Phytochemistry, 1973, Vol. 12, pp. 2181 to 2187. Pergamon Press. Printed in England.

BIOSYNTHESIS OF α-SPINASTEROL FROM (2-^{14}C,(4*R*)-4-$^{3}H_1$) MEVALONIC ACID BY *SPINACEA OLERACEA* AND *MEDICAGO SATIVA*

WILFRED L. F. ARMAREGO*, L. JOHN GOAD and TREVOR W. GOODWIN
Department of Biochemistry, The University, P.O. Box 147, Liverpool L69 3BX

(*Received* 12 *March* 1973. *Accepted* 2 *April* 1973)

Key Word Index—*Spinacea oleracea*; Chenopodiaceae; *Medicago sativa*; Leguminoseae; phytosterol biosynthesis; α-spinasterol; stigmast-7-enol; 24-methylcholest-7-enol; incorporation of (2-^{14}C, (4*R*)-4-$^{3}H_1$) mevalonic acid; mechanism of side chain alkylation.

Abstract—Leaves of *Spinacea oleracea* and *Medicago sativa* were incubated with (2-^{14}C, (4*R*)-4-$^{3}H_1$) mevalonic acid and the sterols isolated. Cycloartenol had a ^{3}H:^{14}C atomic ratio of 6:6 whilst oxidation to cycloartenone resulted in a ratio of 5:6 showing that tritium was present in the 3α-position and that the cycloartenol was symmetrically labelled. Separation of the 4-demethyl sterols gave α-spinasterol and a mixture of stigmast-7-enol and 24-methylcholest-7-enol, which had ^{3}H:^{14}C atomic ratios of 3:5. Ozonolysis of α-spinasteryl acetate gave the terminal side chain fragment as 2-ethyl-3-methyl butanoic acid. The acid contained ^{14}C but no tritium thus showing that the C-24 hydrogen of cycloartenol is lost during the alkylation reactions leading to the C-24 ethyl group of α-spinasterol.

