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STEREOSPECIFICITY OF STEROL BIOSYNTHESIS IN *CALENDULA OFFICINALIS* FLOWERS

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Abstract—Flowers of *Calendula officinalis* were incubated with mevalonic acid doubly labelled with ^{14}C in position 2 and ^{3}H in positions 2R, 2S, 4R or 5R,S and the [$^{3}H/^{14}C$] ratios determined in squalene β-sitosterol, stigmasterol, Δ^7-sterols and stigmastan-3 β-ol The results indicated that in the biosynthesis of these sterols· formation of the Δ^7 double bond is associated with elimination of hydrogen from the 7β position, formation of the Δ^5 double bond with elimination of hydrogens from the 5 and 6α positions, and formation of the Δ^{22} double bond with elimination of the 22-pro-S and 23 hydrogens Demethylation in position 4 is associated with elimination of hydrogen from the 3α position whereas demethylation in position 14 occurs without hydrogen loss from position 15 Alkylation in position 24 is associated with hydrogen elimination from this position

INTRODUCTION

EARLIER studies[1,2] on the flowers of *Calendula officinalis* demonstrated the presence of β-sitosterol, stigmasterol and unidentified Δ^7-sterols. GLC of the sterols isolated from the green parts of the plant (Turowska–Adler, unpublished results) showed that in the Δ^7-sterols fraction stigmast-7-en-3β-ol is present with small quantities of ergost-7-en-3β-ol, whereas in the Δ^5-sterols fraction β-sitosterol is accompanied by campesterol (less than 5%). Small amounts of stigmastan-3β-ol also occur. In our present work the stereospecificity of biosynthesis of these sterols in *C. officinalis* flowers was investigated using mevalonic acid (MVA) preparations doubly labelled with ^{14}C in position 2 and with ^{3}H in positions 2R, 2S, 4R or 5R,S.

RESULTS AND DISCUSSION

Preliminary experiments[3] demonstrated that the lactone of [2-^{14}C] MVA is two to three times more efficiently incorporated than the sodium salt into triterpenes and sterols in the flowers of *C. officinalis*, the most favourable incubation time being 120–130 hr. It was also found that the optimal dose of [2-^{14}C] MVA lactone is 50 μCi/g of fresh flowers. After feeding with the doubly labelled MVA preparations, a nonsaponifiable fraction was isolated into which 20–35% of the precursor radioactivity was incorporated. From this fraction pure squalene, β-sitosterol, stigmasterol, the Δ^7 sterol fraction and stigmastan-3β-ol were isolated and the radioactivity ratios [$^{3}H/^{14}C$] determined.

The [$^{3}H/^{14}C$] ratios of the four different preparations of labelled MVA used in our experiments together with the values obtained for squalene isolated after feeding with these

[1] KASPRZYK, Z AND TUROWSKA, G (1969) *Bull Acad Polon Sci, Ser Sci Chim* **17**, 397
[2] PYREK, J ST (1969) *Chem Commun* 107
[3] ŚLIWOWSKI, J (1973) Ph D Thesis, Warsaw

preparations are given in Table 1 The results demonstrate that the [$^3H/^{14}C$] ratios in squalene biosynthesized from MVA labelled with ^{14}C in position 2 and ^3H in positions 2R, 2S, or 5R,S, were about 20% lower than those of the original MVA In contrast, for squalene biosynthesized from [2-^{14}C,4R,4-^3H] MVA the ratio was essentially the same as in the precursor The lower ratio in squalene biosynthesized from [2-^{14}C,5R,S,5-$^3H_2$] MVA was not 18 but 10% because of the elimination of one ^3H atom during condensation of two farnesyl pyrophosphate units to squalene [4-6] Therefore, in all results obtained with this precursor a ratio of 11.6 was assumed in squalene

TABLE 1 [$^3H/^{14}C$] RATIOS OF MVA PREPARATIONS AND OF SQUALENE ISOLATED FROM *C. officinalis* FLOWERS AFTER INCUBATION WITH THE PREPARATIONS

MVA preparation	Compound	Radioactivity of the sample [(dpm) × 10^{-3}] ^3H	^{14}C	[$^3H/^{14}C$] ratios	Isotopic effects (%)
[2-^{14}C,2R,2-^3H]	MVA Precursor	194315	66005	2.94	–
	Squalene	36	16	2.37	20
[2-^{14}C,2S,2-^3H]	MVA Precursor	275176	96182	2.86	–
	Squalene	335	151	2.22	22
[2-^{14}C,5R,S,5-$^3H_2$]	MVA Precursor	345798	68800	5.03	–
	Squalene	536	131	4.11	18 (10*)
[2-^{14}C 4R,4-^3H]	MVA Precursor	243611	89563	2.72	–
	Squalene	306	115	2.66	2

