Biosynthesis of Isoprenoids. Part III.[1] Mechanism of Alkylation during Biosynthesis of Stigmasterol in Tissue Cultures of Higher Plants

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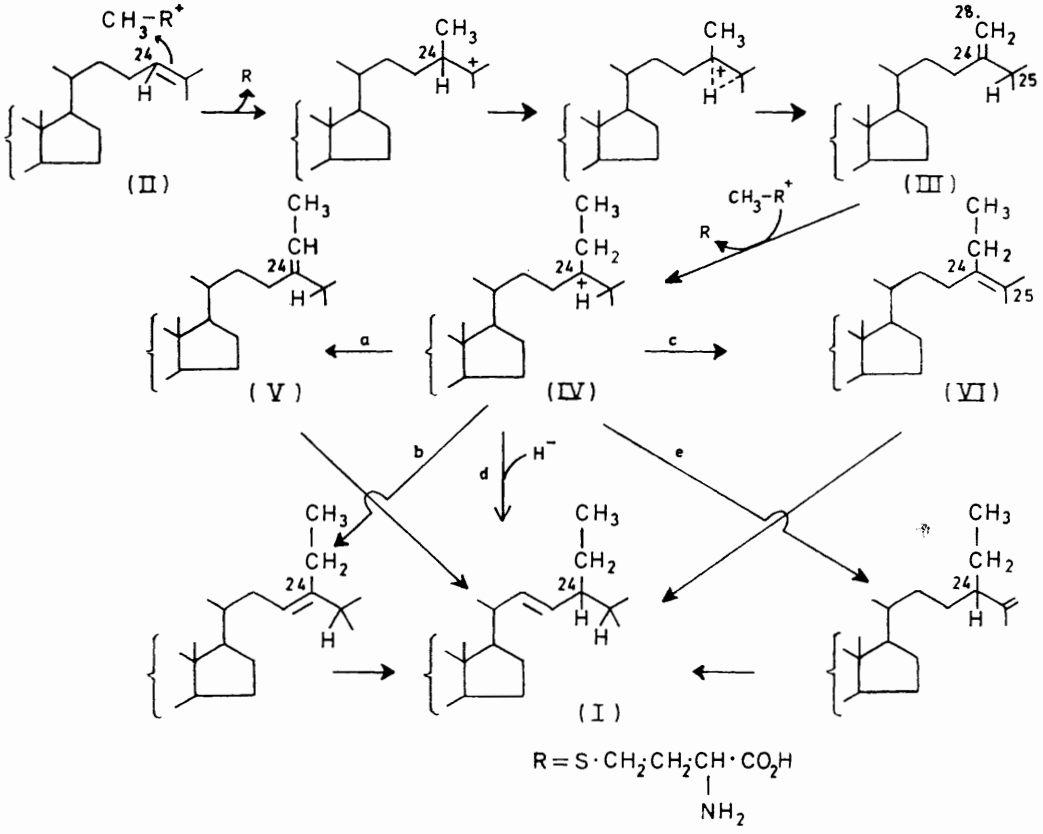
The C-24 hydrogen atom of the Δ^{24} precursor is eliminated during biosynthesis of stigmasterol in tissue cultures of *Nicotiana tabaccum* and *Dioscorea tokoro*. Thus the $\Delta^{24(25)}$ intermediate is involved in stigmasterol biosynthesis.

PHYTOSTEROLS having an alkyl group at C-24 are usually present both in intact plants and tissue cultures, and these compounds are thought to play a physiological role in plant cells, although the nature of this role is not yet known. Moreover, conversion of 24-methyl- and ethyl-sterols into ecdyson *via* cholesterol in insects has been demonstrated[2] and Lederer has suggested[3] that the process of dealkylation at C-24 in insects may in part be the reverse of C-24 alkylation in plants.