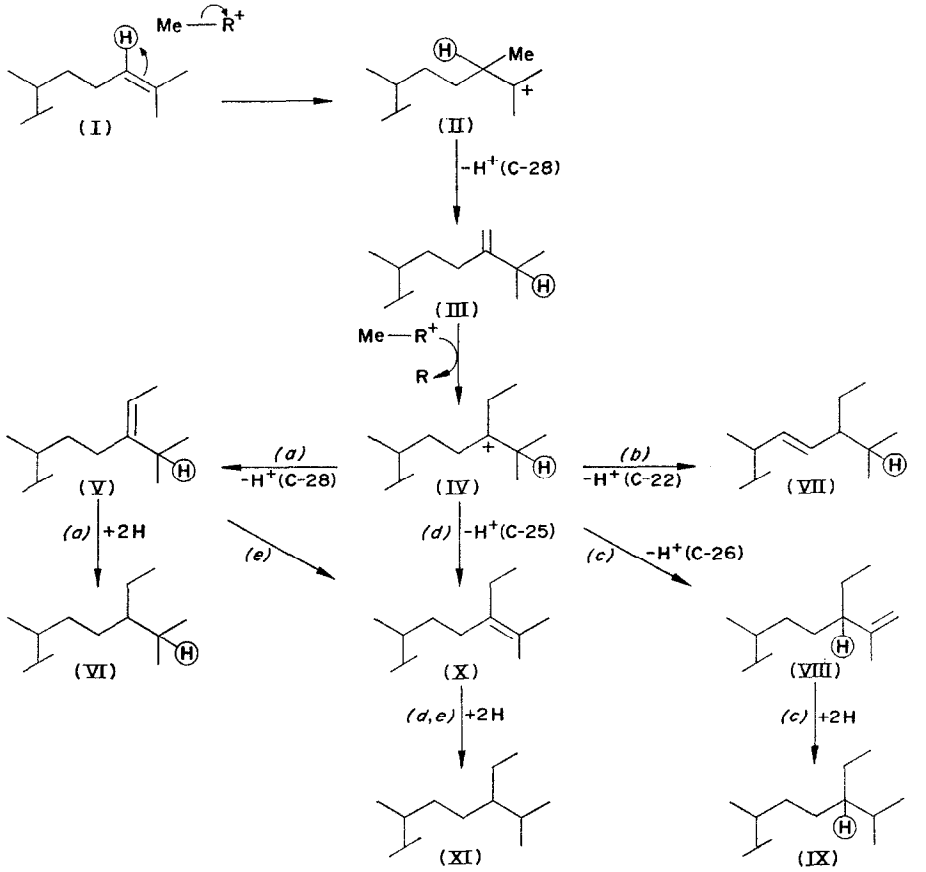
INTRODUCTION

IN STUDIES on phytosterol biosynthesis considerable attention has been focussed on the introduction of the C-24 alkyl group.[1,2] It was initially suggested that an ethyl group (VI) arose via a 24-ethylidene sterol (V) (Route *a*, Scheme 1). In higher plants this gained support from the occurrence of 24-ethylidene sterols,[2] the labelling of isofucosterol (side chain V) from radioactive precursors[3–6] and evidence for the conversion of isofucosterol into sitosterol (side chain VI).[5,6] The operation of route *a* for poriferasterol (XIIa) production by the chrysophyte *Ochromonas malhamensis* has been demonstrated by the use of (CD_3)—methionine,[7] the conversion of 24-ethylidene sterols into poriferasterol[8] and by the labelling pattern obtained with (2-^{14}C, (4*R*)-4-$^{3}H_1$) mevalonic acid (MVA).[9] This species of MVA gives cycloartenol in higher plants[10] and algae with the labelling as shown in XIII. In *O. malhamensis*[9] this cycloartenol was metabolised to give poriferasterol (XIIa) with a ^{3}H:^{14}C atomic ratio of 3:5 and in which the C-24 tritium atom of (XIII) had migrated to

* Present address: Medical Chemistry, The John Curtin School of Medical Research, The Australian National University, Box 4 P.O. Canberra, A.C.T. Australia 2600.

[1] LEDERER, E. (1969) *Quart Rev. (London)*, **23**, 453.
[2] GOAD, L. J. and GOODWIN, T. W. (1972) *Progr. Phytochem.* **3**, 113.
[3] RAAB, K. H., DE SOUZA, N. J. and NES, W. R. (1968) *Biochim. Biophys Acta* **152**, 742.
[4] GOAD, L. J., GIBBONS, G. F., BOLGER, L. M., REES, H. H. and GOODWIN, T. W. (1969) *Biochem. J.* **114**, 885.
[5] VAN ALLER, R. T., CHIKAMATSU, H. C., DE SOUZA, N. J., JOHN, J. P. and NES, W. R. (1968) *Biochem. Biophys Res. Commun.* **31**, 842.
[6] BAISTED, D. J. (1969) *Phytochemistry* **8**, 1697.
[7] SMITH, A. R. H., GOAD, L. J., GOODWIN, T. W. and LEDERER, E. (1967) *Biochem. J.* **104**, 56C.
[8] KNAPP, F. F., GREIG, J. B., GOAD, L. J. and GOODWIN, T. W. (1971) *Chem. Commun.* 707.
[9] SMITH, A. R. H., GOAD, L. J. and GOODWIN, T. W. (1972) *Phytochemistry* **11**, 2775.
[10] REES, H. H., GOAD, L. J. and GOODWIN, T. W. (1968) *Biochem. J.* **107**, 417.

C-25 (XIIa) as required by route *a*. However alternative mechanisms have been revealed. In a slime mould, *Dictyostelium discoideum*, route *b* has been suggested[11] to give stigmast-22-en-β-ol (side chain VII) while it appears that route *c* involving a 25-methylene intermediate (VIII) may be operative in some Chlorococcales[12,13] and higher plants.[14] Stigmasterol (XIIb) biosynthesized by *Nicotinia tabacum* and *Dioscorea tokoro* from (2-^{14}C, (4*R*)-4-$^{3}H_1$) MVA had a ^{3}H:^{14}C atomic ratio of 2:5 with no tritium located at either C-24

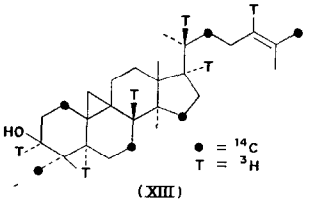


SCHEME 1.

