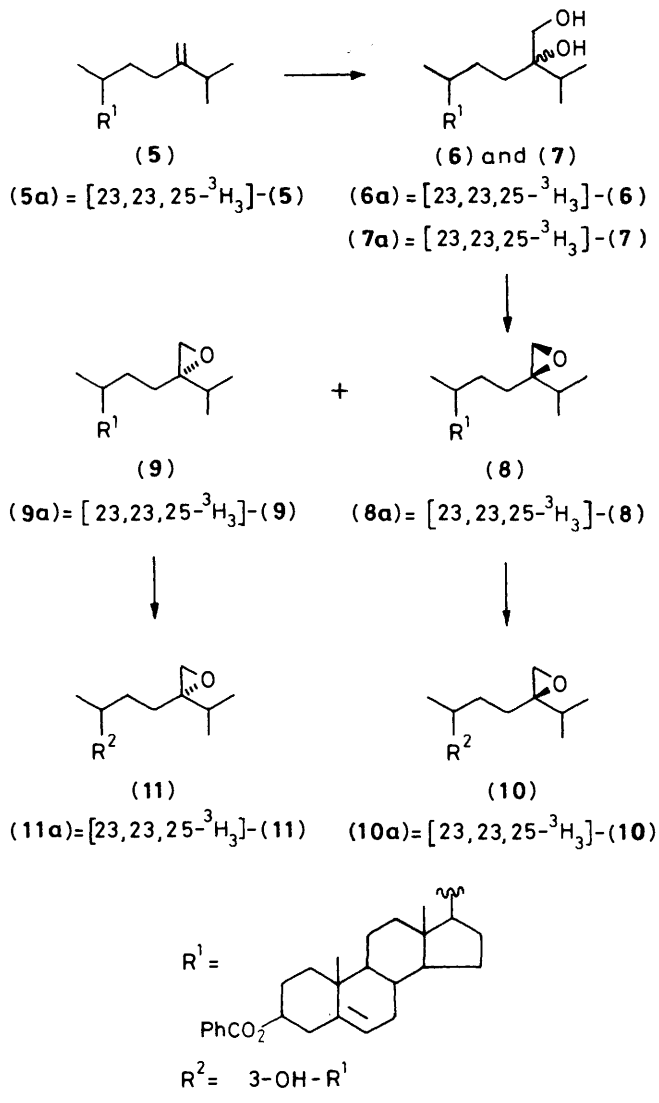
Results and Discussion

The diastereoisomeric (24*R*)- and (24*S*)-24,28-epoxyergost-5-en-3β-ols (**10**) and (**11**) were synthesized from 24-methylene-3-cholesterylbenzoate[4] (**5**), as shown in Scheme 2. Compound (**5**) was transformed into a diastereoisomeric mixture of the 24,28-diols (**6**) and (**7**) by treatment with OsO_4 (1 mol equiv.) in benzene–pyridine; mesylation of the 24,28-diols (**6**) and (**7**), followed by mild base treatment,[5] then afforded a mixture of the 24,28-epoxides (**8**) and (**9**). Unfortunately, all attempts to separate compounds (**8**) and (**9**) on a multi-milligram scale (by t.l.c. or column chromatography) failed and the mixture could only be separated on a few-milligram scale by preparative h.p.l.c. to yield an epoxide with higher R_f (**8**), and one with lower R_f (**9**). The two 24-epimers (**8**) and (**9**) can be distinguished by their ^{1}H n.m.r. spectra at 200 MHz (see Table 1), which show significant differences in the signals due to 28-CH_2 and to 26- and 27-Me.

