Biosynthesis of Phytosterols in *Calendula officinalis* Flowers from (2*R*)- and (2*RS*)-[2-^{14}C, 2-^{3}H]-Mevalonic Acid. The Incorporation of a 15α-Tritium Atom into Sitosterol

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Summary It is proven that only the 2-*pro-R* hydrogen of MVA is retained at the 15α-position of sitosterol biosynthesized by excised petals of *Calendula officinalis* flowers.

RECENTLY results indicating the possible retention of both the 2-*pro-R* and 2-*pro-S* hydrogen atoms of mevalonic acid at C-15 of sterols biosynthesized in marigold flowers (*Calendula officinalis*) were reported.[1] This could imply that the biosynthesis of phytosterols from cycloartenol, which is thought to be an intermediate in higher plants,[2,3] proceeds by a different route from the biosynthesis of sterols from lanosterol in rat livers[4] and yeast preparations.[5]

We have established that cycloartenol (or an intermediate with an anionic terminus at C-19) is a key precursor of phytosterols in the pea.[3] In addition we showed that the end result of events around C-14 and C-15 in the biosynthesis of sitosterol in germinating peas[6] was the same as in cholesterol[4] and in sterols[5] biosynthesized in rat livers and yeast homogenates, respectively. In view of the obvious inconsistency of the results for *Calendula* flowers we reinvestigated the biosynthesis of phytosterols in this plant.

Calendula officinalis plants (*cv.* Radio) of the same variety as previously used[1] were grown from seeds in an environmental chamber (8 weeks). Petals (120, *ca.* 1·18 g fresh wt.) isolated from several flowers, were placed vertically in an open weighing glass, with the ends previously attached to the flowers immersed in an aqueous solution of (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]MVA (11 μCi of ^{14}C; ^{3}H:^{14}C ratio 11·3). The assembly was then placed in the environmental chamber and when the MVA solution was absorbed (*ca.* 6 h) water was supplied as needed. After 6 days the petals were collected and saponified to yield a non-saponifiable residue (5·57 × 10^{6} d.p.m. of ^{14}C).

TABLE

Specific activities of ^{14}C and $^{3}H:^{14}C$ ratios of metabolites and their transformation products (see text)[a]

No.	Compound	Expt. A: (3*RS*,2*R*)-[2-^{14}C,2-^{3}H]MVA ^{14}C Specific activity	$^{3}H:^{14}C$ Ratio Isotopic	$^{3}H:^{14}C$ Ratio Atomic	Expt. B: (3*RS*,2*RS*)-[2-^{14}C,2-$^{3}H_2$] ^{14}C Specific activity	$^{3}H:^{14}C$ Ratio Isotopic	$^{3}H:^{14}C$ Ratio Atomic
1	MVA-amide	18·10	11·09		12·4	20·76	
2	Squalene 6HCl	8·50	9·14		30·1	13·33	
3	(**1**)	22·80	8·90	5.00 : 5	7·26	14·30	8·00 : 5
3a	(**1**) after bromination and debromination	22·88	8·87		7·23	14·36	
4	(**2**)	7·58	8·90	5·00 : 5	7·20	14·44	8·07 : 5
5	(**3**)	7·54	7·57	4·25 : 5	7·17	12·94	7·27 : 5
6	(**4**)	7·71	7·53	4·23 : 5	7·20	13·01	7·30 : 5
7	(**5**)	7·61	7·37	4·14 : 5	3·36	12·75	7·16 : 5
8	(**6**)	7·54	8·92	5·01 : 5	7·25	14·48	8·10 : 5
9	Stigmasterol acetate				6·66	12·97	7·25 : 5

[a] The results are the average of at least three crystallizations in which the ^{14}C specific activity and $^{3}H:^{14}C$ remained constant (±3%). Specific activity × 10^4 in d.p.m. per mmol. The MVA and squalene were counted as benzhydrylamide and hexahydrochloride, respectively. The results are significant to ±3%. The atomic ratios are calculated on the basis of atomic ratio of sitosteryl acetate (assumed to be 5-^{3}H : 5-^{14}C in expt. A and 8-^{3}H : 5-^{14}C in expt. B).

Sitosterol acetate —i→ 5α-stigmastanol acetate —ii→ 5α-stigmast-14-en-3β-ol acetate —iii→ 5α-stigmastane-3β,14α,15β-triol 3-acetate
(**1**) (**2**) (**3**) (**4**)

—iv→ 15-oxo-5α-stigmastane-3β,14α-diol 3-acetate.
(**5**)

SCHEME. i: EtOAc; $HClO_4$; PtO_2; H_2. ii: C_6H_6; $C_6H_5ICl_2$; *hν*. iii: a, $CHCl_3$; *m*-ClC_6H_4COOOH; b, Me_2CO; H_2O; HIO_4. iv: CrO_3–pyridine.

An analogous experiment (102 petals; 1·19 g fresh wt.) with (3*RS*,2*RS*)-[2-^{14}C,2-$^{3}H_2$]MVA (19 μCi of ^{14}C; $^{3}H:^{14}C$ ratio 20·7) was carried out and a non-saponifiable residue ($9·87 \times 10^6$ d.p.m. of ^{14}C) was obtained. The '*R*' and '*RS*' squalenes and sitosteryl acetates were extensively purified by chromatography, derivatization, and crystallization to yield homogenous metabolites.[1,5]

The calculated and observed $^{3}H:^{14}C$ atomic ratios of MVA benzhydrylamide, squalene hexahydrochloride, and sitosteryl acetate (Table; expts. A and B, entries 1—3) show significant discrepancies. Such discrepancies were previously noted and are considered to be related to the metabolism of polyprenoids in plants.[1,6]

The obtained '*R*' and '*RS*' sitosteryl acetates were then submitted to the sequence of transformations outlined in the Scheme. The transformation of the derived '*R*' and '*RS*' 5α-stigmastanyl acetate to 5α-stigmast-14-en-3β-ol acetate and 5α-stigmast-9(11)-en-3β-ol acetate was carried photochemically in the presence of $C_6H_5ICl_2$.[7] We have proven that the C-14 dehydrogenation of 5α-cholestan-3β-ol acetate involved the overall abstraction of the *cis* 14α and 15α hydrogen atoms.[5] It may be assumed with certainty that the C-14(15) dehydrogenation of 5α-stigmastan-3β-ol acetate will also proceed *via* the removal of the 14α and 15α hydrogen atoms.

For the discussion of the results we will assume that the specimens of sitosteryl acetates have the indicated $^{3}H:^{14}C$ atomic ratios (Table; expts. A and B, entries 3). These ratios are based on results on the biosynthesis of sterols in several species (rat, pea, yeast, *etc.*).

The results show that the '*R*' sitosteryl acetate (Table; expt. A) and '*RS*' sitosteryl acetate (Table; expt B) contain tritium only in the 15α position (*ca.* 0·75 atom) and are devoid of tritium at the 15β position.

In view of these results it must be concluded that the previous observations were in error.[1] It is also apparent that the end result of the biosynthetic events at C-14 and C-15 in *Calendula* flowers, rat liver homogenates,[4] yeast homogenates,[5] and in germinating peas[6] are analogous. Very likely the loss of a hydrogen derived from 2-*pro-S* of MVA from C-15 occurs in the course of the formation of the C-14(15) double bond. However, since 5α-stigmast-8(14),-15,24(28)-trien-3β-ol was isolated from *Vernonia anthelminitica* plant,[8] this hypothesis requires experimental verification.

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