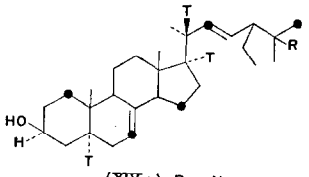
or C-25.[15] In this case the C-24 tritium of the precursor cycloartenol (XIII) was lost in the alkylation process and the formation of a $\Delta^{24(25)}$ intermediate (X) from the carbonium ion (IV) is suggested (route *d*). In another study[16] administration of (2-^{14}C, (4*R*)-4-$^{3}H_1$) MVA to *Camelia sinensis* gave α-spinasterol with a ^{3}H:^{14}C atomic ratio of 2·44:5 and from which the

[11] ELLOUZ, R. and LENFANT, M. (1971) *European J. Biochem.* **23,** 544.
[12] TOMITA, Y., UOMORI, A. and MINATO, H. (1970) *Phytochemistry* **9,** 555; TOMITA, Y., UOMORI, A. and SAKURAI, E. (1971) *Phytochemistry* **10,** 573.
[13] GOAD, L. J., KNAPP, F. F., LENTON, J. R. and GOODWIN, T. W. (1972) *Biochem. J.* **129,** 219.
[14] BOLGER, L. M., REES, H. H., GHISALBERTI, E. L., GOAD, L. J. and GOODWIN, T. W. (1970) *Biochem. J.* **118,** 197.
[15] TOMITA, Y. and UOMORI, A. (1970) *Chem. Commun.* 1416.
[16] SHARMA, R. K. (1970) *Phytochemistry* **9,** 565.

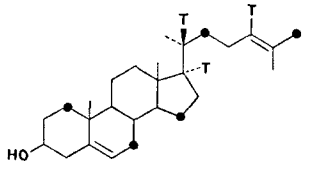
side chain was cleaved[17] by ozonolysis to give 2-ethyl-3-methyl butanoic acid with a $^3H:^{14}C$ atomic ratio of 0·5:1. This half an atom equivalent of tritium was thought to result from the simultaneous operation of two alkylation mechanisms, one involving loss and the other retention of the C-24 hydrogen of cycloartenol. Desmosterol (XV) or a related C_{27} compound was apparently considered[16] as an α-spinasterol precursor and presumably accounts for the indicated[16] loss of tritium from C-5 of the α-spinasterol although loss of the C-5 tritium should not occur by the routes usually suggested[2] for the conversion of cycloartenol into a sterol such as α-spinasterol. If in fact the C-5 tritium of cylcoartenol (XIII) is retained in the derived α-spinasterol it would be anticipated to have a $^3H:^{14}C$ atomic ratio of either 3:5 (XIVa) or 4:5 (XIVb) depending upon the nature of the C-24 alkylation mechanism employed.



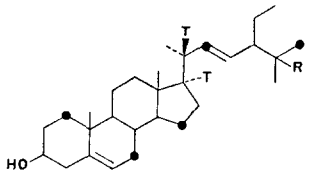
(XIII)



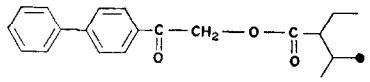
(XIVa) R = H
(XIVb) R = T



(XV)



(XIIa) (24*R*), R = T
(XIIb) (24*S*), R = H



(XVI)

Preliminary studies carried out in our laboratory by Dr. F. F. Knapp indicated that the Δ^7 sterols biosynthesised by *Spinacea oleracea* and *Medicago sativa* from (2-^{14}C, (4*R*)-4-$^3H_1$) MVA had $^3H:^{14}C$ atomic ratios of approximately 3:5 but difficulty in separating the sterol mixtures prevented the determination of accurate ratios. We now describe the results of a re-examination of this problem which was undertaken in view of the reports discussed above.

RESULTS AND DISCUSSIONS

The major sterol of both *M. sativa*[18] and *S. oleracea*[19] has previously been reported as α-spinasterol. However re-examination of sterols from these plants by GLC and MS showed them to be mixtures of α-spinasterol, stigmast-7-enol and 24-methylcholest-7-enol in the approximate proportions 34:10:1. As noted previously[20] these sterols are difficult to separate but we found that after acetylation resolution into α-spinasteryl acetate and a mixture of stigmast-7-enyl and 24-methylcholest-7-enyl acetate could be achieved by TLC on $AgNO_3$-Kieselgel when developed with ethanol-free redistilled chloroform.