* Calculated on the basis of a 11.6 atomic ratio in squalene

Isotopic effects produced by ^3H atoms localized in positions 5R,S, 2R or 2S of MVA may be additionally explained by the lower affinity of MVA molecules labelled with ^3H in position 5 for the enzymes responsible for phosphorylation to MVA pyrophosphate and by isopentenyl–dimethylallyl pyrophosphate isomerase activity[6,7] as well as by the lower affinity of MVA molecules labelled with ^3H in position 2 (both 2R or 2S) toward the enzymatic system catalyzing MVA pyrophosphate transformation to isopentenyl pyrophosphate [8] The lack of a distinct decrease in the [$^3H/^{14}C$] ratio in squalene obtained from [2-^{14}C,4R,4-^3H] MVA, as noted by Goodwin *et al* [9,10] indicates an absolute stereospecificity of the reaction of squalene biosynthesis from MVA towards the position 4-pro-R of this precursor and also to an unchanged affinity of molecules labelled with ^3H toward the enzymes catalyzing the biosynthesis of squalene.

In Table 2 the [$^3H/^{14}C$] ratios are listed for the individual sterols isolated from *C. officinalis* flowers after feeding with the labelled MVA preparations. The results are best discussed according to the various types of reaction which can be distinguished in the biosynthesis of plant sterols.

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[8] POPJAK G *ibid* pp 17–34
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Stereospecificity of the formation of the Δ^7 double bond

According to the generally accepted scheme of plant sterol biosynthesis,[6,7,11] isomerisation of the Δ^8 double bond formed as the result of the opening of the cyclopropane ring of cycloeucalenol[12] leads to the formation of compounds with a double bond in position Δ^7(Δ^7-sterols) which are then transformed to $\Delta^{5,7}$ dienes. Reduction of the Δ^7 double bond leads to Δ^5-sterols (β-sitosterol, campestrol). Position 7 of sterols is derived from position 2 in MVA.[7] The values of the [^{3}H/^{14}C] ratio obtained for Δ^7 sterols isolated after feeding with [2-^{14}C,2R,2-^{3}H] MVA (^{3}H/^{14}C ratio *ca* 5:5) and with [2-^{14}C,2S,2-^{3}H] MVA (^{3}H/^{14}C ratio *ca* 4:5) indicate that, during the formation of the Δ^7 double bond a stereospecific elimination of ^{3}H atoms from the 7α position occurs (elimination of the ^{3}H atom originating from position 2-pro-S of MVA). In both cases the loss of one ^{14}C atom is associated with demethylation in position 4, because the 4α methyl group is also derived from C-2 of MVA.[7]

TABLE 2 [^{3}H/^{14}C] RATIOS IN STEROLS ISOLATED FROM *C officinalis* FLOWERS AFTER FEEDING WITH [2-^{14}C,2R,2-^{3}H] MVA (1), [2-^{14}C,2S,2-^{3}H] MVA (2), [2-^{14}C,5RS,5-$^3H_2$] MVA (3) AND [2-^{14}C,4R,4-^{3}H] MVA (4)

MVA (Sterol acetates)	Radioactivity [(dpm) × 10^{-3}] ^{3}H	^{14}C	[^{3}H/^{14}C] ratio	Normalized [^{3}H/^{14}C] atomic ratio*	Theoretical [^{3}H/^{14}C] atomic ratio
(1) β-Sitosteryl	2673	1151	2 32	4 91 5	1 1
Stigmasteryl	1594	693	2 30	4 87 5	1 1
Δ^7-Steryl	106	46	2 30	4 86 5	1.1
Stigmastan-3β-yl	58	25	2 34	4 94 5	1·1
(2) β-Sitosteryl	1825	1106	1 65	3 72 5	4 5
Stigmasteryl	345	275	1 26	2 85.5	3.5
Δ^7-Steryl	54	30	1 79	4 04 5	4:5
(3) β-Sitosteryl	8186	1809	4 53	10 09 5	2.5
Stigmasteryl	2987	737	4·06	9 04 5	9:5
Δ^7-Steryl	60	13	4 66	10·40.5	2 1
Stigmastan-3β-yl	16	3	4 71	10·51 5	2 1
(4) β-Sitosteryl	1337	1097	1 22	2 28 5	2.5
β-Sitosterol†	328	280	1 17	2 20 5	2 5
β-Sitosterone†	28	24	1 19	2·23 5	2 5
Stigmasteryl	439	351	1·25	2 34 5	2 5
Stigmasteryl	104	84	1 24	2 32 5	2 5
Stigmasterone†	12	10	1 22	2 28 5	2 5
Δ^7-Steryl	14	9	1 53	2 88 5	3·5
Stigmastan-3β-yl	6	4	1 72	3 23 5	3·5

