Phytochemistry, 1975, Vol. 14, pp. 1523–1528. Pergamon Press. Printed in England.

SITOSTEROL BIOSYNTHESIS IN *HORDEUM VULGARE*

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(*Received* 26 *September* 1974)

Key Word Index—*Hordeum vulgare*; Gramineae; barley; biosynthesis; side chain alkylation mechanism; phytosterols; sitosterol; stigmasterol, 28-isofucosterol.

Abstract—Excised barley embryos cultured on a nutrient medium containing methionine-[CD_3] incorporated deuterium into the newly biosynthesized sterols. Two deuterium atoms were present in 24-methylenecycloartanol, 24-methylenelophenol and campesterol and a maximum of four deuterium atoms were incorporated into 24-ethylidenelophenol, stigmasterol and sitosterol. Mevalonic acid-[2-^{14}C(4*R*)4-$^{3}H_1$] was utilized by the barley embryos to give 28-isofucosterol with a ^{3}H–^{14}C atomic ratio of 3:5 and stigmasterol and sitosterol with a ^{3}H–^{14}C atomic ratio of 2:5. 24-Methylenelophenol and 24-ethylidenelophenol were isolated from barley seed and 24-ethylidenelophenol-[2,4-$^{3}H_3$] was incorporated into sitosterol by barley seedlings. These results show that in the production of sitosterol a 24-ethylidenesterol intermediate is produced and it is suggested that this is isomerized to give a $\Delta^{24(25)}$ sterol prior to reduction to the saturated C_{29} sterol side chain.

INTRODUCTION

The C-24 ethyl group of phytosterols arises by a double transmethylation from the methyl group of methionine and several alternative pathways have been proposed for these reactions [1–3]. Information on the various pathways has been obtained by feeding either methionine-[CD_3] or mevalonic acid-[2-^{14}C(4*R*), 4-$^{3}H_1$] which labels cycloartenol (side chain **1**, Scheme 1) at C-24 with tritium [4,5]. With these two substrates phytosterols are obtained with various labelling patterns in the side chain in accord with the alkylation mechanism employed (Scheme 1). Thus the Chrysophyte alga, *Ochromonas malhamensis*, produced poriferasterol (side chain **7**) containing four deuterium atoms [6] and tritium at C-25 [5]. This indicated the intermediacy of a 24-ethylidenesterol (**5**, route *a*), a conclusion supported by the demonstration that *O. malhamensis* can convert various 24-ethylidenesterols into poriferasterol [7,8]. By contrast the Chlorophytes *Chlorella vulgaris* [9], *C. ellipsoidea* [10] and *Trebouxia* sp. [11] incorporated five deuterium atoms into C_{29} sterols (side chains **9** and **10**) so precluding the involvement of 24-ethylidene intermediates. Other evidence [11–13] suggests that in these algal species side chain alkylation proceeds via 25-methylene intermediates (**8**, route *d*). During ergosterol biosynthesis in yeast a 24-methylene intermediate is produced [14,15] and the alkylation mechanism proceeds with the migration of the C-24 hydrogen to C-25 [16].

The alkylation mechanisms operating in higher plants have been investigated using only mevalonic acid-[2-^{14}C (4*R*)-4-$^{3}H_1$] [2]. In *Clerodendrum campbellii* [17] the hydrogen at C-24 of (**1**) migrates to C-25 of (**3**) in the stabilization of cation (**2**) and then remigrates back to C-24 of (**8**) during the second alkylation (route *d*). However in several other higher plants the C-24 hydrogen of (**1**) is eliminated in the elaboration of stigmasterol [18] and spinasterol [19] (side chain **13** or **16**) and sitosterol [20] (side chain **12** or **15**) and the stabilization of cation **4** to give a $\Delta^{24(25)}$ intermediate (**11**) has been suggested [18]