Incubation of *S. oleracea* and *M. sativa* leaves with (2-^{14}C, (4*R*)-4-$^3H_1$) MVA gave labelled squalene, 4,4-dimethyl sterols and 4-demethyl sterols which were separated by TLC. The 4-demethyl sterol fraction was acetylated, diluted with a carrier steryl acetate

[17] SHARMA, R. K. (1970) *Current Sci.* **39**, 466.
[18] HART, M. C. and HEYE, F. W. (1932) *J. Biol. Chem.* **95**, 311.
[19] FERNHOLZ, E. and MOORE, M. L. (1939) *J. Am. Chem. Soc.* **61**, 2467.
[20] CLARK-LEWIS, J. W. and DAINIS, I. (1967) *Australian J. Chem.* **20**, 1961.

mixture obtained from *M. sativa*, and separated into the component steryl acetates by $AgNO_3$-TLC. The α-spinasteryl acetate was crystallized to constant specific radioactivity and a constant $^3H:^{14}C$ ratio (Table 1). The mixture of stigmast-7-enyl and 24-methylcholest-7-enyl acetates was also crystallized to a constant $^3H:^{14}C$ ratio (Table 1) and further analysed by GLC with sample trapping which showed the two compounds to have similar $^3H:^{14}C$ ratios.

Isolation of the labelled squalene fraction and conversion into the hexahydrochloride after addition of carrier squalene showed that the incorporation of radioactivity was low and an accurate $^3H:^{14}C$ ratio could not be obtained. The $^3H:^{14}C$ ratio of the starting mevalonic acid applied to the plant tissue was therefore used for normalizing the $^3H:^{14}C$ atomic ratios presented in Table 1. The radioactivity in the 4,4-dimethyl sterol fraction was relatively high and this material was diluted with cycloartenol carrier, acetylated and purified by $AgNO_3$–TLC. Elution of the cycloartenyl acetate band and crystallization to constant specific radioactivity (Table 1) gave a $^3H:^{14}C$ atomic ratio of 6:6 consistent with previous reports.[2,10]

The $^3H:^{14}C$ atomic ratio of 3:5 for the α-spinasteryl acetate indicated that no tritium was located at C-24 or C-25. To confirm this point the labelled α-spinasteryl acetates obtained from the two plants were ozonized and the products oxidized with Jones' reagent. The acidic fractions were diluted with carrier 2-ethyl-3-methyl butanoic acid and converted into the *p*-phenyl phenacyl ester[11] (XVI), purified by TLC and crystallized to constant specific radioactivity. In both cases the radioactive ester (XVI) contained ^{14}C but was devoid of tritium. A tritium atom, if present at C-24 of the α-spinasterol should not have been lost during the preparation of (XVI) since the reaction conditions were essentially similar to those used previously[11] when a tritium at this position was retained in the ester (XVI).

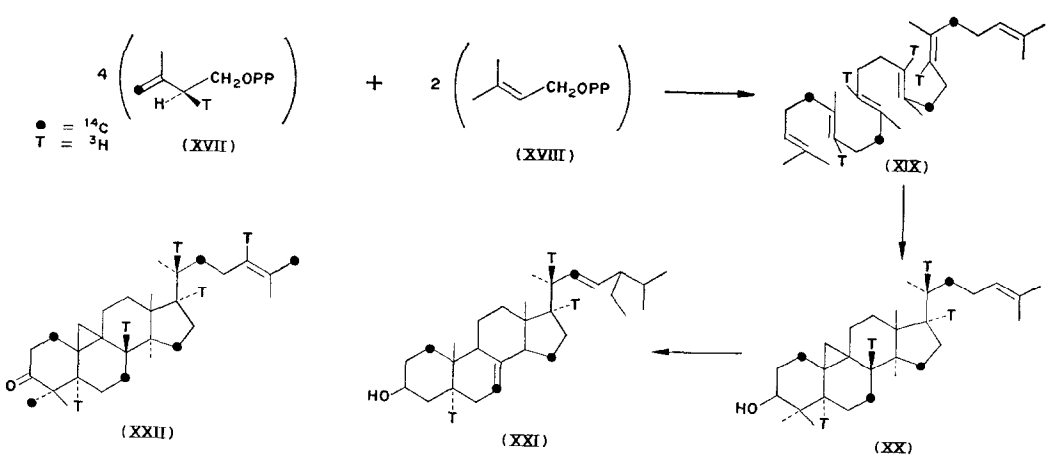
TABLE 1. INCORPORATION OF [2-^{14}C, (4*R*)-4-$^3H_1$] MEVALONIC ACID

