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BIOSYNTHESIS OF SITOSTEROL IN BARLEY SEEDLINGS

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; sitosterol; stigmasterol; 28-isofucosterol; stigmasta-5,24-dien-3β-ol; clerosterol; phytosterol biosynthesis; synthesis of ^{14}C-labelled sterols.

Abstract—A method is described for the chemical synthesis of stigmasta-5,24-dien-3β-ol-[26-^{14}C] and (24*S*)-24-ethylcholesta-5,25-dien-3β-ol-[26-^{14}C] (clerosterol). 28-Isofucosterol-[7-$^{3}H_2$] fed to developing barley seedlings (*Hordeum vulgare*) was incorporated into sitosterol and stigmasterol confirming the utilisation of a 24-ethylidene sterol intermediate in 24α-ethyl sterol production in this plant. Also, the use of mevalonic acid-[2-^{14}C(4*R*)-4-$^{3}H_1$] verified the loss of the C-25 hydrogen of 28-isofucosterol during its conversion into sitosterol and stigmasterol in agreement with the previously postulated isomerisation of the 24-ethylidene sterol to a $\Delta^{24(25)}$-sterol prior to reduction. However, feeding stigmasta-5,24-dien-3β-ol [26-^{14}C] to barley seedlings gave very low incorporation into sitosterol. Attempts to trap radioactivity from mevalonic-[2-^{14}C(4*R*)-4-$^{3}H_1$] in stigmasta-5,24-dien-3β-ol when this unlabelled sterol was administered to barley seedlings gave only a very small incorporation although both 28-isofucosterol and sitosterol were labelled.

INTRODUCTION

Higher plants produce mixtures of sterols in which sitosterol (**1**), stigmasterol (**2**) and 24-methylcholesterol often predominate [1]. Sitosterol (**1**) and stigmasterol (**2**) have the 24α-configuration but the 24-methylcholesterol found in many higher plants is now recognised to be a mixture of the 24α-epimer, campesterol (**3**) and the 24β-epimer, dihydrobrassicasterol (**4**) [2–4].

Scheme 1 outlines the mechanisms now considered to be operative in higher plants for the production of the various phytosterol side chains. Methylation of a Δ^{24}-precursor (**5**), probably cycloartenol, leads, by alternative methods of stabilisation of the cation **6**, to either a 25-methylene (**7**) or a 24-methylene (**8**) compound from which the 24β- or 24α-methyl side chains **9** and **11** respectively, can be produced by reduction as indicated [5]. A second transmethylation step leads from the 24-methylene (**8**) intermediate via cation **12** to a 24*Z*-ethylidene sterol (**13**). Evidence for the role of 24*Z*-ethylidene compounds in 24α-ethylsterol (**15** and **16**) biosynthesis has been provided by the isolation from higher plants of several sterols with side chain **13** [1], the ready labelling of such compounds by ^{14}C-precursors [6–9], their conversion into sitosterol [9–11] and the incorporation of 4 deuterium atoms from methionine-[CD_3] into sitosterol by barley seedlings [11].

The participation of a $\Delta^{24(25)}$ intermediate (**14**), produced by isomerisation of the 24-ethylidene sterol (**13**), has been postulated [5] to explain the observed loss of the C-25 hydrogen of the 24-ethylidene compound (**13**) during its conversion into the 24α-ethyl sterols [11–14]. The possible participation of $\Delta^{24(25)}$ sterols (**10**, **14**) in 24α-alkyl sterol (**11**, **15**) biosynthesis is supported by the isolation of ergosta-5,24-dien-3β-ol from *Withania somnifera* [15] and the tentative identification of a sterol with side chain **14** in the sunflower [16]. To obtain further information on the operation of the proposed route **8** → **13** → **14** → **15** → **16** we have synthesised ^{14}C-labelled stigmasta-5,24-dien-3β-ol (**18**) and now report the results of feeding this sterol (**18**), and also labelled 28-isofucosterol (**17**), to developing barley seedlings.

RESULTS AND DISCUSSION

Synthesis of stigmasta-5,24-dien-3β-ol[26-^{14}C] (**18**)

To investigate the role of stigmasta-5,24-dien-3β-ol (**18**) in phytosterol biosynthesis it was necessary to prepare this sterol radioactively labelled. The starting material chosen was (24*S*)-24-ethylcholesta-5,25-dien-3β-ol (**19**, clerosterol) which can be readily isolated from the seaweed *Codium fragile* [17]. Acetylation and treatment with osmium tetroxide followed by sodium periodate [18, 19] produced 3β-acetoxy-(24*S*)-27-nor-24-ethylcholesta-5-en-25-one (**20**).

