C-25 Prochirality in the Fragmentation Reaction Catalysed by Fucosterol Epoxide Lyase from the Silkworm, *Bombyx mori*

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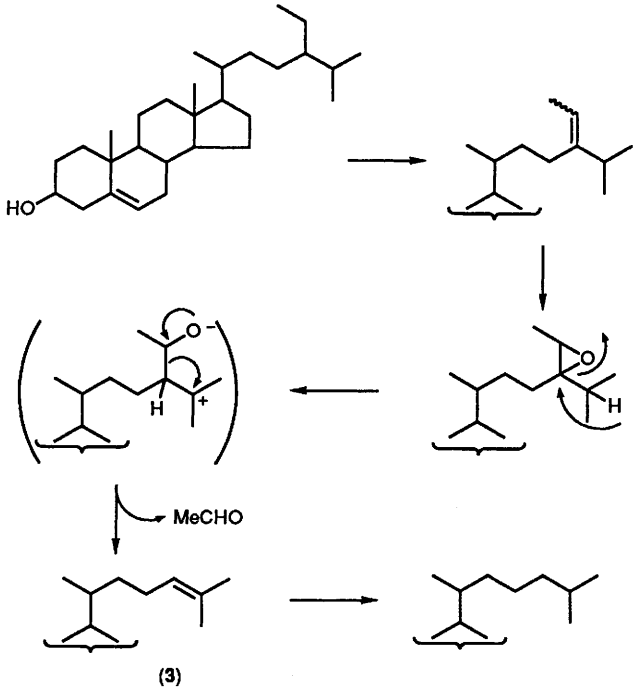
The fate of the diastereotopic methyl groups on the prochiral C-25 centre of fucosterol epoxide and isofucosterol epoxide during enzymatic conversion into desmosterol has been investigated. Incubation of stereospecifically ^{13}C-labelled fucosterol (24*R*,28*R*)-epoxides and isofucosterol (24*R*,28*S*)-epoxides with a cell-free preparation obtained from the guts of larvae of the silkworm, *Bombyx mori*, followed by ^{13}C NMR analysis of the product has shown that the reaction is stereospecific, where the *pro-S* and *pro-R* methyl groups of the epoxides turn stereospecifically into (*Z*)- and (*E*)-methyl groups of desmosterol, respectively.

It is well known that phytophagous insects metabolize plant sterols such as sitosterol to cholesterol by cleaving the C-24–C-28 bond.[1] The carbon–carbon bond-cleavage reaction is essential to insects, since they have no capacity of *de novo* sterol biosynthesis. The pathway of the conversion of sitosterol into cholesterol in insects is depicted in Scheme 1, in which a



(3)

Scheme 1. Dealkylation pathway from sitosterol to cholesterol in phytophagous insects.

fragmentation reaction of an epoxide intermediate with the migration of hydrogen from the C-25 to the C-24 position is characteristic.[2] We previously described the preparation of a cell-free system, active for the conversion of fucosterol epoxide into desmosterol, from the guts of larveae of *Bombyx mori*,[3] and substrate specificity of fucosterol epoxide isomers.[4] We recently found that the cell-free preparation (the term 'fucosterol epoxide lyase' was first introduced by Prestwich *et al.*[5]) is able to convert isofucoserol (24*R*,28*S*)-epoxide (**2**) as well as fucosterol (24*R*,28*R*)-epoxide (**1**) into desmosterol (**3**). By contrast, negligible conversion for (24*S*,28*S*)- and (24*S*,28*R*)-epoxide isomers was observed (unpublished results).† To get further insight into the mechanism of this unique fragmentation reaction, cryptic stereochemistry of the isopropyl group of compounds (**1**) and (**2**) during the conversion into (**3**) was investigated. Some of the results of this work have appeared in preliminary form.[6]

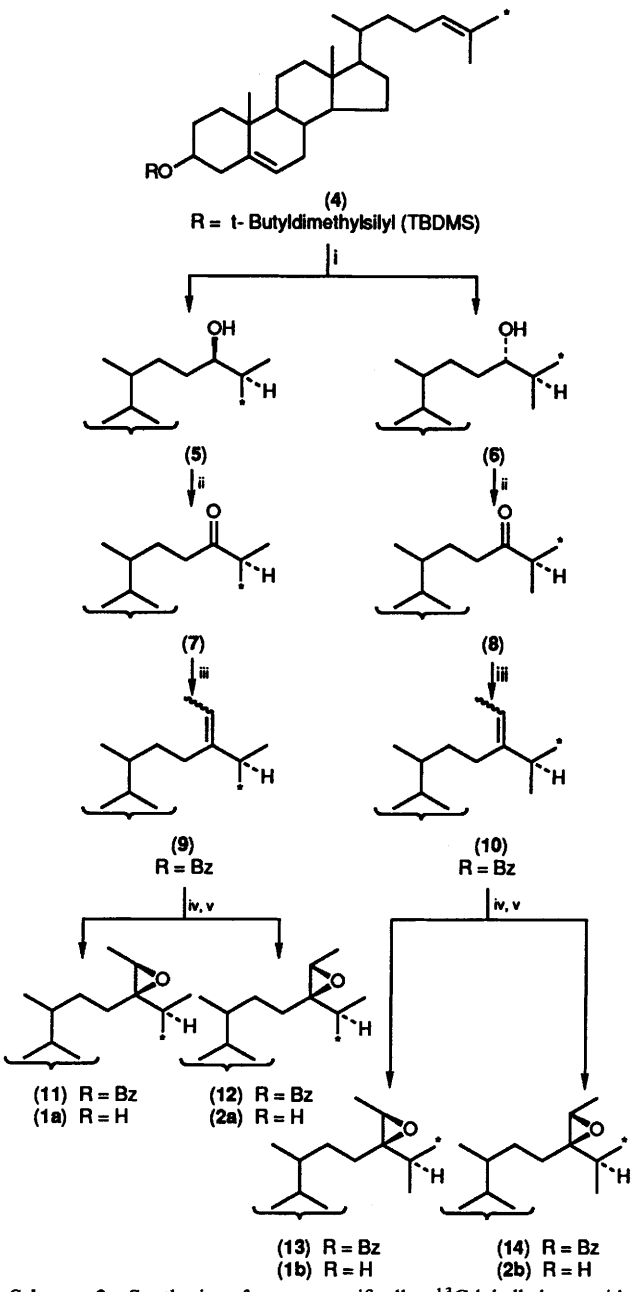
To follow the fate of the diastereotopic methyl (C-26 and C-27) groups advantage was taken of ^{13}C NMR spectroscopy. This technique would allow the determination of the position of the label by simple measurements of the ^{13}C NMR spectra of incubation products. Therefore, our initial studies were focused on the synthesis of stereospecifically ^{13}C-labelled epoxides (**1**) and (**2**) having the predetermined configuration at the C-25 centre. Synthesis of the requisite labelled epoxides, [*pro-S*-Me-^{13}C]-(24*R*,28*R*)- (**1a**), [*pro-R*-Me-^{13}C]-(24*R*,28*R*)- (**1b**), [*pro-S*-Me-^{13}C]-(24*R*,28*S*)- (**2a**), and [*pro-R*-Me-^{13}C]-(24*R*,24*S*)- (**2b**) epoxides, is illustrated in Scheme 2.