	Spinacea oleracea			*Medicago sativa*		
	$^3H:^{14}C$ ratio	$^3H:^{14}C$ atomic ratio	Specific radioactivity (dpm of ^{14}C/mg)	$^3H:^{14}C$ ratio	$^3H:^{14}C$ atomic ratio	Specific radioactivity (dpm of ^{14}C/mg)
Mevalonic acid	6·64	1:1	—*	8·93	1:1	—†
α-Spinasteryl acetate	4·09	3·07:5	5430	5·50	3·08:5	372
1st Crystallization	3·96	2·98:5	5300	5·65	3·16:5	362
2nd Crystallization	3·96	2·98:5	5490	5·58	3·12:5	400
3rd Crystallization	3·95	2·97:5	5220	5·65	3·16:5	365
Stigmast-7-enyl and 24-methylcholest-7-enyl acetate mixture	4·07	3·06:5	7540	—	—	—
1st Crystallization	3·92	2·95:5	7490	5·48	3·01:5	1240
2nd Crystallization	3·92	2·95:5	7200	5·45	3·01:5	1240
Cycloartenyl acetate	6·71	6·06:6	3210	8·60	5·78:6	456
1st Crystallization	6·59	5·95:6	3220	8·81	5·91:6	423
2nd Crystallization	6·63	5·90:6	3210	8·58	5·76:6	377
3rd Crystallisation	6·55	5·92:6	3280	8·71	5·85:6	369

* Total used ~12 μCi of ^{14}C.
† Total used ~2·5 μCi of ^{14}C.

Feeding experiments with *Camelia sinensis* gave only a low incorporation of (2-^{14}C, (4*R*)-4-$^{3}H_1$) MVA into the isolated α-spinasteryl acetate (0·012% incorporation) and mixture of stigmast-7-enyl and 24-methylcholest-7-enyl acetates (0·047% incorporation). Consequently accurate $^{3}H:^{14}C$ ratios could not be determined but the approximate $^{3}H:^{14}C$ atomic ratio of 2·86:5 obtained for the α-spinasteryl acetate was higher than the value of 2·44:5 reported earlier[16] for α-spinasterol from this species.

Non-symmetrical labelling of monoterpenes biosynthesised from radioactive MVA is now well documented for several higher plants.[21] Such labelling, which is observed with low levels of incorporation, apparently occurs via the condensation of labelled isopentenyl pyrophosphate (XVII) derived from the administered MVA, with unlabelled dimethylallyl pyrophosphate (DMAPP, XVIII) from an endogenous pool. The possibility of such a situation arising during phytosterol biosynthesis from MVA does not appear to have been considered before but its occurrence could in some cases affect the interpretation of results where dual labelled mevalonic acids are used. This is illustrated in Scheme 2 which shows that the intervention of a large pool of endogenous DMAPP (XVIII) would yield cycloartenol (XX) from (2-^{14}C, (4*R*)-4-$^{3}H_1$) MVA via squalene (XIX) unlabelled at C-26, C-30, H-3 and H-24. However both the squalene (XIX) and the cycloartenol (XX) would still have $^{3}H:^{14}C$ ratios identical to the starting MVA so that a simple determination of $^{3}H:^{14}C$ ratios would not reveal the operation of this phenomenon. Subsequent metabolism of the cycloartenol (XX), for example to α-spinasterol (XXI), would give sterol containing only *four* positions labelled with ^{14}C and which could therefore lead to erroneous conclusions of tritium content if the $^{3}H:^{14}C$ atomic ratio were calculated on the assumption that the sterol contained *five* ^{14}C atoms as normally expected.[2] It was therefore desirable to check this point in the present work and this was achieved by determining the tritium content at C-3 of the cycloartenol biosynthesised by the *S. oleracea* leaves. The cycloartenol obtained by hydrolysis of the purified cycloartenyl acetate was oxidized to cycloartenone (XXII) with a loss of one sixth of the tritium. This result demonstrated symmetrical labelling of the cycloartenol (XIII) and showed that endogenous unlabelled DMAPP had not been incorporated significantly into the squalene and sterols. This conclusion was further substantiated



SCHEME 2.