The mechanism of alkylation at C-24 during phytosterol biosynthesis has, therefore, been studied by several groups.[3] The 24-methyl group of ergosterol has been shown to arise from methionine, and the ethyl group at C-24 in β-sitosterol[4] and α-spinasterol[5] and the ethylidene group of fucosterol[6] are also derived by double transmethylation from methionine. The first transfer (Scheme 1) of a methyl group to a 24,25-double bond in compound (II) leads to 24-methylene compound (III) with migration of a hydrogen atom from C-24 to C-25[7,8] and the second transmethylation to the 24,28-double bond of the intermediate (III) forms carbonium ion (IV), which can conceivably be stabilized by several routes (a, b, c, d, and e). It was thought that an ethylidene compound (V) such as fucosterol is a precursor for the 24-ethyl-sterol, and this has been proved



SCHEME 1

[1] Preliminary report, Y. Tomita and A. Uomori, *Chem. Comm.*, 1970, 1416.
[2] M. J. Thomson, J. A. Suoboda, J. N. Kaplains, and W. E. Robbins, *Proc. Roy. Soc.*, 1972, *B*, **180**, 203.
[3] E. Lederer, *Quart. Rev.*, 1969, **23**, 453.
[4] M. Castle, G. Blondin, and W. R. Nes, *J. Amer. Chem. Soc.*, 1963, **85**, 3306.
[5] S. Bader, L. Guglielmetti, and D. Arigoni, *Proc. Chem.*, 1964, 16.
[6] V. Villanueva, M. Barbier, and E. Lenderer, *Bull. Soc. chim. France*, 1964, 1423.
[7] K. H. Raab, J. De Souza, and W. R. Nes, *Biochim. Biophys. Acta*, 1968, **152**, 742.
[8] M. Akhtar, P. F. Hunt, and M. A. Parvez, *Biochem. J.*, 1967, **103**, 1616.

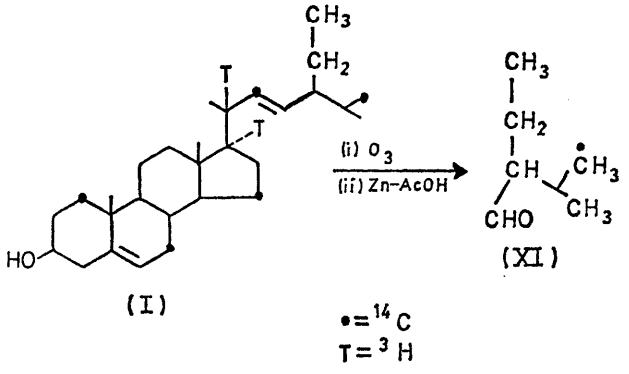
in the case of poriferasterol synthesized by *Ochromonas malhamensis* (route a).[9] On the other hand, Lenfant *et al.* showed that stigmast-22-en-3β-ol in *Dictyostelium discideum* is synthesized by a mechanism which does not involve a C-24 ethylidene intermediate (route b, c, d, or e).[10] In previous reports, we have shown that the C-24 ethylidenesterol (V) is not involved in the biosyntheses of chondrillasterol, Δ^7-chondrillastenol, and poriferasterol in Chlorella and suggested a $\Delta^{24(25)}$ intermediate (VI) for their biosynthesis.[11,12]

The above findings obtained in micro-organisms were obtained from incorporation of [*methyl*-$^2H_3$]methionine, but the mechanism of alkylation during phytosterol biosynthesis in higher plants has not yet been clarified, since incorporation of [*methyl*-$^2H_3$]methionine is too small for the number of deuterium atoms in the ethyl group to be determined, even in tissue cultures.

Therefore, we report here a study of the mechanism of alkylation during stigmasterol biosynthesis, made on the basis of hydrogen elimination at C-24.

Cells of *Nicotiana tabaccum* tissue cultures grown in Linsmaier–Skoog medium containing (3*R*)-[2-^{14}C,4-*pro*-*R*-^{3}H]mevalonic acid (VII) were extracted and a phytosterol mixture was isolated from unsaponifiable lipid. Stigmasterol (I) was obtained as an acetate from the mixture by preparative t.l.c. on silver nitrate–silica gel and was finally recrystallized to constant specific radioactivity after addition of carrier stigmasteryl acetate.