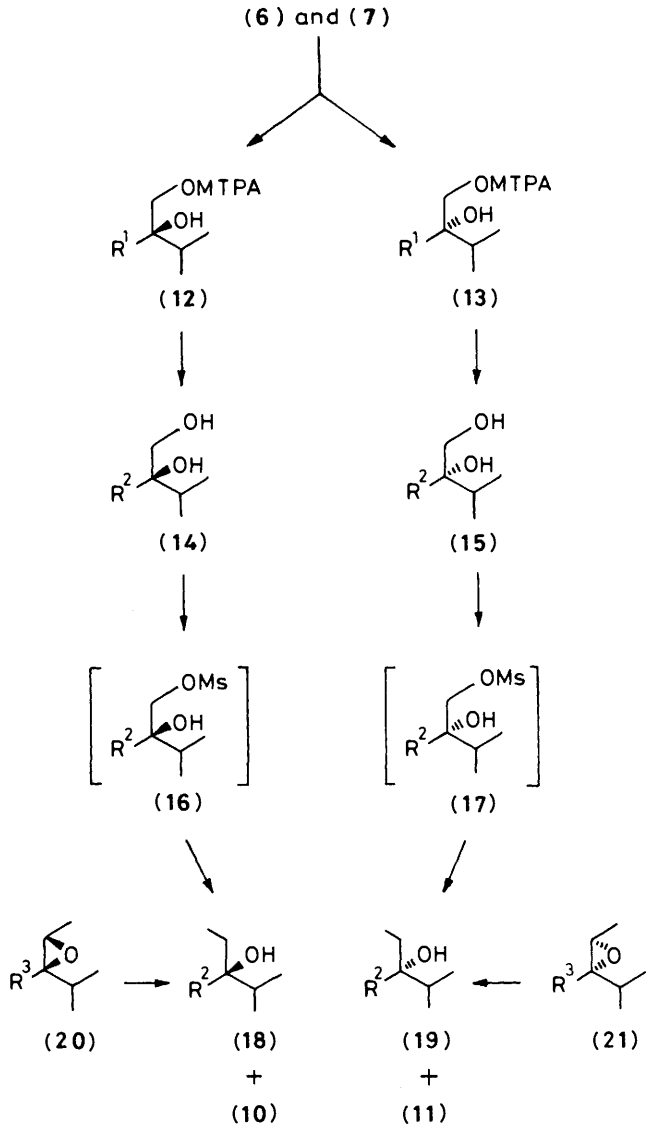
The benzoyl epoxides (**8**) and (**9**) were hydrolysed[2] respectively to the corresponding 3β-ols (**10**) and (**11**), which showed the same significant differences in their ^{1}H n.m.r. spectra (see Table 1). As the amounts of separated 24,28-epoxides (**10**) and (**11**) obtained were too small to be used for the assignment of the configuration at C-24, this was determined as follows (Scheme 3): a mixture of the 24,28-dihydroxy-3-benzoates (**6**) and (**7**) was treated with (*R*)-α-methoxy-α-trifluoromethyl-α-phenylacetyl (MTPA) chloride[6] and the 28-MTPA esters (**12**) and (**13**) obtained were carefully separated by preparative t.l.c. and separately hydrolysed to give the 3,24,28-triols (**14**) and (**15**). The triol (**14**), obtained from the MTPA derivative with lower R_F (**12**), was treated with methanesulphonyl chloride (1 equiv.) and the crude reaction mixture was directly treated with lithium dimethylcuprate, affording a small amount of the 24,28-epoxide (**10**) (200 MHz ^{1}H n.m.r. spectrum in Table 1) together with (24*R*)-stigmast-5-ene-3β,24-diol (**18**); the configuration at C-24 of the latter was deduced from the fact that it is identical with the compound obtained by $LiAlH_4$ reduction of the known[3] (24*R*,28*S*)-24,28-epoxystigmast-5-en-3β-ol (**20**) (200 MHz ^{1}H n.m.r. in Table 1).