The starting material, [*E*-^{13}C]desmosterol t-butyldimethylsilyl (TBDMS) ether (**4**), was prepared from a steroidal C-24 aldehyde and sodium [1-^{13}C]propionate.[7,‡] Hydroboration of alkene (**4**) afforded a 1:1 mixture of chromatographically separable 24-epimeric alcohols, a less polar [*pro-S*-Me-^{13}C]-(24*R*)-alcohol (**5**) (35%) and a more polar [*pro-R*-Me-^{13}C]-(24*S*)-alcohol (**6**) (35%). Their C-24 stereochemistry was predicted by comparison of the ^{13}C chemical shifts of the C-26 and C-27 methyl groups of (**5**) and (**6**) with those of the corresponding 3-benzoates[8] (see the first four rows of the Table) and confirmed by direct TLC comparison of the 3,24-dibenzoate derived from compound (**5**) with authentic specimens[9] of the (24*R*)- and (24*S*)-3,24-dibenzoate. The C-25 stereochemistry of compounds (**5**) and (**6**) was therefore established to be *R*§ (*pro-S*-Me-^{13}C labelled) and *S*§ (*pro-S*-Me-^{13}C labelled), respectively, on the basis of the well known *cis*-addition mechanism of hydroboration. Pyridinium chlorochromate (PCC) oxidation of compound

† In contrast to our previous observation, conversion of the (24*S*,28*S*)-isomer into desmosterol was not detected when desmosterol was analysed for by GLC. We are grateful to Professor G. D. Prestwich, State University of New York at Stony Brook, for suggesting this possibility.

‡ Since the (*E*)- and (*Z*)-methyl signals of non-labelled desmosterol were observed in the ratio 1.8:1 under our NMR conditions, the location of the ^{13}C label of compound (**4**) in ref. 7 should be read as 85% for (*E*) and 15% for (*Z*).

§ A sequence rule that the ^{13}C atom is superior to the ^{12}C atom is applied.



Scheme 2. Synthesis of stereospecifically ^{13}C-labelled epoxides. *Reagents and conditions:* i, BH_3–THF, then H_2O_2–NaOH, silica gel Lobar column separation; ii, PCC; iii, (i) CH_2=CHMgBr; (ii) Bu_4NF; (iii) Ac_2O–pyridine; iv, (i) Ph_3P–I_2; (ii) $LiAlH_4$; (iii) BzCl–pyridine; (iv) MCPBA, then HPLC separation; v, KOH–MeOH.

(**5**) gave [*pro-S*-Me-^{13}C]-24-oxocholesterol derivative (**7**) (92%). Similarly, the isomer (**6**) afforded [*pro-R*-Me-^{13}C]-compound (**8**). The *pro-S* (δ_C 18.30) and *pro-R* (δ_C 18.37) methyl carbon signals of ketone (**7**) were observed in an 85:15 ratio, while those of the isomeric ketone (**8**) were found in the opposite ratio. The ratios are consistent with those of alcohols (**5**) and (**6**) as well as that of alkene (**4**), thus indicating that no epimerization took place at the C-25 chiral centre during the oxidation.

The ketones (**7**) and (**8**) were converted into stereospecifically ^{13}C-labelled 24(28)-olefin by the published method[10] with a slight modification. Thus, a series of reactions of compound (**7**), *i.e.* vinyl Grignard addition, desilylation, acetylation, triphenylphosphine–iodine treatment, $LiAlH_4$ reduction, and benzoylation, afforded [*pro-S*-Me-^{13}C]-24(28)-olefinic compound (**9**) (60%). The olefin was found to be a *ca.* 4:1 mixture of the 24(28)*E*- and 24(28)*Z*-isomer (fucosterol and isofucosterol benzoates, respectively) by ^{13}C NMR as well as ^1H NMR spectroscopy, in which 25-H for the *Z*-isomer was characteristically observed at δ_H 2.8.[11] Controlled epoxidation of alkene (**9**) with 3-chloroperoxybenzoic acid (MCPBA) (1.2 mol equiv.) gave a diastereoisomeric mixture of four 24,28-epoxides (74%). HPLC (normal phase) separation of the mixture obtained from compound (**9**) is shown in Figure 1. Among them, [*pro-S*-Me-^{13}C]-(24*R*,28*R*)-epoxide benzoate (**11**) (26%) and [*pro-S*-Me-^{13}C]-(24*R*,28*S*)-epoxide benzoate (**12**) (6%) were separated.

The [*pro-R*-Me-^{13}C]-ketone (**8**) was similarly converted *via* olefin (**10**) into [*pro-R*-Me-^{13}C]-(24*R*,28*R*)-epoxide benzoate (**13**) and [*pro-R*-Me-^{13}C]-(24*R*,28*S*)-epoxide benzoate (**14**). At this stage location of the ^{13}C label was checked by ^{13}C NMR spectroscopy. As can be seen in Figure 2, the label was found to reside 85% at *pro-S*-methyl and 15% at *pro-R*-methyl compounds (**11**) and (**12**), and *vice versa* in compounds (**13**) and

Table. ^{13}C Chemical shifts (125 MHz; $CDCl_3$) of the diastereotopic *pro-S* and *pro-R* methyl groups on the C-25 prochiral centre.

Compound	*pro-S*-Methyl	*pro-R*-Methyl
(24*R*)-24-Hydroxycholesterol (3-OTBDMS)	17.29	18.96
(3-OBz)[a]	17.2	18.8[b]
(24*S*)-24-Hydroxycholesterol (3-OTBDMS)	19.12	16.75
(3-OBz)[a]	19.0[b]	16.7
24-Oxocholesterol (3-OTBDMS)	18.30	18.37
Fucosterol (3-OBz)	22.17	22.27
Isofucosterol (3-OBz)	21.05	22.14
(24*R*,28*R*)-Epoxide (3-OBz)	17.78	18.44
(24*S*,28*S*)-Epoxide (3-OBz)	18.18	18.01
(24*S*,28*R*)-Epoxide (3-OBz)	17.72	18.87
(24*R*,28*S*)-Epoxide (3-OBz)	18.91	17.80

[a] In ref. 9. [b] The chemical shifts for C-21 and C-27 in ref. 9 should be reversed.