Using this precursor (**20**) it was originally intended to employ the Wittig reaction using $^{14}CH_3I$ and thus regenerate (24*S*-24-ethylcholesta-5,25-dien-3β-yl acetate (**19a**) labelled with ^{14}C in the 25-methylene group; isomerisation of **19a** with iodine [17, 20] would then produce the desired stigmasta-5,24-dien-3β-yl acetate-[26-^{14}C]. However it was found that yields were unacceptably low when the Wittig synthesis was attempted on the scale desired for the radiochemical synthesis to produce **18** of a reasonably high sp. act. Accordingly an alternative approach was adopted employing the Grignard reaction. The tetrahydropyranosyl ether of 3β-hydroxy-(24*S*)-27-nor-24-ethylcholest-5-en-25-one was reacted with Grignard reagent generated from $^{14}CH_3I$ to give 3β-tetrahydropyranosyl-(24*S*)-24-ethylcholest-5-en-25-ol-[26-^{14}C] from which treatment with

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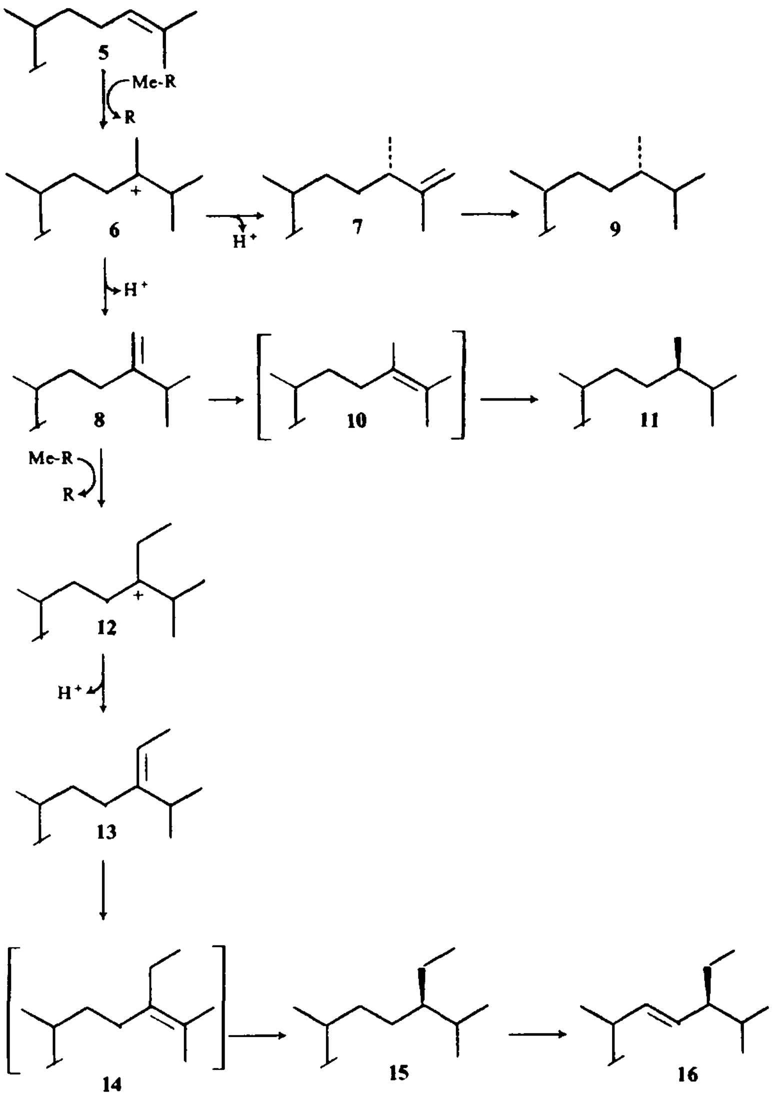
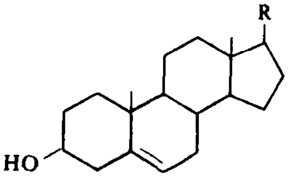


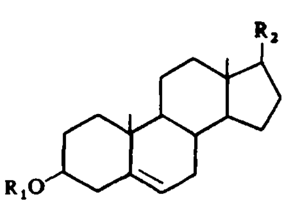
Scheme 1. Proposed biosynthetic routes for the production of the phytosterol side chains of higher plants.

THF/HCl gave (24*S*)-24-ethylcholest-5-en-3β,25-diol-[26-^{14}C] (**21**) in *ca* 20% radiochemical yield. Acetylation of **21** gave 3β-acetoxy-(24*S*)-24-ethylcholest-5-en-25-ol-[26-^{14}C] (**21a**) which was dehydrated by treatment with $POCl_3$ to give two products which were separated by PLC on $AgNO_3$–Si gel and identified as stigmasta-5, 24-dien-3β-yl acetate-[26-^{14}C] (**22**) and (24*S*)-24-ethylcholesta-5,25-dien-3β-yl acetate-[26-^{14}C] (**19a**) respectively. In several test dehydration reactions of compound **21a** the proportions of the two products were always found to be in the order of 70% compound **19a** and 30% compound **22**, showing that the dehydration conditions favoured the production of the $\Delta^{25(26)}$ compound rather than the anticipated $\Delta^{24(25)}$ sterol [21]. An alternative method of synthesis of compound **18** has recently been described by Sucrow [22].