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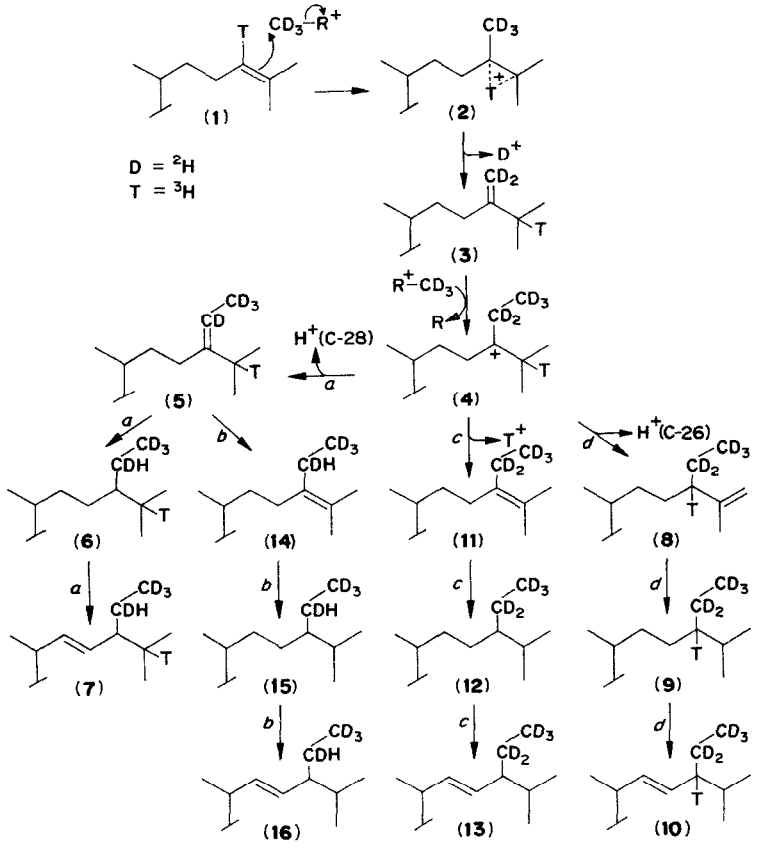


Scheme 1. Possible mechanisms for the formation of the phytosterol side chain indicating the labelling expected from methionine-[CD_3] and mevalonate-[(4*R*)-4-$^3H_1$].

as shown in route *c*. There is however considerable evidence [2] for the widespread occurrence and conversion of 24-ethylidenesterols (**5**) into 24-ethylsterols in higher plants and the alternative possibility, route *b*, involving the isomerization of a 24-ethylidene intermediate (**5**) into a Δ^{24} compound has been considered [19]. It is clear from Scheme 1 that routes *a*, *b*, *c* and *d* can be readily differentiated by the incorporation of both methionine-[CD_3] and mevalonic acid-[2-^{14}C(4*R*)-4-$^3H_1$] into the C_{29} sterols of a higher plant. The incorporation of labelled mevalonic acid into higher plant sterols is easily achieved [2] but the incorporation of methionine-[CD_3] into higher plant sterols in sufficient amounts for MS analysis is more difficult. We have found that although *Larix decidua* shoots and *Zea mays* leaves absorbed a solution of methionine-[CD_3] it was poorly incorporated into the sterols and excessive dilution by the non-deuterated endogenous sterols made impracticable the determination of the number of deuteriums incorporated. To overcome this problem we have utilized developing excised barley embryos. These can be cultured on a nutrient medium [21] and because the shoots and roots increase greatly in size during the culture period they presumably synthesize a relatively large quantity of new sterol which will not be excessively diluted by the endogenous sterol of the ungerminated embryo. Since the embryos retain the scutellum, the tissue responsible for the absorption of amino acids and other nutrients released in the endosperm during seed germination [22], this should facilitate the uptake of methionine-[CD_3] added to the amino acid-nutrient medium upon which the embryos are cultured. This paper reports the incorporation of methionine-[CD_3] and mevalonic acid-[2-^{14}C(*R*)-

4-$^3H_1$) into the sterols of the developing barley seedings and provides evidence for the operation of route *b* in a higher plant tissue.