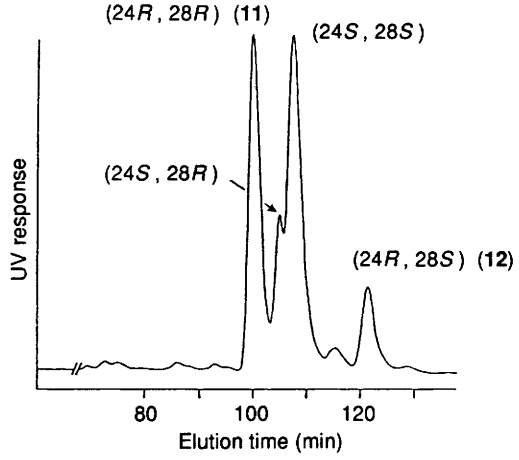


Figure 1. HPLC separation of the diastereoisomeric mixture of epoxide benzoates. Conditions: column, a Shim-pack CLC SIL (15 × 0.6 cm i.d.); eluting solvent hexane–dichloromethane (2:1); flow rate 2 ml min^{-1}; detected with a UV monitor at 230 nm. It was previously known that the (24*R*,28*R*)-epoxide is more mobile than the (24*S*,28*S*)-isomer (Y. Fujimoto and N. Ikekawa, *J. Org. Chem.*, 1980, **45**, 566; N. Ikekawa, Y. Fujimoto, A. Takasu, and M. Morisaki, *J. Chem. Soc., Chem. Commun.*, 1980, 709). The elution order of isofucosterol (24*R*,28*S*)- and (24*R*,28*S*)-epoxide benzoates was determined in the present work by conversion of the stereochemically defined isomeric acetates (ref. 14) into the corresponding benzoates.

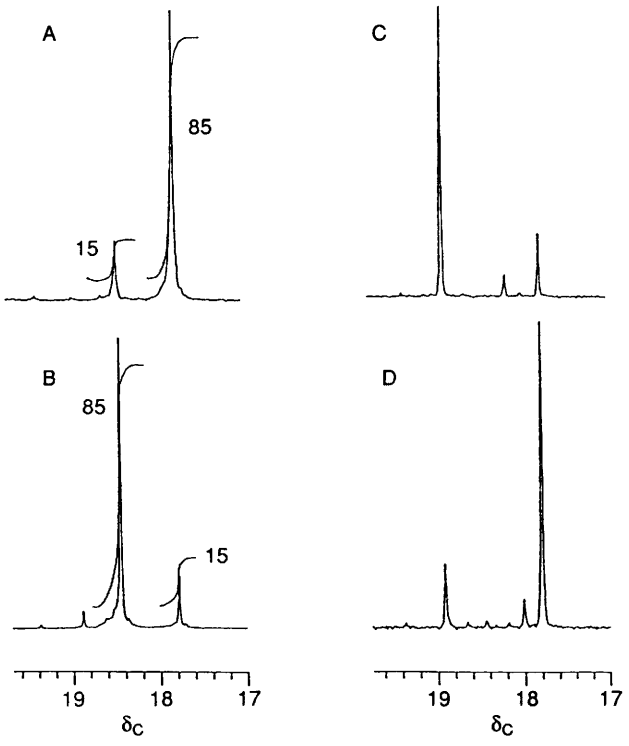


Figure 2. ^{13}C NMR spectra (125 MHz; $CDCl_3$) of the stereospecifically ^{13}C-labelled epoxide benzoates. A: [*pro-S*-Me-^{13}C]-(24*R*,28*R*)-epoxide benzoate (**11**), B: [*pro-R*-Me-^{13}C]-(24*R*,28*R*)-epoxide benzoate (**13**), C: [*pro-S*-Me-^{13}C]-(24*R*,28*S*)-epoxide benzoate (**12**), D: [*pro-R*-Me-^{13}C]-(24*R*,28*S*)-epoxide benzoate (**14**). Small peaks at δ_C 18.91 in B, 18.18 in C, and 18.01 in D are due to *pro-S*-Me of (24*R*,28*S*)-, *pro-S*-Me of (24*S*,28*S*)-, and *pro-R*-Me of (24*S*,28*R*)-epoxide, respectively (see the Table).

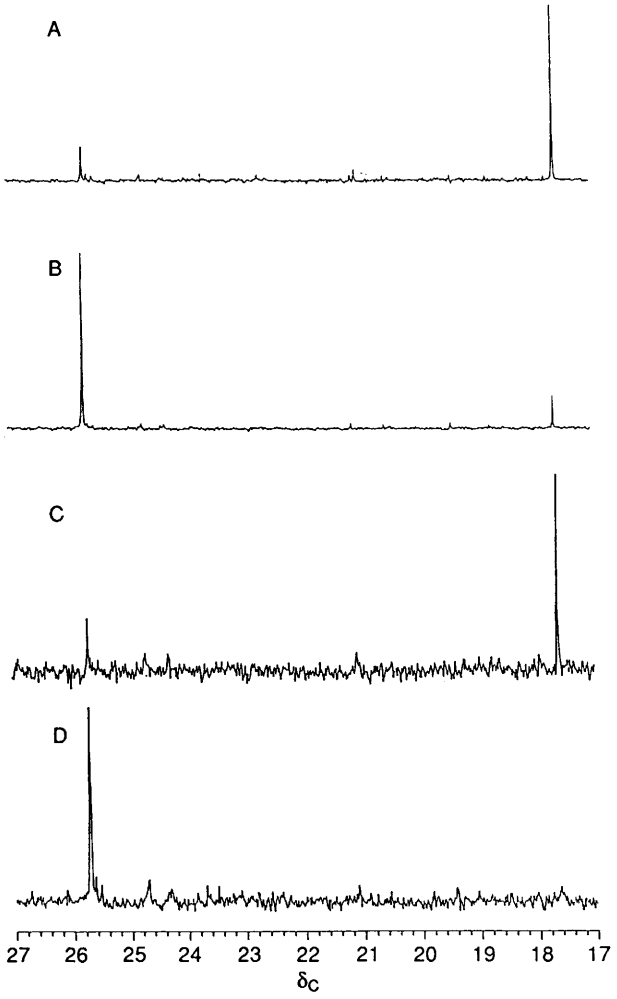


Figure 3. ^{13}C NMR spectra (125 MHz; $CDCl_3$) of the sterol fraction. A: from [*pro-S*-methyl-^{13}C]-(24*R*,28*R*)-epoxide (**1a**), B: from [*pro-R*-methyl-^{13}C]-(24*R*,28*R*)-epoxide (**1b**), C: from [*pro-S*-methyl-^{13}C]-(24*R*,28*S*)-epoxide (**2a**), D: from [*pro-R*-methyl-^{13}C]-(24*R*,28*S*)-epoxide (**2b**). Chemical shifts of the (*Z*)- and (*E*)-methyl of desmosterol are at δ_C 17.6 and 25.7, respectively. For comparison of the peak-intensity ratio of (*Z*)- and (*E*)-methyl signals, footnote † might be taken into account.