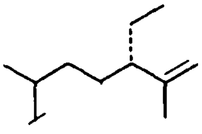


1 R = side chain **15**
2 R = side chain **16**
3 R = side chain **11**
4 R = side chain **9**
17 R = side chain **13**
18 R = side chain **14**

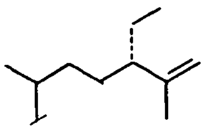
Since labelled (24*S*)-24-ethylcholesta-5,25-dien-3β-ol (**19**, clerosterol) was required for other investigations on algal sterol biosynthesis, the product (**19a**) of the above synthesis was analysed by high resolution PMR at 220 MHz in an attempt to ascertain if it had retained the (24*S*)-configuration of the parent compound since C-24 epimerisation was possible during the synthetic sequence. Unfortunately the (24*R*)-epimer of **19** has not been isolated from natural sources nor synthesised chemically and was therefore unavailable for comparative purposes. However, it is now well established [2, 3, 23, 24] that all other pairs of C-24 epimers available are distinguishable by the chemical shifts of the side chain Me groups in the high resolution PMR spectra. The spectrum of the synthetic (24*S*)-24-ethylcholesta-5,25-dien-3β-yl-acetate (**19a**) was superimposable upon that of the naturally occurring



19 $R_1 = H, R_2 =$



19a $R_1 = Ac, R_2 =$



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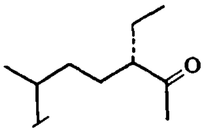

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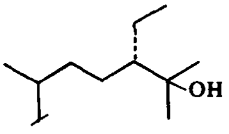
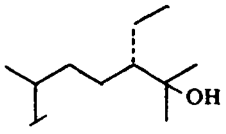
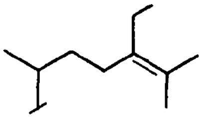


21a $R_1 = Ac, R_2 =$



22 $R_1 = Ac, R_2 =$



compound thus providing reasonable evidence for the conclusion that the (24*S*)-epimer predominated.

Feeding labelled compounds to barley embryos

In previous work [11] we demonstrated the ability of barley embryos to utilise labelled 24-ethylidene lophenol for sitosterol biosynthesis. However, this compound is a 4α-methyl Δ^7-sterol and therefore it was possible that side chain reduction may have occurred at some intermediate stage of ring modification to establish the Δ^5-bond and that the enzyme(s) responsible may show a high specificity for an intermediate other than a Δ^5-sterol. Before investigating the potential of stigmasta-5,24-dien-3β- ol (**18**) as a sitosterol precursor we therefore considered it desirable to demonstrate the conversion of 28-isofucosterol (**17**), its presumed precursor, into sitosterol (**1**).

Barley embryos were obtained from sterilised seeds and grown in a defined growth medium to produce seedlings as described previously [11]. After 8 days culture in the presence of 28-isofucosterol-[7-$^3H_2$] (**17**) the sterols were isolated from the barley seedlings and shown by GLC to contain 24-methylcholesterol (**3** and **4**), stigmasterol (**2**) and sitosterol (**1**) in the proportions observed previously [11], the 28-isofucosterol (**17**) content was somewhat elevated, presumably owing to the uptake and accumulation of this compound which was added to the growth medium. Addition of carrier sterols, acetylation and PLC on $AgNO_3$–Si gel gave the acetates of sitosterol (**1**) and stigmasterol (**2**) which were both labelled. Crystallisation of each to constant sp. act. confirmed the conversion of the 28-isofucosterol-[7-$^3H_2$] into these sterols. The incorporation of label into 24α-ethyl sterols (**1** and **2**) was *ca* 0.2% based upon the amount of 28-isofucosterol-[7-$^3H_2$] added initially to the medium or *ca* 0.7% based upon the radioactivity recovered in the sterols which included absorbed, but unmetabolised, precursor (**17**).

Since some radioactivity (~0.3% of that recovered) was associated with the materials of low polarity that chromatographed on TLC at the solvent front in the same region as squalene it was thought advisable to check that incorporation of label into sitosterol had not occurred by metabolism of the 28-isofucosterol-[7-$^3H_2$] and reincorporation of the tritium by *de novo* sterol biosynthesis. Oxidation of the labelled sitosteryl acetate (1730 dpm/mg) gave 3β-acetoxy-stigmast-5-en-7-one (61 dpm/mg). Thus *ca* 95% of tritium was located at C-7 which is in accord with a direct bioconversion of the 28-isofucosterol-[7-$^3H_2$] into sitosterol-[7-$^3H_2$] rather than extensive randomisation and reincorporation of the tritium. The nature and origin of the radioactive material associated with the hydrocarbon fraction remains unclear.