RESULTS AND DISCUSSION

Barley embryos were first cultured in the presence of methionine-[CD_3] and the sterols isolated. GLC and MS analysis of the 4,4-dimethylsterol fraction revealed the presence of cycloartenol (*m/e* 426) and 24-methylenecycloartanol (*m/e* 440) in the approximate ratio of 1:2. The incorporation of two deuterium atoms into the side chain of 24-methylenecycloartanol (**3**) was shown by a MS molecular ion at *m/e* 442. Analysis of the 4α-monomethylsterols showed the presence of 24-methylenelophenol (60%, *m/e* 412) and 24-ethylidenelophenol (30%, *m/e* 426) which included molecular species with two (*m/e* 414) and four (*m/e* 430) deuterium atoms respectively. The 4-demethylsterols were a mixture of campesterol (30%) stigmasterol (20%) and sitosterol (50%). MS showed that the content of deuterium in these compounds was somewhat lower than in the 24-methylene- and 24-ethylidene-sterols, possibly owing to greater dilution by the unlabelled sterols present originally in the ungerminated embryo. We have observed previously (unpublished results) that deuterated sterols tend to elute slightly faster than the corresponding nondeuterated sterols during GLC analysis. Prior to MS analysis the three 4-demethylsterols were therefore purified and enriched in the deuterated species by collecting the leading portions of the sterol peaks as they eluted during preparative GLC. MS analysis (Fig. 1) clearly demonstrated the incorporation of two deuterium atoms into campesterol (M^+ at *m/e* 400 and 402) and molecular species containing none, one, three and four deuteriums in the stigmasterol (M^+ at *m/e* 412, 413, 415 and 416) and sitosterol (M^+ at *m/e* 414, 415, 417 and 418). These results provided convincing evidence for the intermediacy of 24-methylene and 24-ethylidene compounds in the biosynthesis of C_{28} and C_{29} sterols respectively.

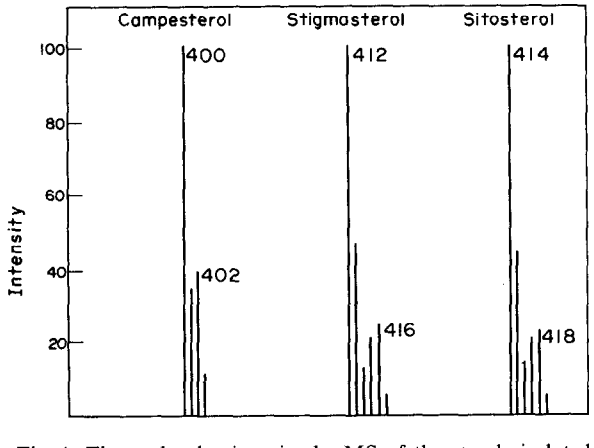
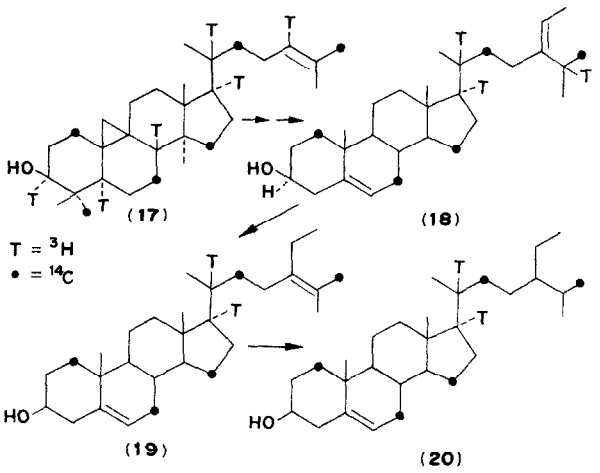


Fig. 1. The molecular ions in the MS of the sterols isolated from *Hordeum vulgare* cultured in the presence of methionine-[CD_3].

Confirmation of the role of 24-ethylidene compounds in the biosynthesis of C_{29} sterols in barley was provided by the conversion of labelled 24-ethylidenelophenol into sitosterol. Barley embryos were cultured in the presence of 24-ethylidenelophenol-[2,4-$^3H_3$] which resulted in 0·56% incorporation of label into the 4-demethylsterol fraction. The labelled sterols were acetylated, the sitosteryl acetate purified by TLC on $AgNO_3$ impregnated Sigel and then crystallized to constant specific radioactivity. Further conclusive evidence for the production of 24-methylene and 24-ethylidene compounds in barley was provided by the isolation and characterization by mp, NMR and MS, of 24-methylenelophenol and 24-ethylidenelophenol. In the latter compound the 24-ethylidene group was shown to have the *E*-configuration by the signal at δ 2·82 ppm for the C-25 hydrogen [23].