(**14**). The chemical shifts of the diastereotopic methyl groups of the epoxide isomers, along with synthetic intermediates in the present work, are listed in the Table. Hydrolysis of each epoxide benzoate with methanolic KOH furnished the target epoxides, (**1a**), (**1b**), (**2a**), and (**2b**).

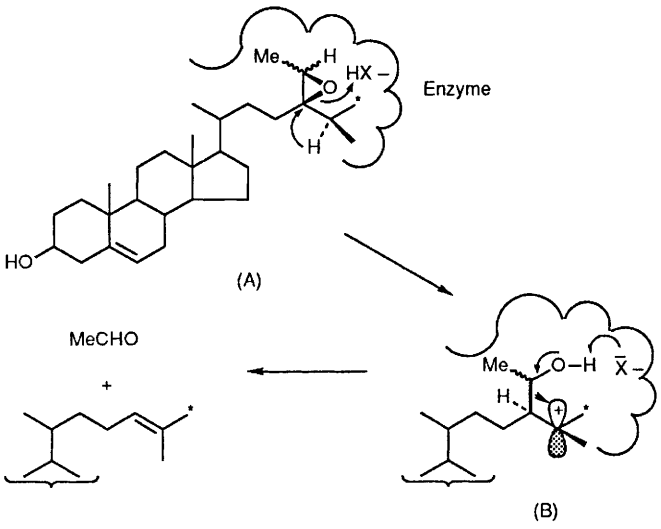
The cell-free system (1 500g supernatant fraction) was prepared from the guts of the fifth instar larvae of *B. mori* as reported previously.[12] The four ^{13}C-labelled epoxides were individually incubated at 30 °C for 3 h. The ^{13}C NMR spectrum[6] of the ethyl acetate extract of the incubation mixture indicated that almost half of the substrate was converted into compound (**3**) as a single product in each case. Gas chromatography–mass spectrometery (GC–MS) analysis of the desmosterol product in the form of its trimethylsilyl (TMS) ether showed that desmosterol TMS ether has a molecular-ion peak at *m/z* 457 (non-labelled sample should be *m/z* 456) and that the isolated yields of compound (**3**) were *ca.* 20% based on the administered epoxide.

The ethyl acetate extract was separated by preparative TLC (PLC) to give a sterol fraction which contained endogenous cholesterol and sitosterol, and the labelled desmosterol as a major component. The fraction was further analysed by ^{13}C NMR spectroscopy as shown in Figure 3. Since the isopropylidene (*E*)- and (*Z*)-methyl resonances of compound (**3**) are known to be at δ_C 25.7 and 17.6, respectively,[7] it is clear from Figures 2 and 3 that the enzyme reaction proceeded in a highly stereospecific manner with respect to the C-25 prochiral centre and that the *pro-S*-methyl group of compounds (**1**) and (**2**) becomes the isopropylidene (*Z*)-methyl of compound (**3**), whereas the *pro-R*-methyl becomes the (*E*)-methyl.

It should be noted that the orientation of the C-29 methyl group does not alter the fate of the diastereotopic methyl groups. Nicotra *et al.* reported that the (24*R*)-epimer of 24-methylenecholesterol (24,28)-epoxide is metabolised to cholesterol much more easily than is the (24*S*)-epimer in an *in vivo* study with *Tenebrio molitor*.[13] Prestwich *et al.* reported that a cell-free preparation from *Manduca sexta* converts fucosterol (24*R*,28*R*)-epoxide, but not (24*S*,28*S*)-isomer, into desmosterol.[5] This evidence suggests the occurrence of an enzyme specific to (24*R*)-epoxides among phytophagous insect species. Strangely, *in vivo* studies with *B. mori*[4] and *T. molitor*,[14] lack of stereoselectivity was found in the conversion of fucosterol and/or isofucosterol (24,28)-epoxide isomers into cholesterol.

A most likely implication of the present results is depicted in Scheme 3. When the epoxide interacts with the lyase enzyme, the isopropyl group seems to be oriented in such a conformation (A) that the C-25 hydrogen is *anti*-periplanar to the C-24–oxygen bond, which can facilitate the migration of the C-25 hydrogen to the C-24 position with an S_N2-type inversion at the C-24 centre. The resulting C-25 electron-deficient species (B) may eliminate acetaldehyde to form the 24(25)-double bond without rotation of the C-24–C-25 single bond.

Boron trifluoride–diethyl ether-catalysed rearrangement of



Scheme 3. Proposed mechanism for the enzymatic conversion of the epoxide into desmosterol. XH implies an acidic residues of an amino acid of the enzyme.

fucosterol epoxides into desmosterol has also been reported.[15] The desmosterol benzoate obtained by treatment of the ^{13}C-labelled epoxide benzoates (**11**) and (**13**) with boron trifluoride–diethyl ether in benzene was shown to have the ^{13}C label distributed essentially equally between the (*E*)- and (*Z*)-methyl groups.[16] The results are in marked contrast with those of the enzymatic reaction.