* The ratios were normalized by assuming a 1 1 atomic ratio in squalene in experiments (1), (2), (4) and 11.6 atomic ratio in squalene in experiment (3)

† Sterols

A similar stereochemistry of the isomerization reaction of the Δ^8 to the Δ^7 double bond has been previously demonstrated in the biosynthesis of poriferasterol in the alga *Ochromonas malhamensis*[13] and in the biosynthesis of α-spinasterol in *Camellia sinensis*.[14] How-

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[14] SHARMA, R K (1970) *Chem Commun* 543

ever, in the biosynthesis of ergosterol in yeast elimination of the ^3H atom from the 7α position occurs [15]

Stereospecificity of the formation of the Δ^5 double bond

The 5 position of sterols is derived from position 4 of MVA, whereas the 6 position originates from position 5 of the same precursor.[7] It was found during the investigations on cholesterol biosynthesis in animals that after incubation with [2-^{14}C,4R,4-^{3}H] MVA one of the ^3H atoms was incorporated in the 5α position of lanosterol and was then eliminated in cholesterol biosynthesis [16] On the other hand, after incubation with [2-^{14}C,5R,5-^{3}H] MVA, the ^3H atom was incorporated in the 6β position and was not eliminated in cholesterol biosynthesis [17]

The [$^3H/^{14}C$] ratios determined for Δ^7-sterols (*ca* 3·5) and for β-sitosterol (*ca* 2 5) isolated after feeding with [2-^{14}C,4R, 4-^{3}H] MVA indicate that in *C officinalis* a H atom is also eliminated from the 5α position. This is derived from 4-pro-R MVA in the biosynthesis of the Δ^5 double bond. On the other hand the $^3H/^{14}C$ ratio of 2.1 obtained for β-sitosterol with [2-^{14}C,5RS,5-$^3H_2$] MVA, a preparation not stereospecifically labelled with ^3H in position 5, points to an elimination of the ^3H atom from position 6 of the sterol, without defining the stereochemistry of this reaction. It would seem probable, therefore, that the synthesis of the Δ^5 double bond in *C officinalis* flowers sterols occurs by *cis*-elimination of two H atoms from the 5α and 6α positions as also occurs in the biosynthesis of cholesterol in animals [7 16] Goodwin *et al* [17] also demonstrated that β-sitosterol isolated from *Larix decidua* leaves after feeding with [2-^{14}C,5R,5-^{3}H] MVA contained six ^3H atoms in its molecule, indicating that the ^3H atom from the 6β position is not eliminated in the formation of the Δ^5 double bond in this plant

Stereospecificity of the formation of the Δ^{22} double bond

It has already been demonstrated that in the biosynthesis of poriferasterol in algae the Δ^{22} double bond is formed as the result of *cis*-elimination of H atoms from positions 22-pro-R and 23-pro-R,[18] and that in ergosterol biosynthesis in fungi removal of H atoms from positions 22-pro-S and 23-pro-S occurs.[19] The stereospecificity of the introduction of Δ^{22} double bond has been also investigated in *Camelia sinensis* by Sharma[14] who proved 22-pro-R hydrogen elimination in the biosynthesis of α-spinasterol.

The [$^3H/^{14}C$] ratio determined for β-sitosterol and stigmasterol isolated from *C officinalis* flowers after feeding with [2-^{14}C,2R-^{3}H] MVA (*ca* 5 5 and 5:5, respectively) and with [2-^{14}C,2S,2-^{3}H] MVA (*ca* 4 5 and 3.5 respectively) indicates that during the transformation of β-sitosterol to stigmasterol the 3H atom from position 22-pro-S derived from [2-^{14}C,2S,2-^{3}H] MVA is stereospecifically eliminated The [$^3H/^{14}C$] ratios determined for β-sitosterol and stigmasterol isolated after feeding with [2-^{14}C,5R,S,5-^{3}H] MVA, 10 5 and 9 5 respectively, prove that in the biosynthesis of stigmasterol the ^3H atom is eliminated from position 23 (elimination of one ^3H atom) These results suggest that the stereochemistry of the introduction of the Δ^{22} double bond of stigmasterol in *C. officinalis* is analo-