Since the incubation with 28-isofucosterol (**17**) established that the side chain reductase system is active with a Δ^5-sterol substrate, feeding of stigmasta-5,24-dien-3β-ol-[26-^{14}C] (**18**) were undertaken. Two experiments were performed, one utilising the free 3β-OH sterol, the other incubation used the acetate. In both experiments GLC analysis of the sterols recovered from the barley seedlings showed the 24-methylcholesterol (**3**) and (**4**), stigmasterol (**2**) and sitosterol (**1**) composition to be in the usual range [11] but there was in addition a considerable amount of stigmasta-5,24-dien-3β-ol (**18**) apparently showing that this sterol had been absorbed from the growth medium by the embryos. However, it was also notable that the amounts of cholesterol (3.6 %) and 28-isofucosterol (8–10 %) appeared to be elevated above the values observed previously.

Analysis of the labelled sterol mixture by GC-RC showed that, in both experiments, more than 95 % of the radioactivity was associated with the mass peak for unchanged stigmasta-5,24-dien-3β-ol-[26-^{14}C] (**18**). Only a minor radioactive peak (5 %) was present with a R_t similar to that of sitosterol (**1**) whilst negligible label was associated with stigmasterol (**2**). Addition of carrier sterols and acetylation of the mixture followed by $AgNO_3$–Si gel TLC confirmed that most of the label was in unchanged starting material (**18**) whilst the sitosterol (**1**) contained very little label (150 dpm and 230 dpm respectively for the two experiments). However, *ca* 20–24 % of the recovered radioactivity had an R_f on $AgNO_3$–Si gel similar to clerosteryl acetate (0.32) and 28-isofucosteryl acetate (0.3). However, the identity of this labelled compound is at present unknown since it could not be either of the above sterols from the GC-RC analysis of the labelled sterol mixture which indicated very little radioactivity in these compounds.

While the present results could be taken to indicate that stigmasta-5,24-dien-3β-ol (**18**) is not converted into sitosterol (**1**) they could equally well be explained by a failure of this labelled sterol to be absorbed by the barley embryos and transferred efficiently to the intracellular site of sterol biosynthesis. Moreover the latter situation would be aggravated by the rather low sp. act. (0.75 μCi/mg) of the sterol so that a small percentage conversion would be difficult to detect. Consequently we do not consider that the very low radioactivity associated with sitosterol (**1**) in these experiments permits any firm conclusions regarding the possible intermediacy of a $\Delta^{24(25)}$ sterol in 24α-ethyl sterol biosynthesis.

As an alternative approach to demonstrate a $\Delta^{24(25)}$-sterol intermediate we have attempted to trap radioactivity from mevalonic acid-[2-^{14}C(4*R*)-4-^{3}H$_1$] in stigmasta-5,24-dien-3β-ol (**18**) by administering the labelled mevalonic acid together with unlabelled **18** to barley embryos. As reported previously [11] the ^{3}H : ^{14}C atomic ratios of the recovered labelled 28-isofucosterol (3.0 : 5), sitosterol (2.4 : 5) and stigmasterol (1.9 : 5) were consistent with the involvement of a $\Delta^{24(25)}$-sterol produced by isomeration of a 24-ethylidene intermediate such as 28-isofucosterol (**17**). The distribution of ^{14}C radioactivity between the sitosterol plus 24-methylcholesterol (~4 %), stigmasterol (~5 %) and 28-isofucosterol (~90 %) was similar to that reported earlier for the culture of barley embryos with labelled mevalonic acid (9 %, 9 %, 82 % respectively for the three sterols, ref. [11]). The radioactivity trapped in stigmasta-5,24-dien-3β-ol (**18**) was very low (480 dpm, 0.5 %). If stigmasta-5,24-dien-3β-ol (**18**) is a true intermediate produced by isomerisation of 28-isofucosterol (**17**) then it might be anticipated that possibly at least comparable amounts of radioactivity would be recovered in stigmasta-5,24-dien-3β-ol (**18**) and the sitosterol (**1**) and stigmasterol (**2**) under the conditions of this experiment with the sterol **18** trap. However, this assumes that sterol **18** added to the growth medium is absorbed and reaches the site of cellular sterol synthesis where it can freely equilibrate with any newly synthesised pool of ^{14}C-labelled sterol **18**. Unfortunately there is no evidence to indicate that this is the case and therefore while the low level of radioactivity present in stigmasta-5,24-dien-3β-ol (**18**) is indicative that it may be produced the results again do not permit a definite conclusion concerning its role as an obligatory precursor. In order to pursue the possible intermediacy of $\Delta^{24(25)}$-sterols it now seems desirable to repeat experiments similar to those described with stigmasta-5,24-dien-3β-ol (**18**) of a considerably higher sp. act. and to utilise cell free preparations to investigate steps **13** → **14** and **14** → **15**.