The fate of the C-24 hydrogen of cylcoartenol (side chain **1**) was determined by cultivating barley embryos in the presence of mevalonic acid-[2-^{14}C(4*R*)-4-$^3H_1$]. Previous work [2,4,6] has established the labelling pattern of cycloartenol (**17**, Scheme 2) biosynthesized from this stereospecifically labelled mevalonic acid. The labelled cycloartenol (**17**), produced by the barley seedlings was purified as the acetate and had the expected ^3H–^{14}C atomic ratio of 6:6 (Table 1). Labelled 28-isofucosterol (**18**) isolated from the same incubation had a ^3H–^{14}C atomic ratio of 3:5 showing that the C-24 tritium of the cycloartenol (**17**) was retained by migration to the C-25 position as previously reported [20,24,25]. However, for sitosterol (**20**) and stigmasterol (**20** but Δ^{22}) the ^3H–^{14}C atomic ratio was approx 2:5 thus showing the loss of the C-24 tritium of cycloartenol (**17**)



Scheme 2. Labelling patterns of sterols biosynthesized from mevalonate-[2-^{14}C(4*R*)-4-^{3}H$_1$].

during the introduction of the C-24 ethyl group as observed during phytosterol biosynthesis in several other higher plants [18–20]. The retention of only four deuterium atoms from methionine-[CD_3] clearly established a 24-ethylidenesterol, such as 28-isofucosterol (**18**), as a precursor of sitosterol (**20**). The loss of the C-25 hydrogen is therefore consistent with the isomerization of the 24-ethylidenesterol (**5**) to a $\Delta^{24(25)}$ compound (**14**) which can then be reduced to give the saturated sterol side chain (**15**, route *b*, Scheme 1). For example the isomerization of 28-isofucosterol (**18**) would give stigmasta-5,24-dien-3β-ol (**19**) with a ^{3}H–^{14}C atomic ratio of 2:5. The ^{3}H–^{14}C atomic ratio of 3:5 observed with 28-isofucosterol indicates that if such an isomerization occurs it cannot be readily reversible. In Chrysophyte algae a 24-ethylidene intermediate is reduced directly without prior isomerization to give a saturated sterol side chain with the 24β-configuration (24*S*) [3]. The occurrence of phytosterols with the 24α-configuration (24*R*) in higher plants may perhaps now be explained by the isomerization of the $\Delta^{24(28)}$ bond to the $\Delta^{24(25)}$ position resulting in the differing stereochemistry of the reductase enzyme.

The labelling results obtained in this work are also consistent with the production and subsequent reduction of a $\Delta^{24(28),25(26)}$ unsaturated side chain sterol. However, the involvement of a Δ^{24} sterol is mechanistically more attractive and gains support from the recent tentative identification of stigmasta-7,24-dien-3β-ol in a higher plant [26]. The incorporation of two deuterium atoms from methionine-[CD_3] into campesterol by barley reveals the intermediacy of a 24-methy-

Table 1. Specific radioactivities and ^{3}H–^{14}C ratios of the sterols isolated from barley seedlings after incubation with mevalonic acid-[2-^{14}C (4*R*) 4-^{3}H$_1$]