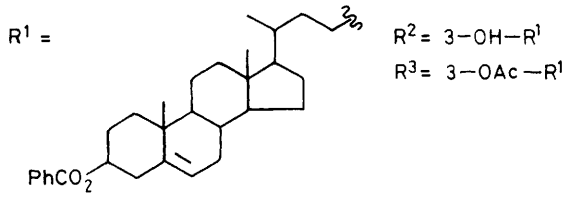
As both the epoxide (**10**) and the diol (**18**) are obtained by stereochemically unambiguous reactions, the epoxide (**10**) could be assigned the (*R*) configuration at C-24. The triol (**15**), derived from the MTPA-derivative with higher R_F (**13**), was treated similarly, allowing us to assign (see Table 1) the (*S*) configuration to C-24 of the epoxide (**11**).

The synthesis outlined in Scheme 2 was repeated starting from 24-methylene[23,23,25-$^3H_3$]cholesteryl benzoate (**5a**) (specific activity: 2.56 × 10^7 d.p.m./mg),[2] to give the [23,23,25-$^3H_3$]-24,28-epoxides (**10a**) and (**11a**). Each of the tritium-labelled epoxides (**10a**) and (**11a**) was mixed with [4-^{14}C]-sitosterol (the radioactivities and the ^{3}H:^{14}C ratios are reported in Table 2) and administered, in separate experiments, to young *Tenebrio molitor* larvae.



Scheme 2.





Scheme 3.

The unsaponifiable material isolated from experiment 1 (Table 2) was separated by SiO_2 column chromatography, yielding a sterol fraction which was benzoylated, purified by preparative t.l.c., and subjected to preparative h.p.l.c. to remove the residual [^{14}C]sitosteryl benzoate from cholesteryl benzoate. The pure labelled cholesteryl benzoate was diluted with cold material, crystallized to constant specific activity, and counted (see Table 2).

Analogously to experiment 1, from the sterol fraction of experiment 2, after benzoylation, preparative t.l.c., and preparative h.p.l.c., pure labelled cholesteryl benzoate was obtained; this was also diluted with cold material, crystallized, and counted (see Table 2).

The data in Table 2 clearly show that only one of the two stereoisomeric 24,28-epoxides, the (24*R*) one, is utilized by the insect. This high degree of stereospecificity is of particular relevance as previous studies[3,8,9] have shown that the dealkylation of several C_{29} phytosterols occurs in the insect *Tenebrio molitor* with low stereospecificity.

Experimental

All m.p.s. are uncorrected. ^{1}H N.m.r. spectra were recorded on a Varian XL-200 or on a Bruker W-P 80 spectrometer using Me_4Si as internal standard. Analytical and preparative t.l.c. were carried out on Merck 60 F_{254} silica gel plates (0.25 mm thickness) and the spots were detected under u.v. light and/or by spraying with 50% aqueous H_2SO_4 and heating at 110 °C for 5 min. Work-up refers to dilution with water, extraction with an organic solvent, washing to neutrality, drying over Na_2SO_4, filtration, and evaporation under reduced pressure. Radioactive samples were counted on a Packard Tri-Carb 3320 liquid-scintillation counter; the samples were dissolved into 10 ml of a solution consisting of 0.65% (w/v) 2,5-diphenyloxazole and 0.013% (w/v) 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene in toluene–dioxane (1:1, v/v). H.p.l.c. was carried out with a Varian 5020 L/C instrument.

Table 1. Significant 200 MHz ^{1}H n.m.r. chemical shifts for compounds (**8**)—(**15**), (**18**), and (**19**) (Me_4Si as internal reference and $CDCl_3$ as solvent unless otherwise stated)