EXPERIMENTAL

General methods. IR spectra were obtained as KBr discs. PMR spectra were measured at 220 MHz in $CDCl_3$ with TMS as internal standard. Mps were determined on a Reichert hot stage and are uncorr. TLC was on Si gel, compounds were detected by spraying either with 10 % aq. H_2SO_4 and heating to 80° or with Rhodamine-6G in Me_2CO and examining under UV light. For preparative work bands were scraped from the plates and eluted with dry Et_2O. GLC employed 1.52 m × 0.64 cm columns of 3 % OV-17 on 100–120 mesh Gas Chrom Q, carrier gas Ar (60 ml/min), temp. 260–280°. RR_t's were: cholesterol 1.0; 24-methylcholesterol 1.32; stigmasterol 1.43; sitosterol 1.66; clerosterol 1.70; 28-isofucosterol 1.80; stigmasta-5,24-dien-3β-ol 1.93. Radioactivity was determined by liquid scintillation counting using 0.7 % butyl-PBD in toluene. For GC-RC an effluent splitter permitted 10 % column effluent to pass to the FID, the remaining 90 % of the effluent passed through a combustion furnace followed by radioassay of the $^{14}CO_2$ produced.

Synthesis of 3β-hydroxy-(24S)-27*-nor-ethylcholest-5-en-25-one.* (24*S*)-24-Ethylcholesta-5,25-dien-3β-ol (**19**, clerosterol) was isolated from the seaweed *Codium fragile* as described previously [17] and acetylated. The (24*S*)-24-ethylcholesta-5,25-dien-3β-yl acetate had mp 124–126° and was fully characterised by GLC, IR, PMR, and MS. (24*S*)-24-Ethylcholesta-5,25-dien-3β-yl acetate (**19a**) (900 mg) was stirred for 5 hr at room temp. with 560 mg OsO_4 in 50 ml C_6H_6 with 0.5 ml C_5H_5N [18, 19]. A soln of 9g Na_2SO_3 in 50 ml H_2O and 70 ml EtOH were then added and the mixture stirred 18 hr. Extraction of the reaction mixture gave 830 mg crude product, mp 168–172°. TLC on Si-gel developed with EtOAc showed two major components which were isolated by PLC. The more polar compound ($R_f = 0.39$) had mp 196–197°; MS *m/e*: 446 (M^+), 431, 419, 417, 414, 401, 399, 359, 277, 275, 259. IR ν_{max}^{KBr} cm^{-1}: 3460 (OH), no acetoxy absorption; identified as (24*S*)-24-ethylcholest-5-en-3β,25,26-triol. The less polar product ($R_f = 0.55$) had mp 172–174° lit. 173–176 [19]. MS *m/e*: 488 (M^+), 446, 428, 415, 413, 396, 386, 372, 357, 273, 271, 232. IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1730 (—OAc); identified as 3β-acetoxy-(24*S*)-24-ethylcholest-5-en-25,26-diol. Clearly some hydrolysis of the 3β-acetate occurs during this reaction, probably upon treatment with Na_2SO_3. Only a trace of unchanged starting material (**19a**) ($R_f = 0.79$) remained, therefore the unpurified reaction mixture was used in the next step. 700 mg of the mixture was dissolved in 40 ml EtOH and 10 ml C_5H_5N, then 500 mg $NaIO_4$ in 2 ml 1.7N H_2SO_4 was added and the mixture stirred 18 hr at room temp. After dilution with H_2O and Et_2O extraction the reaction product was acetylated (C_5H_5N–Ac_2CO). GLC of the reaction products (655 mg, mp 122°) showed 7 % of the original (24*S*)-24-ethylcholesta-5,25-dien-3β-yl acetate and 90 % of the desired 3β-acetoxy-(24*S*)-27-nor-24-ethylcholest-5-en-25-one (**20**). Column chromatography on Al_2O_3 (grade III) and elution with petrol–Et_2O (97 : 3) gave the starting material while petrol–Et_2O (17 : 3) gave pure 3β-acetoxy-

(24*S*)-27-nor-ethylcholest-5-en-25-one, mp 124° (lit. [18] 131–133°); IR ν_{max}^{KBr} cm^{-1}: 1730 (OAc), 1710 (C=O). Refluxing in K_2CO_3–MeOH–H_2O gave 3*β*-hydroxy-(24*S*)-27-nor-24-ethylcholest-5-en-25-one, mp 118° lit. [18] 117–120°; IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1710 (C=O) MS *m/e*: 414 (M^+), 396; pure by GLC.