Compound	Crystallization number	Specific radioactivity (dpm of ^{14}C/mg)	Observed ^{3}H:^{14}C ratio	Normalized ^{3}H:^{14}C atomic ratio
Mevalonic acid	—	—	6·78	—
Cycloartenyl acetate	1	110	6·68	5·91:6
	2	106	6·85	6·06:6
	3	105	6·90	6·11:6
	4	107	6·74	5·96:6
	5	105	6·89	6·10:6
28-Isofucosteryl acetate	1	762	4·15	3·06:5
	2	745	4·23	3·12:5
	3	767	4·17	3·08:5
	4	753	4·27	3·15:5
	5	762	4·17	3·08:5
Stigmasteryl acetate	1	229	3·02	2·23:5
	2	228	3·08	2·27:5
	3	228	3·16	2·33:5
	4	237	3·07	2·26:5
	5	233	3·11	2·29:5
Sitosteryl acetate	1	753	3·03	2·23:5
	2	742	2·99	2·20:5
	3	733	3·01	2·22:5
	4	751	3·02	2·23:5
	5	787	2·97	2·19:5

lenesterol. Although the present results provided no evidence for isomerization of a Δ^{24} intermediate in C_{28} sterol production it is notable that ergosta-5,24-dien-3β-ol has now been isolated from *Withania somnifera* [27].

EXPERIMENTAL

Materials and methods. Mevalonic acid-[2-^{14}C] (17·5 mCi/mmol) and mevalonic acid-[(4*R*)4-$^{3}H_1$] (250 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, and mixed to give the mevalonic acid-[2-^{14}C(4*R*)-4-$^{3}H_1$]. 24-Ethylidenelophenol-[2,4-$^{3}H_3$] (5·92 mCi/mmol) was synthesized as described previously [8]. Methionine-[CD_3] was synthesized [28] by Dr. F. F. Knapp. Sterol extraction and purification, TLC, GLC, MS and radioactivity assay by liquid scintillation counting were as described previously [5,7,19].

Culture of barley embryos. Barley seed was dehusked and sterilized by gentle shaking for 1·5 hr in 50% H_2SO_4. The seeds were then washed with sterile dist. H_2O (10 × 500 ml). Embryos were dissected from the seed endosperms under sterile conditions, placed into covered sterile dishes (9·0 cm dia × 5·0 cm deep) containing sterile culture media (4·0 ml), and incubated at 23° in the light for 6 days.

Incorporation of methionine-[CD_3]. Barley embryos (80) were cultured in Rapport's mineral medium [21] supplemented with 5·0 mM L-glutamic acid (Na salt), 5·0 mM L-asparagine, 10·0 mM L-methionine-[CD_3], 5·0 mM mevalonic acid, 20·0 mM sucrose and 10 μM GA which was sterilized by Millipore (0·45 μm) filtration. Hydroxymethylglutaryl-CoA reductase is a control point in sterol synthesis in mammals [29] and it may play a similar role in plants although this is not yet established. Therefore unlabelled mevalonic acid was added to the media in order to by-pass this possible rate-limiting step and so stimulate maximal sterol production. GA was added to promote rapid growth of the seedlings. Non-saponifiable lipid (18·3 mg) from the seedlings (4·0 g fr. wt) was subjected to TLC (Si gel–$CHCl_3$) and the 4,4-dimethyl, 4α-methyl and 4-demethylsterols eluted and analysed by GLC and MS. The 4-demethylsterols were purified by preparative GLC on 2% OV-17 and the leading edges of the peaks trapped and their MS determined.

Incorporation of 24-ethylidenelophenol-[2,4-$^{3}H_3$]. 24-Ethylidenelophenol-[2,4-$^{3}H_3$] (10 μCi) was solubilized in 0·05% Triton X-100 (2 ml) by sonication, sterilized by filtration and administered to barley embryos. The non-saponifiable lipid (16·9 mg) was extracted and the 4-demethylsterols ($1·25 \times 10^5$ dpm) purified twice by TLC (Sigel–$CHCl_3$), acetylated, and the sitosteryl acetate (5700 dpm) isolated by TLC (10% w/w $AgNO_3$ impregnated Si gel–EtOH–free $CHCl_3$). After addition of carrier sitosteryl acetate (11·1 mg) the mixture was crystallized to constant specific radioactivity (512, 480, 491, 504, 502 dpm/mg).