Experimental

M.p.s were determined on a hot-stage microscope and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on a JEOL FX-200 (200 MHz for ^1H and 50 MHz for ^{13}C) or JEOL GSX-500 (500 MHz for ^1H and 125 MHz for ^{13}C) spectrometer for solutions in $CDCl_3$. ^1H Chemical shifts are reported relative to tetramethylsilane as internal standard, while ^{13}C chemical shifts are expressed relative to $CDCl_3$ (δ_C 77.0). GC–MS analysis was done on a Shimadzu GC-MS DF 9020 spectrometer equipped with a OV-1 capillary column (Shimadzu CBP-1, 15 m). Typical retention times for the trimethylsilyl derivatives of cholesterol, desmosterol, and sitosterol were 6.0, 6.5, and 8.4 min, respectively (oven temperature was increased at the rate of 2 °C min^{-1} from 265 °C). Column chromatography was carried out on Kieselgel 60 (Merck, 70–230 mesh). PLC was performed on pre-coated Kieselgel 60 F_{254} glass plates (Merck, 0.25 mm). Parallel experiments were carried out with non-labelled materials and analytical samples were prepared from non-labelled samples in most cases in order to economize on the use of labelled materials. THF refers to tetrahydrofuran.

Hydroboration of [E-^{13}C]*Desmosterol TBDMS Ether* (**4**).—BH_3–THF (1M-solution in THF; 0.44 ml) was added to a stirred solution of the silyl ether (**4**) (200 mg) in dry THF (2.0 ml). After 1 h at 0 °C, the mixture was treated with water (0.14 ml), 3M-NaOH (0.20 ml), and H_2O_2 (30%; 0.20 ml) and then was stirred at room temperature for 5 h. Extractive (Et_2O) work-up gave a crude product, which was chromatographed on a Lobar column (Merck, LiChroprep Si60) with benzene–hexane (2:1) as eluant to give the *more mobile* (24R)-*alcohol* (**5**) (72 mg, 35%) and the *less mobile* (24S)-*alcohol* (**6**) (72 mg, 35%). For the isomer (**5**): m.p. 141–143 °C (from MeOH); δ_H 0.06 (6 H, s, $SiMe_2$), 0.68 (3 H, s, 18-H_3), 0.89 (9 H, s, Bu^t), 0.91 (*ca.* 2.5 H, dd, J_{HH} 6.8, $^1J_{CH}$ 125 Hz, *pro-S*-Me), 1.00 (3 H, s, 19-H_3), 3.30 (1 H, m, 24-H), 3.48 (1 H, m, 3-H), and 5.32 (1 H, m, 6-H); δ_C 17.29 and 18.96 (*ca.* 6:1 ratio). For the *non-labelled sample*: m.p. 138–142 °C (from MeOH) (Found: C, 76.5; H, 11.7. $C_{33}H_{60}O_2Si$ requires C, 76.68; H, 11.70%).

For the isomer (**6**): m.p. 149.5–151 °C (from MeOH); δ_H 0.06 (6 H, s, $SiMe_2$), 0.68 (3 H, s, 18-H_3), 0.89 (9 H, s, Bu^t), 0.89 (*ca.* 2.6 H, dd, J_{HH} 6.8, $^1J_{CH}$ 125 Hz, *pro-R*-Me), 1.00 (3 H, s, 19-H_3), 3.30 (1 H, m, 24-H), 3.48 (1 H, m, 3-H), and 5.32 (1 H, m, 6-H); δ_C 16.75 and 19.12 (*ca.* 6:1 ratio). For the *non-labelled sample*: m.p. 154–155 °C (from MeOH) (Found: C, 76.6; H, 11.7%).

Stereochemical Assignment of Alcohols (**5**) *and* (**6**).—(i) For comparison purposes the ^{13}C chemical shifts of the diastereotopic methyl groups, reported in ref. 9, are included in the Table.

(ii) A solution of the non-labelled, less polar isomer (**5**) (3 mg) and Bu_4NF (1M solution in THF; 48 μl) in THF (0.3 ml) was stirred at room temperature overnight. Extractive (Et_2O) work-up gave a crude product, which was chromatographed on silica gel. Elution with hexane–EtOAc (10:1) afforded the 3,24-diol (2 mg) as a solid, which was treated with benzoyl chloride (5 μl) and 4-(dimethylamino)pyridine (DMAP) (catalytic amount) in pyridine (0.2 ml) at room temperature overnight. Usual work-up gave a crude product, which was chromatographed on silica gel. Elution with hexane–Et_2O (15:1) afforded the 3,24-dibenzoate as a solid (2 mg). The dibenzoate was identical with the authentic (24*R*)-isomer,[9] but not the (24*S*)-isomer, by TLC [developed three times with hexane–benzene (1:1) as a developing solvent, R_F 0.35 for (24*R*)-dibenzoate, 0.31 for (24*S*)-dibenzoate].

[pro-S-Me-^{13}C]-24-*Oxocholesterol* 3-*TBDMS Ether* (**7**).—A solution of the alcohol (**5**) (58 mg), NaOAc (3.6 mg), and PCC (50 mg) in dry CH_2Cl_2 (2.5 ml) in the presence of molecular sieves 3 Å (3 g) was stirred for 3 h. Dilution with dry diethyl ether and filtration through a column of Florisil afforded a crude product, which was chromatographed on silica gel. Elution with hexane–EtOAc (10:1) gave the ketone (**7**) (53 mg, 92%), m.p. 142.5–144 °C (from MeOH); δ_H 0.06 (6 H, s, $SiMe_2$), 0.67 (3 H, s, 18-H_3), 0.89 (9 H, s, Bu^t), 1.00 (3 H, s, 19-H_3), 1.09 (*ca.* 2.5 H, dd, J_{HH} 6.8, $^1J_{CH}$ 128 Hz, *pro-S*-Me), 1.09 (*ca.* 2.5 H, dd, J_{HH} 6.8, $^3J_{CH}$ 5.4 Hz, *pro-R*-Me), 3.48 (1 H, m, 3-H), and 5.31 (1 H, m, 6-H); δ_C 18.30 and 18.37 (6:1 ratio). For the *non-labelled sample*: m.p. 146–147 °C (from MeOH); δ_H 1.09 (6 H, d, *J* 6.8 Hz, 26- and 27-H_3) (Found: C, 76.9; H, 11.4. $C_{33}H_{58}O_2Si$ requires C, 77.04; H, 11.28%).

[pro-R-Me-^{13}C]-24-*Oxocholesterol* 3-*TBDMS Ether* (**8**).—The alcohol (**6**) (58 mg) was oxidized as described above to give the ketone (**8**) (57 mg, 92%), m.p. 142.5–144 °C (from MeOH); δ_H 0.06 (6 H, s, $SiMe_2$), 0.67 (3 H, s, 18-H_3), 0.89 (9 H, s, Bu^t), 1.00 (3 H, s, 19-H_3), 1.09 (*ca.* 2.5 H, dd, J_{HH} 6.8, $^1J_{CH}$ 127 Hz, *pro-R*-Me), 1.09 (*ca.* 2.5 H, dd, J_{HH} 6.8, $^3J_{CH}$ 5.4 Hz, *pro-S*-Me), 3.48 (1 H, m, 3-H), and 5.31 (1 H, m, 6-H); δ_C 18.30 and 18.37 (1:5 ratio).