It has been demonstrated that C_{27} sterol, such as cholesterol (IX), derived from (3*R*)-[2-^{14}C,4-*pro*-R-^{3}H]-mevalonic acid (VII) is labelled with three tritium atoms,



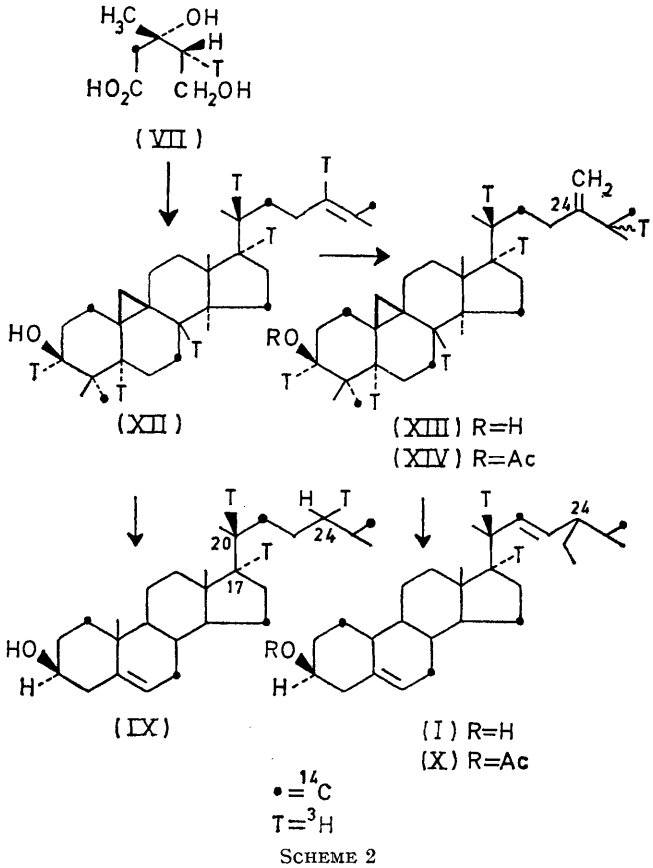
at C-17, C-20, and C-24;[13,14] the stigmasteryl acetate (X) obtained here, however, was labelled with two ^{3}H and five ^{14}C. To determine the locations of the tritium atoms the stigmasteryl acetate was decomposed with ozone and the side chain fragment (XI) was obtained as its dimedone derivative. As shown in the Table, 95% of the tritium present at C-24 of the C_{30} precursor was lost during transmethylation. Cycloartenol (XII) and

The ^{3}H/^{14}C ratios for cycloartenol, 24-methylenecycloartanol, and stigmasterol derived from[2-^{14}C,4-*pro*-*R*-^{3}H]mevalonic acid, and their degradation products

	^{3}H/^{14}C	^{3}H : ^{14}C (Atomic) Exp.	Theor.
Cycloartenyl acetate [a]	9·15	6·03 : 6	6 : 6
24-Methylenecycloartanyl acetate [a]	9·08	5·98 : 6	6 : 6
24-Oxocycloartanol [a]	7·52	4·95 : 6	5 : 6
Stigmasteryl acetate [a]	3·90	2·14 : 5	2 : 5
Stigmasteryl acetate [b]	5·40	2·17 : 5	2 : 5
Dimedone derivative of (VII) [a]	0·45	0·049 : 1	0 : 1

[a] Derived from mevalonic acid, ^{3}H/^{14}C = 9·1 in *N. tabaccum*.
[b] Derived from mevalonic acid, ^{3}H/^{14}C = 12·4 in *D. tokoro*.