Synthesis of 3β-acetoxy-(24S)-24-ethylcholest-5-en-25-ol (**21a**). 3*β*-Hydroxy-(24*S*)-27-nor-24-ethylcholest-5-en-25-one (79 mg) was dissolved in CH_2Cl_2 (0.7 ml), dihydropyrane (0.2 ml) added together with a few drops of $POCl_3$ and the mixture stirred at room temp. for 40 min. TLC of the reaction product on Si gel ($CHCl_3$) showed a major spot (R_f = 0.75) for the tetrahydropyranosyl steryl ether and a minor spot (R_f ≑ 0.30) for unchanged starting material. Purification on neutral Al_2O_3, grade II, eluted with petrol–Et_2O (22:3) gave 3*β*-tetrahydropyranosyl-(24*S*)-27-nor-24-ethylcholest-5-en-25-one (83 mg), mp 121–122° MS *m/e*: 498 (M^+), 414, 396 (100%) 381, 339, 273, 255, 213, 84. For the Grignard reaction MeI (68 mg) in 0.4 ml dry Et_2O was mixed with 500 μCi $^{14}CH_3I$ (Radiochemical Centre, Amersham) and the soln transferred with a syringe to the dry reaction flask containing 20 mg Mg. When the Grignard reagent was formed 80 mg 3*β*-tetrahydropyranosyl-(24*S*)-27-nor-24-ethylcholest-5-en-25-one in 2 ml dry Et_2O was added. After work up the product was warmed for 40 min in THF–HCl to cleave the THP–ether. TLC on Si gel ($CHCl_3$) showed one major spot (R_f = 0.2) for the product (24*S*)-24-ethylcholest-5-en-3*β*-25-diol (**21**) which was purified by chromatography on neutral Al_2O_3, grade III. Elution with petrol–Et_2O (13:7) gave **21**, 48.5 mg, 36μCi, (20% theoretical radiochemical yield), mp 142° (lit. [18] 144–147°) IR ν_{max}^{KBr} cm^{-1}: 3450 (OH); pure by TLC and GLC. Acetylation (C_5H_5N, Ac_2CO 18 hr room temp.) gave 3*β*-acetoxy-(24*S*)-24-ethylcholest-5-en-25-ol (**21a**) mp 133°, IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1740(—OAc); MS *m/e*: 412 (M^+-acetate), 394, 379, 354, 339, 273, 255, 253, 213; pure by GLC.

Synthesis of stigmasta-5,24-dien-3β-ol (**18**) *and* (*24S*)*-24-ethylcholesta-5,25-dien-3β-ol* (**19**, *clerosterol*). 3*β*-Acetoxy-(24*S*)-24-ethylcholest-5-en-25-ol (**21a**, 55 mg) was dissolved in 3.6 ml C_5H_5N containing 0.72 ml $POCl_3$ and left at room temp. for 20 hr. GLC of the products showed that dehydration was complete and the 3*β*-acetates of **18** and **19** were obtained in the proportions 3 : 7. The two steryl acetates were separated by TLC on 10% $AgNO_3$–Si gel developed with pure $CHCl_3$. Stigmasta-5,24-dien-3*β*-yl acetate (**22**), 6.2μCi; sp. act. 0.75μCi/mg; mp 118–120° (lit. [25] mp 133–134°). GLC: less than 1% of compound **19a**. MS *m/e* (rel. int.): 394 (M^+-acetate, 100) 296(95), 281(20), 253(24) 213(10); PMR ($CDCl_3$); *δ* 0.68 (*s*, 3H, C-18), 1.02 (*s*, 3H, C-19), 0.93 (*t*, 3H, C-29), 1.62 (*s*, 6H, C-26 and C-27), 2.02 (*s*, 3H, MeCO), 4.63 (*m*, 1H, C-3α), 5.37 (*m*, 1H, C-6). (24*S*)-24-Ethylcholesta-5,25-dien-3*β*-yl acetate (**19a**), 14.1μCi, sp. act. 0.75μCi/mg; mp 125–127° (lit. [18] mp 124–127°); pure by GLC analysis; MS *m/e* (rel. int.): 454 (M^+, 5), 394 (M^+-acetate, 100), 313 (8), 255 (7), 228 (5), 213 (6); PMR ($CDCl_3$): 0.66 (*s*, 3H, C-18), 1.01 (*s*, 3H, C-19), 0.79 (*t*, 3H, C-29), 0.89 (*d*, 3H, C-21), 1.56 (*s*, 3H, C-27), 2.02 (*s*, 3H, MeCO), 4.65 (*m*, 1H, C-3α) 4.64 and 4.72 (1H each, C-26), 5.36 (*m*, 1H, C-6).