[pro-2-Me-^{13}C]-24(28)-*Olefin Benzoate* (**9**).—A solution of the ketone (**7**) (53 mg) in dry THF (1.0 ml) was added dropwise to vinylmagnesium bromide (1M solution in THF; 0.21 ml) at room temperature. The mixture was heated at 50 °C for 2.5 h. Extractive (Et_2O) work-up gave a crude product, which was chromatographed on silica gel. Elution with hexane–EtOAc (15:1) afforded the allyl alcohol (52 mg, 93%); δ_H 0.06 (6 H, s, $SiMe_2$), 0.67 (3 H, s, 18-H_3), 0.89 (9 H, s, Bu^t), 0.85 and 0.91 (*ca.* 2.5 H, dd, J_{HH} 6.8, $^1J_{CH}$ 125 Hz, *pro-S*-Me of C-24 epimers), 1.00 (3 H, s, 19-H_3), 3.46 (1 H, m, 3-H), 5.1–5.25 (2 H, m, 29-H_2), 5.32 (1 H, m, 6-H), 5.80 (0.5 H, dd, *J* 17.3 and 10.7 Hz, 28-H), and 5.81 (0.5 H, dd, *J* 17.6 and 10.7 Hz, 28-H); δ_C 16.51 and 17.59 (1:1

ratio). For the *non-labelled sample*: m.p. 124.5–131.5 °C (from MeOH) (Found: C, 77.3; H, 11.4. $C_{35}H_{62}O_2Si$ requires C, 77.49; H, 11.44%).

A solution of the allyl alcohol (90 mg) and Bu_4NF (1M solution in THF; 0.38 ml) in THF (1.0 ml) was stirred at room temperature overnight. Extractive (Et_2O) work-up gave a crude product, which was chromatographed on silica gel. Elution with hexane–EtOAc (1:1) afforded the 3,24-diol (70 mg, 98%); δ_H 0.67 (3 H, s, 18-H_3), 0.85 and 0.91 (*ca.* 2.5 H, dd, J_{HH} 6.8, $^1J_{CH}$ 125 Hz, *pro-S*-Me of C-24 epimers), 0.92 (3 H, d, *J* 6.8 Hz, 19-H_3), 1.00 (3 H, s, 21-H_3), 3.50 (1 H, m, 3-H), 5.1–5.25 (2 H, m, 29-H_2), 5.35 (1 H, m, 6-H), 5.80 (1 H, dd, *J* 17.3 and 10.7 Hz, 28-H), and 5.81 (1 H, dd, *J* 17.6 and 10.7 Hz, 28-H); δ_C 16.50 and 17.58 (1:1 ratio). For *non-labelled sample*: m.p. 158.5–160.5 °C (from acetone) (Found: C, 81.0; H, 11.4. $C_{29}H_{48}O_2$ requires C, 81.31; H, 11.21%).

A mixture of the diol (68 mg), acetic acid anhydride (26 µl), DMAP (catalytic), dry pyridine (0.5 ml) was stirred at room temperature for 4 h. Extractive (Et_2O) work-up gave a crude product, which was chromatographed on silica gel. Elution with hexane–EtOAc (10:1) afforded the 3-acetate (74 mg, 99%); δ_C 0.67 (3 H, s, 18-H_3), 0.85 and 0.91 (*ca.* 2.5 H, dd, J_{HH} 6.8, $^1J_{CH}$ 125 Hz, *pro-S*-Me of C-24 epimers), 0.92 (3 H, d, *J* 6.4 Hz, 21-H_3), 1.02 (3 H, s, 19-H_3), 2.03 (3 H, s, Ac), 4.60 (1 H, m, 3-H), 5.1–5.25 (2 H, m, 29-H_2), 5.37 (1 H, m, 6-H), 5.80 (0.5 H, dd, *J* 17.3 and 10.7 Hz, 28-H), and 5.81 (0.5 H, dd, *J* 17.6 and 10.7 Hz, 28-H); δ_C 16.48 and 17.56 (1:1 ratio). For the *non-labelled sample*: m.p. 148.5–157 °C (from acetone) (Found: C, 79.4; H, 10.8. $C_{31}H_{50}O_3$ requires C, 79.15; H, 10.64%).

A solution of I_2 (202 mg) in dry benzene (6.0 ml) was added to a stirred solution of PPh_3 (207 mg) in benzene (3.0 ml) at room temperature. After 20 min, a solution of the 3-acetate (72 mg) in dry benzene (3.0 ml) was added and the mixture was stirred for 4.5 h. Extractive (Et_2O) work-up (washed with aq. $Na_2S_2O_3$ to remove residual iodine) gave a crude product, which was chromatographed on silica gel. Elution with hexane–EtOAc (10:1) afforded the allyl iodide (86 mg).

A mixture of the iodide and $LiAlH_4$ (75 mg) in dry THF (3.0 ml) was heated at reflux for 2 h. Extractive (Et_2O) work-up gave a crude product, which was chromatographed on silica gel. Elution with hexane–EtOAc (6:1) afforded the olefin (40 mg, 63%); δ_H 0.69 (3 H, s, 18-H_3), 0.98 (*ca.* 2.5 H, dd, J_{HH} 7.0, $^1J_{CH}$ 125 Hz, *pro-S*-Me), 0.9–1.01 (*ca.* 6 H, m, 21-H_3 and *pro-R*-Me), 1.01 (3 H, s, 19-H_3), 1.57 (3 H, d, *J* 6.6 Hz, 29-H_3), 2.81 (0.19 H, m, 25-H of the *Z*-isomer), 3.52 (1 H, m, 3-H), 5.18 (1 H, q, *J* 6.8 Hz, 28-H), and 5.35 (1 H, m, 6-H); δ_C 21.05, 22.17, and 22.27 (*ca.* 1:6:1 proportions). For the *non-labelled sample* (Found: C, 84.4; H, 11.7. $C_{29}H_{48}O$ requires C, 84.40; H, 11.72%).