Incorporation of mevalonic acid-[2-^{14}C(4*R*)-$^{3}H_1$]. Barley embryos (80) were cultured in the presence of mevalonic acid-[2-^{14}C (4*R*) 4-$^{3}H_1$] (2·0 μCi of ^{14}C) and the non-saponifiable lipids (17·2 mg) extracted. TLC (Si gel–$CHCl_3$) gave the 4,4-dimethylsterols ($4·96 \times 10^4$ dpm of ^{14}C) and the 4-demethylsterols ($1·79 \times 10^5$ dpm of ^{14}C). Carrier cylcoartenol (5 mg) was added to the 4,4-dimethylsterols, the mixture acetylated, and cycloartenyl acetate ($2·3 \times 10$ dpm of ^{14}C) purified by TLC (10% w/w $AgNO_3$ impregnated Si gel) and crystallized to constant specific radioactivity (Table 1) after addition of more cycloartenyl acetate (20 mg). Carrier 28-isofucosterol (2 mg), stigmasterol (2 mg) and sitosterol (2 mg) were added to the 4-demethylsterols, the mixture acetylated and separated by TLC (10% $AgNO_3$ impregnated Si gel developed 2× with EtOH-free $CHCl_3$). 28-Isofucosteryl acetate (R_f 0·10–0·16, $4·54 \times 10^4$ dpm of ^{14}C), stigmasteryl acetate (R_f 0·36–0·42, $4·92 \times 10^3$ dpm of ^{14}C) and sitosterol acetate (R_f 0·48–0·54, $4·71 \times 10^4$ dpm of ^{14}C) were eluted and crystallized to constant specific radioactivity after addition of further carrier steryl acetate (Table 1).

Identification of 24-methylenelophenol and 24-ethylidenelophenol in barley grain. Barley grain (5 kg) was ground and the total lipid extracted with EtOH (12 l.). Saponification gave the non-saponifiable lipid (6·29 g) from which the 4α-methylsterols (109 mg) were isolated by chromatography on alumina [30]. Acetylation followed by TLC on 10% $AgNO_3$ impregnated Sigel-C_6H_6–hexane (2:3) gave 24-methylenelophenyl acetate (33 mg) and 24-ethylidenelophenyl acetate (54 mg). 24-Methylenelophenyl acetate: mp 126°; MS: *m/e* 454, 439, 394, 379, 370, 327, 269, 227; NMR ($CDCl_3$): δ0·54 (3H, *s*, C-18), 0·84 (3H, *s*, C-19), 0·84 (3H, *d*, C-30) 0·95 (3H, *d*, C-21), 1·04 (6H, *d*, C-26, C-27), 2·04 (3H, *s*—OAc) 4·40 (1H, *bm*, C-3α) 4·65 and 4·70 (2H, *S*, C-28), 5·16 (1H, *m*, C-7). 24-Ethylidenelophenyl acetate: mp 144°; MS: *m/e* 468, 453, 408, 393, 370, 327, 269, 267, 227; NMR ($CDCl_3$): δ0·54 (3H, *s*, C-18), 0·84 (3H, *s*, C-19), 0·85 (3H, *d*, C-30) 0·94 (3H, *d*, C-21), 0·97 (6H, *d*, C-26, C-27), 1·58 (3H, *d*, C-29), 2·04 (3H, *s*,—OAc), 2·82 (1H, *septet*, C-25), 4·40 (1H, *bm*, C-3α), 5·10 (1H, *q*, C-28), 5·16 (1H, *m*, C-7).

Sterol nomenclature: Campesterol: (24*R*)-24-methylcholest-5-en-3β-ol; cycloartenol: 9β, 19-cyclo-5α-lanost-24-en-3β-ol; 24-ethylidenelophenol: 4α-methyl-5α-stigmasta-7,*Z*-24(28)-dien-3β-ol; 24-methylenecycloartanol: 4,4,14α-trimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol; 24-methylenelophenol: 4α-methyl-5α-ergosta-7,24(28)-dien-3β-ol; poriferasterol: (24*R*)-24-ethylcholesta-5,*E*-22-dien-3β-ol; sitosterol: stigmast-5-en-3β-ol; spinasterol: 5α-stigmasta-7,*E*-22-dien-3β-ol; stigmasterol: stigmasta-5,*E*-22-dien-3β-ol.

Acknowledgements—We thank the SRC for financial support and the PCMU, Harwell, England for the determination of 100 MHz NMR spectra.

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