Compound	18-Me	19-Me	21-Me	26,27-Me	28-CH_2	29-Me	25-CH
(**8**)	0.679	1.057	0.915 (d, *J* 7.0)	0.893, 0.952 (d, *J* 7.0) (d, *J* 7.0)	2.517, 2.574 (ABq, *J* 4.5)		
(**9**)	0.679	1.057	0.914 (d, *J* 7.0)	0.914, 0.941 (d, *J* 7.0) (d, *J* 7.0)	2.539, 2.565 (ABq, *J* 4.5)		
(**10**) from (**8**)	0.675	1.006	0.920 (d, *J* 6.4)	0.900, 0.960 (d, *J* 7.0) (d, *J* 6.8)	2.530, 2.586 (ABq, *J* 4.6)		
(**10**) from (**14**)	0.675	1.008	0.918 (d, *J* 6.5)	0.899, 0.958 (d, *J* 7.0) (d, *J* 6.8)	2.533, 2.589 (ABq, *J* 4.6)		
(**11**) from (**9**)	0.676	1.005	0.918 (d, *J* 7.0)	0.918, 0.949 (d, *J* 7.0) (d, *J* 7.0)	2.547, 2.577 (ABq, *J* 4.4)		
(**11**) from (**15**)	0.676	1.008	0.918 (d, *J* 7.0)	0.918, 0.950 (d, *J* 7.0) (d, *J* 7.0)	2.546, 2.577 (ABq, *J* 4.6)		
(**12**)	0.672	1.070	0.917 (d, *J* 7.0)	0.858, 0.917 (d, *J* 7.0) (d, *J* 7.0)	4.205, 4.311 (ABq, *J* 11.2)		
(**13**)	0.674	1.070	0.917 (d, *J* 7.2)	0.876, 0.898 (d, *J* 7.2) (d, *J* 6.8)	4.191, 4.341 (ABq, *J* 11.2)		
(**14**)[a]	0.666	1.065	1.049 (d, *J* 6.6)	1.205, 1.228 (d, *J* 7.0) (d, *J* 6.8)	3.945, 4.039 (ABq, *J* 10.8)		2.250 (sept, *J* 7.0)
(**15**)[a]	0.658	1.066	1.051 (d, *J* 6.1)	1.224, 1.233 (d, *J* 6.9) (d, *J* 7.0)	3.983, 4.045 (ABq, *J* 10.8)		2.281 (sept, *J* 7.0)
(**18**) from (**14**)	0.682	1.007	0.942 (d, *J* 6.4)	0.885, 0.891 (d, *J* 6.8) (d, *J* 6.8)		0.864 (t, *J* 7.6)	1.755 (sept, *J* 6.8)
(**18**) from (**20**)	0.683	1.008	0.943 (d, *J* 6.4)	0.888, 0.891 (d, *J* 6.6) (d, *J* 6.6)		0.865 (t, *J* 7.6)	1.756 (sept, *J* 6.8)
(**19**) from (**15**)	0.682	1.008	0.939 (d, *J* 6.2)	0.888, 0.888 (d, *J* 6.8) (d, *J* 6.8)		0.852 (t, *J* 7.5)	1.733 (sept, *J* 6.8)
(**19**) from (**21**)	0.684	1.010	0.941 (d, *J* 6.3)	0.890, 0.890 (d, *J* 6.6) (d, *J* 6.6)		0.853 (t, *J* 7.6)	1.733 (sept, *J* 6.8)

[a] [$^2H_5$]Pyridine as solvent.

Table 2. Total radioactivities and ^3H:^{14}C ratios of the administered precursors and of the isolated cholesteryl benzoates

	Administered precursors		Recovered cholesteryl benzoate	
Exp.	Compounds	^3H:^{14}C	^{14}C(d.p.m.)	^3H:^{14}C
1	(**10a**) + [4-^{14}C]sitosterol (1.53×10^7 d.p.m. of ^3H) (2.18×10^6 d.p.m. of ^{14}C)	7.02	1.18×10^5	10.36
2	(**11a**) + [4-^{14}C]sitosterol (6.78×10^6 d.p.m. of ^3H) (1.01×10^6 d.p.m. of ^{14}C)	6.71	3.97×10^4	0.09