[21] BANTHORPE, D. V., CHARLWOOD, B. V. and FRANCIS, M. J. O. (1972) *Chem. Rev.* **72**, 115.

by the presence of ^{14}C in the 2-ethyl-3-methyl butanoic acid derived from the α-spinasterol side chain. Consequently the absence of tritium in the terminal portion of the α-spinasterol side chain arises from the C-24 alkylation mechanism and not from non-symmetrical labelling. Although the three tritium atoms remaining in the α-spinasterol were not located by degradation they are presumably located as shown in (XIVa) by analogy with other sterols.[2,9,22]

Hydrogen migration from C-24 to C-25 after biological transmethylation of a Δ^{24} sterol (I) to give a 24-methylene sterol (III) has been demonstrated in several organisms.[2] Further methylation of (III) produces the carbonium ion (IV) from which a 24-ethylidene (V) and 24-ethyl sterol (VI) could be derived as shown by route *a*. However this would lead to the retention of the C-24 hydrogen of (I) and this clearly does not occur in α-spinasterol (XIVa) production by *S. oleracea* or *M. sativa* nor for the C_{29} sterols synthesized by other species of higher plant.[15,23] It is suggested[15] that carbonium ion (IV) is stabilized (route *d*) by loss of the C-25 hydrogen to give a Δ^{24} sterol (X) followed by reduction to a C-24 ethyl sterol (XI). However the possibility that a 24-ethylidene compound (V) is first formed and then is isomerised (route *e*) to a Δ^{24} sterol (X) is equally consistent with the data available and we have now obtained evidence[24] for the operation of this mechanism in *Hordeum vulgare* (barley).

EXPERIMENTAL

General procedures. M.ps were determined on a Reichert hot stage apparatus. IR spectra were measured on a Perkin Elmer 237 spectrometer as KBr discs. MS were obtained on an AEI MS12 instrument and accurate masses were determined on an AEI MS9 spectrometer (by Dr. J. Clark, University of Salford). NMR spectra were at 100 MH_z in $CDCl_3$ with tetramethyl silane as internal standard. TLC was on 0·5 mm thickness plates of Kieselgel-H. GLC analysis was performed on 1·5 m × 6·4 mm columns of either 3% OV1 or 3% OV17 on Gaschrom Q. In GLC trapping experiments a 1:10 splitter was employed and samples were trapped in glass capillary tubes at ambient temp. Radioactivity measurements were made on a Beckmann LS200 spectrometer using the external standardization method to correct for quenching. [(4*R*)-4-$^3H_1$] Mevalonic acid (MVA) was a mixture of [(3*R*)-(4*R*)-4$^3H_1$] MVA and [(3*S*)-(4*S*)-4$^3H_1$] MVA, (134 mCi/mmol) and [(3*RS*)-2-^{14}C] MVA (5·85 mCi/mmol) purchased from the Radiochemical Centre, Amersham and mixed in ratios of about 8:1 to give the samples of [2-^{14}C, (4*R*)-4-$^3H_1$] MVA used in this work.