Administration of 28-*isofucosterol*-[7-$^3H_2$] (**17**) *to barley seedlings. Hordeum vulgare* (barley) seeds were surface sterilised and the embryos dissected and grown in a nutrient growth medium as previously described [11]. 0.05% Triton X 100 was incorporated in the media to help solubilise added sterols 28-Isofucosterol-[7-$^3H_2$] (10μCi/mg) was synthesised as reported [26] and its purity checked by GLC and TLC prior to use. 28-Isofucosterol-[7-$^3H_2$] (10μCi, 1 mg) was dissolved in 0.3 ml Et_2O and 0.1 ml of the soln injected into each of 3 culture flasks containing a total of 80 barley embryos. After 8 days when the seedlings had grown considerably, the incubation was ended and the non-saponifiable lipid obtained by the usual method employing ethanolic KOH [11]. PLC on Si gel ($CHCl_3$–EtOH, 49:1) gave the 4-desmethyl sterol mixture (4.5 mg, 5.75 × 10^6 dpm). The squalene and 4,4-dimethyl zones of the plate were monitored to check for randomisation of label and were found to contain less than 0.3% of the recovered radioactivity. GLC analysis showed the sterol mixture to comprise 24-methylcholesterol (23%), stigmasterol (12%), sitosterol (39%) and 28-isofucosterol (26%). *Ca* 2 mg of each of these sterols was added and the mixture acetylated and separated on 10% $AgNO_3$–Si gel developed with $CHCl_3$–Et_2O (97:3) to give sitosteryl plus 24-methylcholesteryl acetates (1.7 × 10^4 dpm), stigmasteryl acetate (2.26 × 10^4 dpm) and 28-iso-fucosteryl acetate (3.05 × 10^6 dpm). To the sitosteryl plus 24-methylcholesteryl acetate mixture was added 16.2 mg sitosteryl acetate and the mixture crystallised to constant sp. act. from MeOH (798, 788, 791, 782, 776 dpm/mg for successive crystallisations). To the labelled stigmasteryl acetate was added 20 mg carrier followed by crystallisation from MeOH (418, 398, 403, 393 dpm/mg for successive crystallisations).

To check if randomisation of tritium had occurred during the incubation the labelled sitosteryl acetate (4.5 mg, 7800 dpm) was subjected to oxidation [24] with CrO_3 (2.3 mg) in HOAc (2.5 ml) to give 3*β*-acetoxystigmast-5-en-7-one (3.4 mg, 210 dpm).

Administration of stimasta-5,24-dien-3β-ol-[26-^{14}*C*] *and its acetate to barley.* (i) Stigmasta-5,24-dien-3*β*-yl acetate-[26-^{14}C] (1.1μCi) was distributed between 3 flasks each containing 25–30 barley embryos as described above. After 10 days growth the non-saponifiable lipid (7 × 10^5 dpm) was isolated. PLC on Si gel developed with $CHCl_3$–EtOH (49:1) gave the 4-desmethyl sterol fraction (5 × 10^5 dpm). GLC analysis showed cholesterol (6%) 24-methylcholesterol (20%), stigmasterol (18%), sitosterol (23%), 28-isofucosterol (8%) and stigmasta-5,24-dien-3*β*-ol (24%). Examination of a further portion by GC-RC revealed that the bulk (~95%) of the radioactivity was associated with the mass peak for the starting stigmasta-5,24-dien-3*β*-ol-ol (**18**). There was no radioactivity associated with the stigmasterol and 28-isofucosterol peaks but a small radioactive peak was apparent with a R_t corresponding to sitosterol. Unlabelled sitosterol (3 mg), stigmasta-5,24-dien-3*β*-ol (2 mg) and clerosterol (3 mg) were added and the labelled sterol mixture acetylated (C_5H_5N–Ac_2O). A portion of the steryl acetates (1 × 10^5 dpm, 6 mg) was subjected to PLC on 10% $AgNO_3$–Si gel developed with pure $CHCl_3$ to give sitosteryl acetate 150 dpm), stigmasta-5,24-dien-3*β*-yl acetate (51 500 dpm) and clerosteryl acetate (16 500 dpm). (ii) 80 Barley embryos were cultured for 12 days in 3 flasks with stigmasta-5,24-dien-3*β*-ol (1μCi). Isolation of the non-saponifiable lipid followed by TLC gave the 4-desmethyl sterols (2.5 mg, 2.3 × 10^5 dpm). GLC analysis: cholesterol (3%), 24-methylcholesterol (24%), stigmasterol (17%), sitosterol (34%), 28-isofucosterol (10%), stigmasta-5, 24-dien-3*β*-ol (12%). GC-RC showed a very similar distribution of radioactivity to the above results (i). Addition of carriers, acetylation and PLC on 10% $AgNO_3$–Si gel gave sitosteryl acetate (230 dpm), stigmasta-5,24-dien-3*β*-ol (56 500 dpm) and clerosteryl acetate (15 000 dpm).