(24R)- *and* (24S)-3β-*Benzoyloxyergost*-5-*ene*-24,28-*diols* (**6**) *and*(**7**).—24-Methylenecholesteryl benzoate(3β-benzoyloxy-24-methylenecholest-5-ene) (**5**) [4] (300 mg) was dissolved in 1% dry pyridine–benzene (20 ml), and OsO_4 (152 mg, 1 mol equiv.) was added. After 2.5 h, H_2S was bubbled into the solution, the resulting precipitate was filtered off and a crude product was obtained from the filtrate by evaporation under reduced pressure. Pure compounds (**6**) and (**7**) were obtained (280 mg) as a diastereoisomeric mixture by chromatography on SiO_2 (H-60) using hexane–ethyl acetate (75:25) as eluant, δ_H (80 MHz; $CDCl_3$) 0.69 (3 H, s, 18-Me), 0.93 (9 H, d, *J* 7 Hz, 26-, 27-, and 21-Me), 1.07 (3 H, s, 19-Me), 3.42 (1 H, d, *J* 11 Hz, 28-CH_A), 3.67 (1 H, d, *J* 11 Hz, 28-CH_B), 4.83 (1 H, m, 3-CH), 5.40 (1 H, m, 6-CH), and 7.3—8.2 (5 H, $C_6H_5CO_2$).

(24R)- *and*(24S)-3β-*Benzoyloxy*-24,28-*epoxyergost*-5-*enes* (**8**) *and* (**9**).—Freshly distilled methanesulphonyl chloride (0.4 ml) was added to an ice-cooled solution of compounds (**6**) and (**7**) (40 mg) in dry pyridine (2 ml); the solution was kept overnight at 0 °C and worked up to afford the crude mesylation product (42 mg); this was directly dissolved in 50% tetrahydrofuran–methanol (4 ml) and, after addition of anhydrous K_2CO_3 (8 mg), the mixture was stirred overnight. The usual work-up gave a mixture of the crude products (**8**) and (**9**), which were purified by preparative t.l.c. (34 mg) (hexane–ethyl acetate 9:1 as eluant). A batch (12 mg) was separated by h.p.l.c. (Merck Hibar Lichrosorb SI 60, 25 cm × 4 mm column, flow rate 1.8 ml/min, solvent hexane–CH_2Cl_2, 40:60) to yield an epoxide (**8**) (5.5 mg) with higher R_t (24.8 min) corresponding to the (24*R*)-anomer [see below for the assignment of configuration; δ_H (200 MHz) in Table 1], and one with lower R_t (23.4 min), epoxide (**9**) (5 mg), corresponding to the (24*S*)-anomer [see below for the assignment of configuration; δ_H (200 MHz) in Table 1].

(24R)-24,28-*Epoxyergost*-5-*en*-3β-*ol* (**10**).—Compound (**8**) (5.5 mg) was dissolved in 0.25% methanolic KOH (1 ml), and kept overnight at room temperature. The methanol was removed under reduced pressure and the usual work-up afforded the product (**10**) (4 mg); δ_H (200 MHz) in Table 1.

(24S)-24,28-*Epoxyergost*-5-*en*-3β-*ol* (**11**).—Compound (**9**) (5 mg) was treated in the same way as (**8**) and afforded the product (**11**) (4 mg); δ_H (200 MHz) in Table 1.

(24R)- *and* (24S)-3β-*Benzoyloxy*-28-(α-*methoxy*-α-*trifluoromethyl*-α-*phenylacetoxy*)*ergost*-5-*en*-24-*ols* (**12**) *and* (**13**).—To a solution of compounds (**6**) and (**7**) (240 mg) in 50% dry pyridine-CCl_4 (3 ml) distilled (*R*)-α-methoxy-α-trifluoromethyl-α-phenylacetyl (MTPA) chloride (216 mg) was added. After 18 h at room temperature, the usual work-up afforded a crude mixture of diastereoisomers (**12**) and (**13**) (320 mg). The (24*R*)- and (24*S*)-anomers were carefully separated by preparative t.l.c. (hexane–CH_2Cl_2–Et_2O, 10:10:1, four elutions), to yield the lower R_F 28-MTPA ester (**12**) (165 mg), m.p. 153—155 °C (from $CHCl_3$–

CH_3OH); δ_H (200 MHz) in Table 1, and the higher R_F 28-MTPA ester (**13**) (140 mg), m.p. 144—146 °C (from Et_2O–CH_3OH); δ_H (200 MHz) in Table 1.