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[17] GOAD, L J, GIBBONS, G F, BOLGER, L M, REES, H H and GOODWIN, T W (1969) *Biochem J* **114**, 885
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gous to that found in the biosynthesis of ergosterol in *Aspergillus fumigatus* namely, H elimination from position 22-pro-S and 23-pro-S

Elimination of methyl group from the 4α and 4β positions

In triterpenes, lanoesterol, cycloartenol and other compounds,[7,9,11,20] biosynthesized from [2-^{14}C,4R,4-^{3}H] MVA the ^{3}H atom is present in the 3α position. The mechanism of demethylation in position 4, postulated for animal organisms[21–23] involves an oxidation of the 3β OH group of a methyl sterol to the corresponding ketone with the subsequent reduction of this group. Such a mechanism of demethylation leads to the elimination from the 3α position of the H atom derived from position 4-pro-R MVA.

The [^{3}H/^{14}C] ratios obtained for β-sitosterol and stigmasterol after incubation of flowers with [2-^{14}C-4R,4-^{3}H] MVA and for their corresponding ketone derivatives are essentially the same (Table 2). This indicates that β-sitosterol and stigmasterol biosynthesized from [2-^{14}C,4R,4-^{3}H] MVA do not contain a ^{3}H atom in the 3α position. Thus, the most probable mechanism of methyl group elimination from position 4 of *C. officinalis* sterols is associated with the formation of 3-keto intermediates, a similar mechanism to that of cholesterol biosynthesis in mammals, as well as in some other higher plants (banana, pea) [10,24]

Elimination of the methyl group from the 14 position

The mechanism of elimination of the methyl group from positions 14 of methylsterols has so far been investigated exclusively in animal material. Two types of demethylation mechanisms have been suggested.[7] One type involves an oxidation of the C-14 methyl group to a carboxyl group, decarboxylation of which leads to formation of a $\Delta^{8,14}$ diene intermediate followed by subsequent reduction of the Δ^{14} double bond. The other type postulates the formation of an intermediate with a single $\Delta^{8(14)}$ double bond.

Caspi *et al.*,[25] Canonica *et al*,[26] Goodwin, Gibbons *et al.*[27] found, when incubating mammalian liver homogenates with [2-^{14}C,2R,2-^{3}H] MVA, that during demethylation from position 14, the ^{3}H atom is eliminated from the 15α position which is derived from position 2-pro-S of MVA. On the other hand, the 15β atom (derived from 2-pro-R MVA) remained in the cholesterol molecule This suggests the formation of compound with $\Delta^{8,14}$ double bonds.

In order to verify which mechanisms of C-14 demethylation are operating in the biosynthesis of *C. officinalis* sterols, sterols were isolated from flowers fed with [2-^{14}C,2R,2-^{3}H] MVA and [2-^{14}C,2S,2-^{3}H] MVA (Table 2). With [2-^{14}C,2R,2-^{3}H] MVA the [^{3}H/^{14}C] ratios in sterols were in all cases approximately 5:5. The loss of one atom of ^{14}C and one of ^{3}H resulted from demethylation in position 4 (elimination of the 4 methyl group). Sterols derived from [2-^{14}C,2S,2-^{3}H] MVA exhibited the following values of the normalized atomic ratio [^{3}H/^{14}C:Δ^{7}-sterol] and β-sitosterol, approx. 4:5, stigmasterol, 3:5. The

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[27] Gibbons, G F, Goad, L J and Goodwin, T W (1968) *Chem Commun* **1212,** 1458

loss of one atom of ^{14}C and one of ^{3}H was also found in all sterols where demethylation occured in position 4 The loss of a second ^{3}H atom on all sterols was associated with the elimination of a ^{3}H atom from the 7β position (formation of Δ^{7} double bond). Additional elimination of a ^{3}H atom in stigmasterol was associated with the synthesis of the Δ^{22} double bond in the side-chain This may suggest that the mechanism of C-14 demethylation in the plant does not involve H elimination from position 15 It is probable therefore, that this process occurs through the formation of an intermediate with a $\Delta^{8(14)}$ double bond