24-methylenecycloartanol (XIII), precursors for phytosterol,[15,16] were isolated as acetates.[17] These acetates



SCHEME 2

were labelled with six ^{3}H and six ^{14}C, and 24-oxocycloartanyl acetate, prepared by degradation of 24-methylenecycloartanyl acetate (XIV) with ozone, lost

[9] A. R. Smith, L. J. Goad, T. W. Goodwin, and E. Lederer, *Biochem. J.*, 1967, **104**, 56c.
[10] M. Lenfant, R. Ellouz, B. C. Das, E. Zissmann, and E. Lederer, *European J. Biochem.*, 1969, **7**, 159.
[11] Y. Tomita, A. Uomori, and H. Minato, *Phytochem.*, 1970, **9**, 555.
[12] Y. Tomita, A. Uomori, and E. Sakurai, *Phytochem.*, 1971, **10**, 573.
[13] J. W. Cornforth, R. H. Cornforth, C. Donninger, Y. Shimizu, S. Ichii, E. Forchielli, and E. Caspi, *J. Amer. Chem. Soc.*, 1965, **87**, 3224.
[14] E. Caspi and L. J. Mulheirn, *Chem. Comm.*, 1969, 1423.
[15] P. Benveniste, L. Hirth, and G. Ourisson, *Phytochem.*, 1966, **5**, 45; M. J. E. Hewlins, J. D. Ehrhardt, L. Hirth, and G. Ourisson, *European J. Biochem.*, 1969, **8**, 184.
[16] J. Hall, A. R. H. Smith, L. J. Goad, and T. W. Goodwin, *Biochem. J.*, 1969, **162**, 129.
[17] P. Benveniste, L. Hirth, and G. Ourisson, *Phytochem.*, 1966, **5**, 31.

one tritium atom on alkaline treatment. Thus, the tritium atom at C-24 of cycloartenol was not lost in 24-methylenecycloartanol and migration of a tritium atom from C-24 to C-25 takes place in the first transmethylation (Scheme 2). Therefore, elimination of the tritium must occur in the second transmethylation. As shown in Scheme 1, if stigmasterol (I) is synthesized by route a, b, d, or e, the hydrogen atom at C-24 derived from the 4-*pro*-*R*-hydrogen of mevalonic acid should be retained at C-25, whereas if stigmasterol is synthesized by route c the hydrogen atom should be eliminated during biosynthesis. Thus stigmasterol is synthesized by route c in the tissue cultures.

Moreover, stigmasteryl acetate isolated from tissue cultures of *Dioscorea tokoro* grown with (3*R*)-[2-^{14}C, 4-*pro*-*R*-^{3}H]mevalonic acid (VII) was labelled with two ^{3}H and five ^{14}C. Tissue cultures of *D. tokoro* were incubated with [24-^{3}H]cycloartenol (XII), and sapogenins and stigmasterol were isolated as described previously.[18] Sapogenins obtained contained radioactivity;[19] but no radioactivity was present in stigmasterol, owing to elimination of the tritium atom at C-24. These results are consistant with those observed in *N. tabaccum* tissue cultures.

Recently, Randall *et al.*[20] showed that the C-24 hydrogen of the Δ^{24} intermediate was eliminated during biosynthesis of β-sitosterol in *Larix decidua* and supported the operation of route c. Therefore, phytosterols having an ethyl group at C-24 in higher plants are synthesized by route c involving a $\Delta^{24(25)}$ intermediate (VI).

EXPERIMENTAL

M.p.s were determined on a hot stage apparatus. Mass spectra were obtained on a Hitachi RMU-6 instrument. [2-^{14}C]Mevalonic acid (10 mCi mmol^{-1}) and [4-*pro*-*R*-^{3}H]-mevalonic acid (250 mCi mmol^{-1}) were purchased from the Radiochemical Centre, Amersham. They were mixed in the proportion of *ca.* 1 : 10 to give the (3*R*)-[2-^{14}C,4-*pro*-*R*-^{3}H]mevalonic acid (VII) used for incubation with tissue cultures of *Nicotiana tabaccum* and *Dioscorea tokoro*. Radioactive measurements were made on a Nuclear Chicago model 720 scintillation counter.

Tissue cultures of *Nicotiana tabaccum* and *Dioscorea tokoro* were cultured on Linsmaier–Skoog agar medium fortified with 2·4D (10^{-6} mmol) and kinetine (0·2 p.p.m.).