(24R)-*Ergost-5-ene*-3β,24,28-*triol* (**14**).—Compound (**12**) (70 mg) was dissolved in 5% methanolic KOH (15 ml) and left at room temperature overnight. The usual work-up afforded the product (**14**) (38 mg), m.p. 195—196 °C (from 20% pyridine–benzene); δ_H (200 MHz) in Table 1.

(24S)-*Ergost-5-ene*-3β,24,28-*triol* (**15**).—Compound (**13**) (63 mg), treated as above, yielded the product (**15**) (35 mg), m.p. 197—199 °C (from 20% pyridine–benzene); δ_H (200 MHz) in Table 1.

(24R)-*Stigmast-5-ene*-3β,24-*diol* (**18**).—(*a*) *From the epoxide* (**20**). (24*R*,28*S*)-3β-Acetoxy-24,28-epoxystigmast-5-ene[3] (**20**) (12 mg) was dissolved in dry Et_2O (10 ml), $LiAlH_4$ (20 mg) was added, and the mixture was stirred at room temperature for 18 h. The usual work-up afforded a crude product (11 mg) which was purified by SiO_2 (H-60) chromatography (hexane–ethyl acetate 8:2, v/v, as eluant) to yield pure (24*R*)-stigmast-5-ene-3β,24-diol (**18**) (8 mg), m.p. 164—166 °C (from $CHCl_3$–CH_3OH); δ_H (200 MHz) in Table 1.

(*b*) *From the triol* (**14**). Freshly distilled methanesulphonyl chloride (10 mg) was added at 0 °C to a solution of (24*R*)-ergost-5-ene-3β,24,28-triol (**14**) (37 mg) in dry pyridine (3 ml). After 18 h at room temperature, the usual work-up afforded a crude mesylation mixture (31 mg), containing the 28-monomethanesulphonate (**16**), which was directly subjected to Me_2CuLi reaction.[7] To an ethereal solution of lithium dimethyl cuprate (prepared from 95 mg of purified CuI) at 0 °C was added an ethereal solution of the above methanesulphonate (31 mg in 5 ml) under argon. The mixture was allowed to warm to 20 °C and was stirred for 8 h. Work-up in the usual manner resulted in a crude mixture which was chromatographed on SiO_2 (H-60) (benzene–ethyl acetate 8:2, v/v, as eluant) to yield, respectively, (24*R*)-24,28-epoxyergost-5-en-3β-ol (**10**) (2.5 mg), δ_H (200 MHz) in Table 1; (24*R*)-stigmast-5-ene-3β,24-diol (**18**) (8 mg), δ_H (200 MHz) in Table 1; and unchanged (24*R*)-ergost-5-ene-3β,24,28-triol (**14**) (19 mg), δ_H (200 MHz) in Table 1.

(24S)-*Stigmast-5-ene*-3β,24-*diol* (**19**).—(*a*) *From the epoxide* (**21**). (24*S*,28*R*)-3β-Acetoxy-24,28-epoxystigmast-5-ene[3] (**21**) (12 mg), treated as described for compound (**20**), afforded pure (24*S*)-stigmast-5-ene-3β,24-diol (**19**), m.p. 180—182 °C (from $CHCl_3$–CH_3OH); δ_H (200 MHz) in Table 1.