Sterols of M. sativa. Freshly collected leaves and stems (2 kg) were homogenized and extracted with EtOH. Concentration of the extract and saponification gave the non-saponifiable lipid (6·8 g). This was subjected to chromatography on alumina (500 g, Brockmann grade III) eluting with mixtures of Et_2O in light petrol. to give the 4-demethyl sterol containing fraction (478 mg) which was crystallized from Et_2O–MeOH to give colourless needles (257 mg). GLC showed three peaks corresponding to stigmast-7-enol, α-spinasterol and 24-methylcholest-7-enol in the ratio of 10:34:1. These were identified further by trapping and measuring their MS. The sterol mixture was acetylated (250 mg) to provide the material used as carrier in subsequent experiments. A portion of the steryl acetate mixture (55 mg) was separated by TLC (Keiselgel H-10% $AgNO_3$ (w/w) developed with EtOH free $CHCl_3$) into α-spinasteryl acetate (33 mg: R_f 0·45; M^+ 454.3784, $C_{31}H_{50}O_2$ requires: M^+ 454.3811; ν_{max} 1738 (C=O), 1250 and 972 (–CH=CH–*trans*) cm^{-1}; NMR (δ, ppm): 0·55 (*s*, 18–Me), 0·82 (*s*, 19-Me), 0·82 (*t*, 29-Me, *J* 6 Hz), 0·85 (*d*, 26- and 27-Me) 4·70 (broad *m*, 3α-H), 3·50 (complex *m*, 7-, 22- and 23-H); m.p. 182–183°, lit,[19] m.p. 183° and a mixture of stigmast-7-enyl acetate and 24-methylcholest-7-enyl acetate (13 mg; R_f 0·55; M^+ 456.3967, $C_{31}H_{52}O_2$ requires: M^+ 456.3940, also *m/e* peak at M^+ 442; ν_{max}: 1730 and 1247 cm^{-1} m.p. 154–155°, lit.[25] m.p. for stigmast-7-enyl acetate 156–157°).

Plant incubations. Leaves (5 g) of freshly collected *S. oleracea* (seedlings, 70–200 cm tall), *M. sativa* and *C. sinensis* (containing terminal buds) were cut into 2–3 mm strips, [2-^{14}C, (4*R*)-4-$^3H_1$] MVA (3·0 ml) added

[22] MULHEIRN, L. J. and CASPI, E. (1971) *J. Biol. Chem.* **246**, 3948.
[23] RANDALL, P. J., REES, H. H. and GOODWIN, T. W. (1972) *J.C.S. Chem. Commun.* 1295.
[24] LENTON, J. R. and GOAD, L. J. unpublished results.
[25] BARTON, D. H. R. and COX, J. D. (1948) *J. Chem. Soc.* 1354.

and the flasks kept at room temp. At 24 hr more H_2O (3·0 ml) was added and after a further 24 hr the incubation was stopped by the addition of 80% EtOH containing 15% KOH (w/v) and the mixture refluxed for 1 hr. Et_2O extraction gave the non-saponifiable lipids.

Sterol purification. Non-saponifiable lipids were separated into squalene, 4,4-dimethyl sterol and 4-demethylsterol fractions by preparative TLC on Kieselgel H developed with pure $CHCl_3$. The 4-demethyl sterol fraction from *M. sativa* was acetylated, diluted with steryl acetate carrier (51 mg) and subjected to TLC on 10% (w/w) $AgNO_3$-Kieselgel H developed with EtOH free $CHCl_3$. The slower moving band gave labelled α-spinasteryl acetate (25·3 mg; 0·36% incorporation of ^{14}C) which was recrystallized from Et_2O–MeOH (Table 1). The faster moving band gave a mixture of labelled stigmast-7-enyl and 24-methylcholest-7-enyl acetates (10:1; 9·2 mg; 0·59% incorporation of ^{14}C) which was recrystallized from Et_2O–MeOH (Table 1). Purity of the two fractions was checked by GLC, m.p. and IR. Similarly the corresponding 4-demethyl sterol from the *S. oleracea* incubation was acetylated, diluted with carrier steryl acetate (35 mg) and separated by $AgNO_3$-Kieselgel TLC into α-spinasteryl acetate (28·1 mg; 0·47% incorporation of ^{14}C) and a mixture of stigmast-7-enyl and 24-methylcholest-7-enyl acetates (10:1; 12·7 mg; 0·79% incorporation of ^{14}C).