A mixture of the olefin (40 mg), benzoyl chloride (30 µl), and DMAP (catalytic) in dry pyridine (0.5 ml) was stirred at room temperature for 5 h. Extractive (Et_2O) work-up gave a crude product, which was chromatographed on silica gel. Elution with hexane–Et_2O (15:1) afforded the olefin benzoate (**9**) (50 mg, 100%) as a solid; δ_H 0.70 (3 H, s, 18-H_3), 0.98 (*ca.* 2.5 H, dd, J_{HH} 7.0, $^1J_{CH}$ 125 Hz, *pro-S*-Me), 0.94–1.03 (*ca.* 6 H, m, 21-H_3 and *pro-R*-Me), 1.07 (3 H, s, 19-H_3), 1.58 (3 H, d, *J* 6.6 Hz, 29-H_3), 2.82 (0.19 H, m, 25-H of the *Z*-isomer), 4.86 (1 H, m, 3-H), 5.18 (1 H, q, *J* 6.8 Hz, 28-H), 5.43 (1 H, m, 6-H), 7.4–8.1 (5 H, m, Ph); δ_C 21.05, 22.16, and 22.28 (*ca.* 1:6:1 proportions). For the *non-labelled sample* (Found: C, 83.5; H, 10.3. $C_{36}H_{52}O_2$ requires C, 83.67; H, 10.14%).

[pro-R-Me-^{13}C]-24(28)-*Olefin Benzoate* (**10**).—The ketone (**8**) (53 mg) was converted into the olefin (**10**) (61 mg) as described above. For the allyl alcohol: δ_H 0.87 (*ca.* 1.3 H, dd, J_{HH} 6.6, $^1J_{CH}$ 125 Hz, *pro-R*-Me of a C-24 epimer) and 0.90 (*ca.* 1.3 H, dd, J_{HH} 5.6, $^1J_{CH}$ 126 Hz, *pro-R*-Me of the other C-24 epimer); δ_C 16.51 and 17.61 (1:1 ratio). For the olefin: δ_H 0.69 (3 H, s, 18-H_3), 0.96 (*ca.* 2.5 H, dd, J_{HH} 7.1, $^1J_{CH}$ 126 Hz, *pro-R*-Me), 0.93–1.04 (*ca.* 6 H, m, 21-H_3 and *pro-S*-Me), 1.01 (3 H, s, 19-H_3), 1.57 (3 H, d, *J* 6.6 Hz, 29-H_3), 2.82 (0.19 H, m, 25-H of the *Z*-isomer), 3.52 (1 H, m, 3-H), 5.18 (1 H, q, *J* 6.8 Hz, 28-H), and 5.35 (1 H, m, 6-H); δ_C 21.14, 22.17, and 22.27 (*ca.* 1:1:6 ratio). For the olefin benzoate (**10**): δ_H 0.70 (3 H, s, 18-H_3), 0.98 (*ca.* 2.5 H, dd, J_{HH} 7.1, $^1J_{CH}$ 126 Hz, *pro-R*-Me), 0.96–1.02 (*ca.* 6 H, m, 21-H_3 and *pro-S*-Me), 1.07 (3 H, s, 19-H_3), 1.58 (3 H, d, *J* 6.6 Hz, 29-H_3), 2.81 (0.19 H, m, 25-H of the *Z*-isomer), 4.86 (1 H, m, 3-H), 5.18 (1 H, q, *J* 6.7 Hz, 28-H), and 5.35 (1 H, m, 6-H); δ_C 21.14, 22.17, and 22.27 (*ca.* 1:1:6 ratio).

[pro-S-Me-^{13}C]-(24R,28R)- *and* (24R,28S)-*Epoxide Benzoates* (**11**) *and* (**12**).—MCPBA (20 mg) was added in several portions to a stirred solution of the olefin benzoate (**9**) (51 mg) in $CHCl_3$ (2.0 ml) cooled in an ice–salt-bath. Extractive ($CHCl_3$) work-up gave a crude product, which was chromatographed on silica gel. Elution with hexane–Et_2O (10:1) afforded a diastereoisomeric mixture of (24,28)-epoxides (39 mg, 74%). The epoxides were separated by HPLC (for conditions, see the caption to Figure 1) to give the (24*R*,28*R*)-epoxide benzoate (**11**) (98% purity; 10 mg, 26%) and (24*R*,28*S*)-epoxide benzoate (**12**) (98% purity; 2.3 mg, 6%).

For the isomer (**11**): δ_H 0.70 (3 H, s, 18-H_3), 0.89 (*ca.* 2.5 H, dd, J_{HH} 6.9, $^1J_{CH}$ 126 Hz, *pro-S*-Me), 0.90–0.96 (*ca.* 6 H, m, 21-H_3 and *pro-R*-Me), 1.07 (3 H, s, 19-H_3), 1.27 (3 H, d, *J* 5.6 Hz, 29-H_3), 2.91 (1 H, q, *J* 5.7 Hz, 28-H), 4.87 (1 H, m, 3-H), 5.42 (1 H, m, 6-H), and 7.4–8.1 (5 H, m, Ph); δ_C 17.78 and 18.44 (6:1 ratio).

For the isomer (**12**): δ_H 0.69 (3 H, s, 18-H_3), 0.92 (3 H, d, *J* 6.3 Hz, 21-H_3), 0.96 (*ca.* 2.5 H, dd, J_{HH} 7.4, $^1J_{CH}$ 125 Hz, *pro-S*-Me), 1.06 (*ca.* 2.5 H, m, *pro-R*-Me), 1.07 (3 H, s, 19-H_3), 1.30 (3 H, d, *J* 5.6 Hz, 29-H_3), 2.95 (1 H, q, *J* 5.6 Hz, 28-H), 4.87 (1 H, m, 3-H), 5.42 (1 H, m, 6-H), and 7.4–8.1 (5 H, m, Ph); δ_C 18.91 and 17.80 (6:1 ratio).

The (24*S*,28*R*)- and (24*S*,28*S*)-isomer were also obtained in *ca.* 80% purity. ^{13}C Chemical shifts of the diastereotopic methyl groups for these isomers are listed in Table 1.

[pro-R-Me-^{13}C]-(24R,28R)- *and* (24R,28S)-*Epoxide Benzoates* (**13**) *and* (**14**).—The olefin benzoate (**10**) was similarly converted into the epoxides. For the isomer (**13**): m.p. 155.5–157.5 °C; δ_H 0.69 (3 H, s, 18-H_3), 0.92 (*ca.* 2.5 H, dd, J_{HH} 7.3, $^1J_{CH}$ 126 Hz, *pro-R*-Me), 0.85–0.96 (*ca.* 6 H, m, 21-H_3 and *pro-S*-Me), 1.07 (3 H, s, 19-H_3), 1.28 (3 H, d, *J* 5.5 Hz, 29-H_3), 2.91 (1 H, q, *J* 5.6 Hz, 28-H), 4.87 (1 H, m, 3-H), 5.42 (1 H, m, 6-H), and 7.4–8.1 (5 H, m, Ph); δ_C 17.78 and 18.44 (1:6 ratio).