(*b*) *From the epoxide* (**15**). Starting from compound (**15**) (35 mg), and following the procedure described for (**14**), the following compounds were obtained after mesylation, reaction with Me_2CuLi, and chromatography of the crude reaction mixture: (24*S*)-24,28-epoxyergost-5-en-3β-ol (**11**) (2 mg), δ_H (200 MHz) in Table 1; (24*S*)-stigmast-5-ene-3β,24-diol (**19**) (8 mg), δ_H (200 MHz) in Table 1; and unchanged (24*S*)-ergost-5-ene-3β,24,28-triol (**15**) (10 mg), δ_H (200 MHz) in Table 1.

(24R)- *and* (24S)-24,28-*Epoxy*[23,23,25-$^3H_3$]*ergost-5-en*-3β-*ols* (**10a**) *and* (**11a**).—3β-Benzoyloxy-24-methylene-[23,23,25-$^3H_3$]cholest-5-ene (**5a**) (30 mg, specific activity 2.56 × 10^7 d.p.m./mg), obtained by benzoylation of 24-methylene-[23,23,25-$^3H_3$]cholesterol,[2] gave a diastereoisomeric mixture (12 mg) of compounds (**8a**) and (**9a**) on reaction with OsO_4, mesylation, and treatment with mild base, as described for the cold material. The two tritium labelled 24,28-epoxy-3-benzoates were carefully separated by h.p.l.c. under the conditions described for the unlabelled compounds (**8**) and (**9**), to yield chromatographically pure compounds (**8a**) and (**9a**).

Hydrolysis of (**8a**) afforded pure (24*R*)-24,28-epoxy[23,23,25-$^3H_3$]ergost-5-en-3β-ol (**10a**) (1.53 × 10^7 d.p.m.); hydrolysis of (**9a**) afforded pure (24*S*)-24,28-epoxy[23,23,25-$^3H_3$]ergost-5-en-3β-ol (**11a**) (6.78 × 10^6 d.p.m.).

Administration of Labelled Precursors and Isolation of the Labelled Cholesteryl Benzoate (*Experiments* 1 *and* 2).—Each 24,28-epoxy[23,23,25-$^3H_3$]ergost-5-en-3β-ol, mixed with [4-^{14}C]sitosterol (Radiochemical Centre, Amersham: the radioactivities and the ^3H:^{14}C ratios are reported in Table 2) was deposited onto 450 mg of finely ground oatmeal and fed to 150 young *T.m.* larvae, 1—1.5 cm long (4.5 g) after 2 days of starvation. 2½ Days later the larvae were sacrificed by freezing and the non-saponifiable fraction recovered as previously described.[2] The unsaponifiable material recovered from Experiment 1 (1.28 × 10^6 d.p.m. of ^{14}C; ^3H:^{14}C ratio 6.82) was separated on SiO_2 (H-60) (hexane-AcOEt, 85:15, as eluant) to yield a sterol fraction (1.05 × 10^6 d.p.m. of ^{14}C; ^3H:^{14}C ratio 5.32) which was benzoylated, purified by preparative t.l.c. (hexane–AcOEt 97:3 as eluant), and subjected to preparative h.p.l.c. (Waters μ-Bondapack-C_{18}, 30 cm × 3.9 mm column, flow rate 2 ml/min, solvent MeOH–H_2O, 97:3) which removed the residual ^{14}C-sitosteryl benzoate from the cholesteryl benzoate. The pure labelled cholesteryl benzoate was diluted with cold material, crystallized to constant specific activity and counted (see Table 2). Analogously to Experiment 1, the non-saponifiable material from Experiment 2 (6.82 × 10^5 d.p.m. of ^{14}C; ^3H:^{14}C ratio 8.39) yielded a sterol fraction (3.77 × 10^5 d.p.m. of ^{14}C; ^3H:^{14}C ratio 0.41) from which, after benzoylation, preparative t.l.c., and preparative h.p.l.c., pure labelled cholesteryl benzoate was obtained; this was diluted with cold material, crystallized, and counted (see Table 2).

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