The 4,4-dimethyl sterol fraction from *S. oleracea* was acetylated, diluted with authentic cycloartenyl acetate (6 mg) and purified by TLC on 10% (w/w) $AgNO_3$-Kieselgel H developed with C_6H_6–light petrol. (1:1, v/v). The recovered cycloartenyl acetate was diluted with more carrier (37·7 mg) and recrystallized from Et_2O–MeOH to give pure (by GLC and i.r.) labelled material (36·8 mg; ν_{max} 1738 and 1246 cm^{-1}; m.p. 123–124°, lit.[26] m.p. 122·5–123·5°). The 4,4-dimethyl sterols from *M. sativa* were treated in a similar manner to give labelled cycloartenyl acetate (35·1 mg). These were recrystallized from Et_2O-MeOH to constant specific radioactivity and ^3H: ^{14}C ratios (Table 1).

p-*Phenyl phenacyl 2-ethyl-3-methyl butanoate from labelled α-spinasteryl acetate.* Ozone was passed through a solution of α-spinasteryl acetate (10 mg; from *S. oleracea*) dissolved in dry CH_2Cl_2 (15 ml) containing pyridine (0·15 ml) at −80° until a blue colour persisted. The solution was then flushed with N_2 to remove excess ozone, concentrated (3 ml) and acetone (5 ml) and Jones' reagent (1 ml) added. After stirring for 10 min H_2O (15 ml) was added and the mixture extracted with Et_2O (5 × 20 ml). The acid recovered after removal of the Et_2O was diluted with carrier 2-ethyl-3-methyl butanoic acid (38·8 mg) and H_2O (5 ml) containing one drop of phenolphthalein solution was added followed by 0·1 M NaOH until the solution was just alkaline. To this mixture was added *p*-phenyl phenacyl bromide (130 mg) in hot EtOH (15 ml), the mixture refluxed for 1·5 hr and then left overnight. The residue, after evaporation, was extracted with Et_2O and evaporated to give the crude ester (103 mg). This was purified by TLC (Kieselgel G(EtOH free $CHCl_3$) and the ester (R_f 0·52) eluted with Et_2O and crystallized from aq. MeOH to give *p*-phenyl phenacyl-2-ethyl-3-methyl butanoate (54·3 mg, 18 d.p.m. of ^{14}C/mg). Because of the low specific radioactivity relatively large amounts of ester were counted to obtain 200 cpm above background. This resulted in quenching in scintillation counting but after quench correction by the external standard method negligible tritium was found in the ester. Two further crystallizations did not alter the ^{14}C specific radioactivity. Similar results were obtained with labelled α-spinasteryl acetate (24 mg) from *M. sativa*. The esters had m.p. 67–68° (lit.[26] m.p. 66–68°), MS: identical to lit;[27] IR: ν_{max} 1738, 1700, 1170 cm^{-1}; NMR: (δ, p.p.m.): 098 (*t*, 2″-Me, *J* 7 Hz), 1·02 (*d*, 4-and 1′-Me, *J* 7 Hz), 1·50–2·40 (*m*, 2- and 3-H and 1″–CH_2), 5·36 (*s*, CH_2–O), 7·30–8·10 (*m*, aromatic H).

Oxidation of labelled cycloartenol to cycloartenone. Labelled cycloartenyl acetate from *S. oleracea* (20·2 mg, ^3H:^{14}C ratio 6·21:1, ^3H:^{14}C atomic ratio 6:6) was converted to cycloartenol (14·7 mg) by stirring for 2 hr in dry Et_2O (6 ml) with excess $LiAlH_4$. The cycloartenol was oxidized in dry acetone (3 ml) with Jones' reagent (0·4 ml) by stirring at room temp. for 4 min. The mixture was diluted with H_2O (4 ml), extracted with Et_2O and the cycloartenone purified by TLC (Kieselgel H developed with $CHCl_3$). The cycloartenone (7·5 mg; m.p. 106°, lit.[26] m.p. 105°; IR: ν_{max} 1714 cm^{-1}; M^+ at 424, $C_{30}HO_{48}$ requires: 424) was crystallized from Et_2O–MeOH to constant specific radioactivity (389 dpm of ^{14}C/mg) and a ^3H:^{14}C ratio of 5·13:1 corresponding to a ^3H:^{14}C atomic ratio of 4·96:6.

Acknowledgements—We are indebted to Dr F. F. Knapp for performing preliminary experiments and for discussions, Mr D. Abramson for a generous gift of cycloartenol and Mr K. Hulme, Nes Botanical Gardens for the supply of plants. This work was financed, in part, by the SRC.

[26] BARTON, D. H. R. (1951) *J. Chem. Soc.* 1444.
[27] LENFANT, M., ELLOUZ, R., DAS, B. C., ZISSMAN, E. and LEDERER, E. (1968) *European J. Biochem.* **7**, 159