Incorporation of [2-^{14}C,4-pro-R-^{3}H]*Mevalonic Acid into Cycloartenol* (XII), *24-Methylenecycloartanol* (XIII), *and Stigmasterol* (I) *in Tissue Cultures of* Nicotiana Tabaccum.—(*a*) Callus of *Nicotiana tabaccum* was cultured in the presence of [2-^{14}C,4-*pro*-*R*-^{3}H]mevalonic acid (^{14}C = 10 μCi; ^{3}H/^{14}C = 9·1) under sterile conditions. After 1 week cells were harvested and extracted twice with boiling methanol, for 4 h each time. The extracts were combined and evaporated to dryness under reduced pressure. The residue was saponified with ethanolic 10% potassium hydroxide for 3 h at the boiling point. The solution, after addition of water, was extracted three times with petroleum and the extracts were washed with water, dried (Na_2SO_4), and evaporated to dryness. The residue (^{14}C = 8·5 μCi) was applied to silica gel plates and developed with n-hexane–ethyl acetate–chloroform (4 : 1 : 1). The bands corresponding to 4,4-dimethylsterols (R_F 0·27) and phytosterols (R_F 0·15) were eluted. The 4,4-dimethylsterol fraction was mixed with a mixture of cycloartenol and 24-methylenecycloartanol, and acetylated with acetic anhydride–pyridine. The acetate mixture was chromatographed on 20% $AgNO_3$-impregnated silica gel plates and developed with n-hexane–chloroform–acetic acid (75 : 20 : 0·5). The bands corresponding to the acetates of cycloartenol (R_F 0·11) and 24-methylenecycloartanol (R_F 0·08) were extracted, then rechromatographed on the same system. Radioactive cycloartenyl acetate was mixed with carrier cycloartenyl acetate (50 mg) and recrystallized three times to constant specific radioactivity (^{3}H/^{14}C = 9·15; ^{3}H : ^{14}C atomic ratio 6·03 : 6).

The radioactive 24-methylenecycloartanyl acetate (XIV) was mixed with carrier 24-methylenecycloartanyl acetate and recrystallized three times to constant specific radioactivity (^{3}H/^{14}C = 9·08; ^{3}H : ^{14}C atomic ratio 5·9 : 6).

(*b*) The phytosterol fraction was acetylated with acetic anhydride–pyridine. The acetate was applied to 20% $AgNO_3$-silica gel plates and developed with n-hexane–chloroform–acetic acid (75 : 25 : 0·5), and stigmasteryl acetate [(X) R_F 0·1] isolated was rechromatographed on the same system. After addition of carrier stigmasteryl acetate (100 mg), the acetate was recrystallized three times to constant specific radioactivity, m.p. 141—142° (^{3}H/^{14}C = 3·90; ^{3}H : ^{14}C atomic ratio 2·14 : 5).

Incorporation of [2-^{14}C,4-pro-R-^{3}H]*Mevalonic Acid into Stigmasterol* (I) *in Tissue Cultures of* Dioscorea Tokoro.—Callus of *D. tokoro* was cultured in the presence of [2-^{14}C,4-*pro*-*R*-^{3}H]mevalonic acid (^{14}C = 10 μCi; ^{3}H/^{14}C = 12·4) under sterile conditions for 1 week. Cells were harvested and extracted twice with boiling ethanol, for 4 h each time. The extracts were combined and saponified with ethanolic 10% potassium hydroxide solution. A mixture of phytosterols (R_F 0·15) was isolated from the unsaponifiable material by preparative silica gel t.l.c. (n-hexane–ethyl acetate–chloroform, 4 : 1 : 1). The phytosterol fraction was acetylated with acetic anhydride–pyridine, and the stigmasteryl acetate (X) was isolated by $AgNO_3$-silica gel t.l.c. as described above. After addition of carrier stigmasteryl acetate (50 mg), the acetate was recrystallized to constant specific radioactivity, m.p. 142—143° (^{3}H/^{14}C = 5·40; ^{3}H : ^{14}C atomic ratio 2·17 : 5).