For the isomer (**14**): δ_H 0.68 (3 H, s, 18-H_3), 0.92 (3 H, d, *J* 6.3 Hz, 21-H_3), 0.96 (*ca.* 2.5 H, m, *pro-S*-Me), 1.05 (*ca.* 2.5 H, dd, J_{HH} 6.7, $^1J_{CH}$ 125 Hz, *pro-R*-Me), 1.07 (3 H, s, 19-H_3), 1.30 (3 H, d, *J* 5.4 Hz, 29-H_3), 2.95 (1 H, q, *J* 5.4 Hz, 28-H), 4.87 (1 H, m, 3-H), 5.42 (1 H, m, 6-H), and 7.4–8.1 (5 H, m, Ph); δ_C 17.80 and 18.91 (6:1 ratio).

The ^{13}C-*Labelled Epoxides* (**1a**), (**1b**), (**2a**), *and* (**2b**).—A mixture of the epoxide benzoate (**11**) (*ca.* 3 mg) and KOH–MeOH solution (5%; 0.5 ml) were stirred at room temperature for 1 h. Extractive (Et_2O) work-up gave a crude product, which was chromatographed on silica gel. Elution with hexane–EtOAc (10:1) afforded the epoxide (**1a**) in good yield. The other benzoates were similarly hydrolysed to give the respective epoxides. For the isomer (**1a**): m.p. 156.5–159 °C (from MeOH); δ_H 0.68 (3 H, s, 18-H_3), 0.89 (*ca.* 2.5 H, dd, J_{HH} 7.0, $^1J_{CH}$ 126 Hz, *pro-S*-Me), 0.89–0.95 (*ca.* 6 H, m, 21-H_3 and *pro-R*-Me), 1.01 (3 H, s, 19-H_3), 1.27 (3 H, d, *J* 5.6 Hz, 29-H_3), 2.91 (1 H, q, *J* 5.6 Hz, 28-H), 3.53 (1 H, m, 3-H), and 5.36 (1 H, m, 6-H); δ_C 17.77 and 18.44 (6:1 ratio).

For the isomer (**1b**): δ_H 0.68 (3 H, s, 18-H_3), 0.89 (*ca.* 2.5 H, dd, J_{HH} 7.0, J_{CH} 126 Hz, *pro-S*-Me), 0.89–0.95 (*ca.* 6 H, m, 21-H_3 and *pro-R*-Me), 1.01 (3 H, s, 19-H_3), 1.27 (3 H, d, *J* 5.5 Hz, 29-H_3), 2.91 (1 H, q, *J* 5.6 Hz, 28-H), 3.52 (1 H, m, 3-H), and 5.35 (1 H, m, 6-H); δ_C 17.77 and 18.44 (1:6 ratio).

For the isomer (**2a**): δ_H 0.67 (3 H, s, 18-H_3), 0.91 (3 H, d, *J* 6.4 Hz, 21-H_3), 0.96 (*ca.* 2.5 H, dd, J_{HH} 7.1, $^1J_{CH}$ 125 Hz, *pro-S*-Me), 1.01 (3 H, s, 19-H_3), 1.06 (*ca.* 2.5 H, J_{HH} 5.0, $^3J_{CH}$ 5.0 Hz, *pro-R*-Me), 1.29 (3 H, d, *J* 5.7 Hz, 29-H_3), 2.93 (1 H, q, *J* 5.6 Hz, 28-H), 3.52 (1 H, m, 3-H), and 5.35 (1 H, m, 6-H); δ_C 17.80 and 18.91 (1:6 ratio).

For the isomer (**2b**): δ_H 0.67 (3 H, s, 18-H_3), 0.93–0.97 (*ca.* 6 H, m, 21-H_3 and *pro-S*-Me), 1.00 (3 H, s, 19-H_3), 1.05 (*ca.* 2.5 H, dd, J_{HH} 7.2, J_{CH} 126 Hz, *pro-R*-Me), 1.29 (3 H, d, *J* 5.7 Hz, 29-H_3), 2.93 (1 H, q, *J* 5.6 Hz, 28-H), 3.52 (1 H, m, 3-H), and 5.35 (1 H, m, 6-H); δ_C 17.80 and 18.91 (6:1 ratio).

Preparation of the Cell-free Extract.—The cell-free preparation was obtained as previously reported.[12] Fifty guts of the fifth instar larvae (on day six or seven, weight 4.1 g/head) of *B. mori* afforded a 1 500g supernatant fraction (the final volume was adjusted to 50 ml, protein concentration 5.5 mg ml^{-1} as estimated by the Lowry–Folin method).

Incubation and Analysis.—An acetone solution (200 μl) of the ^{13}C-labelled epoxide [500 μg for (**1a**) and (**1b**), 400 μg for (**2a**) and (**2b**)] was added to each test tube containing the above preparation (5 ml). Four incubations were done for each substrate, thus the total weight of the substrate amounted to 2.0 and 1.6 mg, respectively, for compounds (**1**) and (**2**). The mixture was shaken at 30 °C for 3 h. One of the test tubes was incubated similarly in the absence of substrate to investigate endogenous sterols in the cell-free preparation. The enzyme reaction was terminated by addition of ethyl acetate (5 ml). The mixture was well mixed in a vortex mixer and was centrifuged. The resulting ethyl acetate layer was saved. This operation was repeated four times and the combined organic layer was filtered through a short column containing silica gel and Na_2SO_4. The filtrate was concentrated and subjected to ^{13}C NMR analysis.

A part of the sample was converted into the TMS ether and was analysed by GC–MS for desmosterol as previously reported.[12] The amount of desmosterol was estimated from a calibration curve obtained for the authentic non-labelled sample. The mass spectrum (70 eV) of desmosterol TMS ether exhibited ions at *m/z*: 457 (M^+), 442 (M − Me), 343 (M − side-chain − 2 H), 328 (M − $Me_3SiOC_3H_4$), 253 (343 − Me_3SiOH), 129 ($Me_3SiOC_3H_4$), 73 ($SiMe_3$), and 70 (C-23–C-27).

The major part of the sample was separated by PLC [developed three times with hexane–Et_2O (2:1)] to give the sterol fraction (R_F 0.42), which contained endogenous cholesterol and sitosterol, and ^{13}C-labelled desmosterol. The ^{13}C NMR spectrum of the fraction was recorded.

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