Feeding mevalonic acid-[2-^{14}*C*(4R)-4-$^3H_1$] *with stigmasta*-5, 24-*dien-3β-ol.* 80 barley embryos were cultured with mevalonic acid-[2-^{14}C(4*R*)-4-$^3H_1$] (30μCi of ^{3}H, 5μCi of ^{14}C) with the addition of unlabelled stigmasta-5,24-dien-3*β*-ol (2 mg) to act as a trap. After 9 days growth the non-saponifiable lipids were isolated (15 mg, 5.8μCi of ^{3}H, 1.1μCi of ^{14}C). TLC on Si gel ($CHCl_3$–EtOH, 49:1) gave fractions containing squalene (1.97 × 10^6 dpm ^{3}H; 3.18 × 10^5 dpm ^{14}C), 4,4-dimethyl sterols (3.84 × 10^6 dpm ^{3}H; 6.57 × 10^5 dpm ^{14}C), 4-desmethyl sterols (6.9 mg; 1.49 × 10^6 dpm ^{3}H; 3.70 × 10^5 dpm ^{14}C), 4-desmethyl sterols (6.9 mg; 1.49 × 10^6 dpm ^{3}H; 3.70 × 10^5 dpm ^{14}C), GLC analysis: 24-methylcholesterol (24%), stigmasterol (10%), sitosterol (40%), 28-isofucosterol (trace), stigmasta-5,24-dien-3*β*-ol (28%). After addition of 1–2 mg of each of these sterols the labelled material was acetylated (C_5H_5N–Ac_2O) (1.1 × 10^6 dpm ^{3}H; 2.66 × 10^5 dpm ^{14}C) and a portion of the steryl acetates subjected to PLC on 10% $AgNO_3$–Si gel developed with $CHCl_3$–Et_2O (97:3) to give sitosteryl plus 24-methylcholesterol acetates (1.2 × 10^4 dpm ^{3}H; 4.3 × 10^3 dpm ^{14}C; ^{3}H: ^{14}C = 2.89:1) stigmasteryl acetate (1.48 × 10^4 dpm ^{3}H; 5.25 × 10^3 dpm ^{14}C; ^{3}H; 14C = 2.81:1), stigmasta-5,24-dien-3*β*-yl acetate (2.23 × 10^3 dpm ^{3}H; 4.8 × 10^2 dpm ^{14}C; ^{3}H: ^{14}C = 4.60:1), 28-isofucosteryl acetate (9.35 × 10^4 dpm ^{3}H; 2.42 × 10^4 dpm ^{14}C; ^{3}H:^{14}C = 3.83:1). To the labelled stigmasteryl acetate was added 10 mg carrier and the mixture crystallised 4 × from MeOH (dpm/mg; observed ${}^3H:{}^{14}C$ ratio; calculated ${}^3H:{}^{14}C$ atomic ratio): 1st cryst. 361, 2.27 : 1, 1.93 : 5; 2nd cryst. 364, 2.23 : 1, 1.90 : 5; 3rd cryst. 363, 2.20 : 1, 1.87 : 5; 4th cryst. 366, 2.21 : 1, 1.88 : 5. Similarly the labelled 28-isofucosteryl acetate was crystallised 5 × after addition of carrier (19 mg): 1st cryst. 150, 3.50 : 1, 2.98 : 5; 2nd cryst. 1150, 3.47 : 1, 2.95 : 5; 3rd cryst. 1140, 3.53 : 1, 3.00 : 5; 4th cryst. 1130, 3.48 : 1, 2.96 : 5; 5th cryst. 1160, 3.47 : 1, 2.95 : 5. The ${}^3H:{}^{14}C$ atomic ratios were calculated from the observed ${}^3H:{}^{14}C$ ratio (5.88 : 1) for the labelled cycloartenol isolated in this incubation.

Nomenclature. Trivial names used in the text are: campesterol (24*R*)-24-methylcholest-5-en-3β-ol; clerosterol: (24*S*-24-ethylcholesta-5,25-dien-3β-ol; cycloartenol; 4,4,14α-trimethyl-9β,19-cyclo-5α-cholest-24-en-3β-ol; dihydrobrassicasterol: ergosta-5, 22-dien-3β-ol; 28-isofucosterol: stigmasta-5,*Z*-24(28)-dien-3β-ol; sitosterol: stigmasta-5-en-3β-ol; stigmasterol: stigmasta-5, 22-dien-3β-ol.

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