Location of Tritium Atoms in 24-*Methylenecycloartanol* (XIII) *Biosynthesized from* [2-^{14}C,4-pro-R-^{3}H]*Mevalonic Acid.*—24-Methylenecycloartanyl acetate (XIV) (30 mg) biosynthesized from [2-^{14}C,4-*pro*-*R*-^{3}H]mevalonic acid (^{3}H/^{14}C = 9·1) in callus of *Nicotiana tabaccum* was dissolved in chloroform (10 ml), and the solution was treated with ozone at −70° for 10 min. The solvent was removed under reduced pressure and zinc dust (300 mg) was added to a solution of the residue in acetic acid (3 ml) with stirring at room temperature.

After 1 h the mixture was filtered. The filtrate was diluted with water and extracted with benzene. The extract was washed with water, dried (Na_2SO_4), and evaporated to dryness. The residue was dissolved in alcoholic 5% potassium hydroxide solution (5 ml) and left

[18] Y. Tomita, A. Uomori, and H. Minato, *Phytochem.*, 1970, **9**, 111.

[19] Y. Tomita and A. Uomori, *Chem. Comm.*, 1971, 284.

[20] P. J. Randall, H. H. Rees, and T. W. Goodwin, *J.C.S. Chem. Comm.*, 1972, 1295.

for 8 h at room temperature. After dilution with water the solution was extracted with benzene. The benzene extract was washed with water, dried (Na_2SO_4), and evaporated to dryness. The residue was acetylated and recrystallized from methanol to constant specific radioactivity, m.p.[21] 121—122° ($^3H/^{14}C$ = 7·52; ^3H : ^{14}C atomic ratio 4·9 : 6).

Location of Tritium Atoms in Stigmasteryl Acetate (X) *Biosynthesized from* [2-^{14}C,4-pro-R-^{3}H]*Mevalonic Acid in* N. Tabaccum.—A solution of stigmasteryl acetate (X) (50 mg) in chloroform (20 ml) was treated with ozone at −70° for 20 min and the solvent was removed under reduced pressure. Zinc dust (300 mg) was added to a solution of the residue in acetic acid (5 ml) with stirring. After 1 h, water was added to the mixture and a side-chain fragment (XI) was isolated by steam distillation. The distillate was neutralized with dilute sodium hydroxide solution, then alcoholic dimedone solution was added and the mixture left for 24 h. The precipitated dimedone derivative was recrystallized from methanol to constant specific radioactivity, as needles, m.p. 127—128°, M^+ 376 ($^3H/^{14}C$ = 0·45; ^3H : ^{14}C atomic ratio 0·049 : 1).

Incorporation of [24-^{3}H]*Cycloartenol into Steroidal Sapogenin and Phytosterol in Callus of* D. Tokoro.—A mixture of [24-$^3H_1$]cycloartenol (0·1 mCi) and water (5 ml) containing Tween 80 (15 mg) was added to callus of *D. tokoro* under sterile conditions. After 1 week, cells (wet wt. 60 g) were harvested and extracted twice with 70% ethanol at the boiling point. The extract was divided into two portions. One portion was saponified with alcoholic 10% sodium hydroxide and stigmasteryl acetate was isolated by preparative 20% $AgNO_3$-silica gel t.l.c. as described above. The acetate was recrystallized twice after addition of carrier acetate (50 mg). The stigmasteryl acetate obtained here contained no radioactivity.

The second portion of the extract was refluxed with 5% hydrochloric acid and sapogenins were extracted with ethyl acetate. Tokorogenin was isolated from the extract as described previously[21] and recrystallized from methanol to constant specific radioactivity after addition of carrier tokorogenin (50 mg) (total radioactivity, 4·12 × 10^4 disint. min^{-1}.

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[21] G. Ohta, *Chem. and Pharm. Bull.* (*Japan*), 1960, **8**, 9.