Introduction of the alkyl group at position 24

β-Sitosterol and stigmasterol obtained after feeding with [2-^{14}C,4R,4-^{3}H] MVA have normalized [$^{3}H/^{14}C$] ratio of approx. 2·5 (2 1–2 3), indicating the elimination of four ^{3}H atoms in the biosynthesis of these sterols ^{3}H atoms derived from position 4-pro-R of MVA located in positions 3,5,8,17,20 and 24 of cycloartenol [7] During sterol biosynthesis in *C officinalis* flowers one ^{3}H atom is removed from position 3 in the process of demethylation at C-4. The [$^{3}H/^{14}C$] ratio for Δ^{7}-sterols amounted approximately to 3·5 as compared with 2·5 for β-sitosterol, indicating that the second ^{3}H atom was eliminated in the course of Δ^{5} double bond formation. The third ^{3}H atom has been probably removed from position 8 as a consequence of the opening of the cyclopropane ring and the formation of the Δ^{8} double bond which is further isomerized to the Δ^{7} double bond.

In order to explain the [$^{3}H/^{14}C$] ratios (*ca* 2.5) obtained for β-sitosterol and stigmasterol, it had to be assumed that, in the process of biosynthesis of these sterols in *C. officinalis* there was also additional elimination of the fourth ^{3}H derived from 4-pro-R of MVA. The explanation that when the alkylation process takes place, an elimination of the ^{3}H atom from position 24 occurs, as postulated by Randall *et al* [28] seemed more plausible. Also Sharma[29] demonstrated that α-spinasterol after incubation with [2-^{14}C,4R,4-^{3}H] MVA had a ratio [$^{3}H/^{14}C$] 2·4:5 and not 3:5 Elimination of the H atom from C-24 during alkylation of Δ^{24}-precursors has also been demonstrated by Tomita *et al* [30] in studies on the biosynthesis of stigmasterol in *Nicotiana tabacum* and *Dioscorea tokoro* These authors suggested that, during alkylation, an intermediate is formed, and 24-ethylenesterols are synthesized without the formation of intermediate 24-ethylidenesterols It appears that a similar alkylation mechanism is operating in the biosynthesis of β-sitosterol in *C. officinalis* flowers.

The [$^{3}H/^{14}C$] ratios determined for stigmasten-3β-ol isolated after incubation with the labelled MVA preparations (Table 2) indicate that this compound is probably formed directly from the reduction of the Δ^{7} double bond in stigmast-7-en-3β-ol.

EXPERIMENTAL

Material Calendula officinalis cv Radio plants were cultivated in a lumistat under stabilized light (3000 lx), 16 hr daily at 24° during daytime and 16° at night

Radioactive precursors [2-^{14}C] MVA (sp act 10 3 mCi) mmol, [3R,2S,2-^{3}H + 3S,2S,2-^{3}H] MVA (sp act 175 mCi/mmol) [3R,2S,2-^{3}H + 3S,2R,2-^{3}H] MVA (sp act 250 mCi/mmol), [3R,4R,4-^{3}H + 3S,4S 4-^{3}H] MVA (sp act 250 mCi/mmol) all in lactone form were supplied by the Radiochemical Centre, Amersham, and the [5R,S,5-^{3}H] MVA, DBED salt (sp act 6740 mCi/mmol by NEN Chemicals, Boston Doubly labelled MVA was prepared by mixing [2-^{14}C] MVA lactone with the appropriate [^{3}H] MVA lactone, except the DBED [5R,S,5-

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$^3H_2$] MVA salt which was mixed with the [2-^{14}C] MVA Na salt The [^{14}C] MVA and [^{3}H-MVA] preparations were combined in such proportions as to obtain [^{3}H/^{14}C] ratios of approx 3 5

Administration of doubly labelled MVA Preparations corresponding to about 50 μCi in H_2O (0 5 ml) were placed in glass vessels and isolated ligular flowers (about 1 g fr wt) were placed vertically in the vessels so that the flowers were partly immersed in the solution. During incubation the flowers were illuminated at 2500 lx for 16 hr a day The solution taken up by the flowers was made up with H_2O and incubation was continued for 120–196 hr

Fractionation of the material The flowers were ground with dry Na_2SO_4 and the powder was extracted with hot EtOH, from the EtOH extract the nonsaponifiable fraction was obtained [31] Hydrocarbon and sterol fractions were obtained by TLC on silica gel impregnated with Rhodamine 6G[32] using hexane–$CHCl_3$–MeOH (20 10.1) About 5 mg of nonlabelled squalene was added to the hydrocarbon fraction and the fraction isolated by TLC using petrol (40–60°) and repurified by chromatography under the same conditions Further purification was carried out after the addition of a further 100 mg of nonlabelled squalene by conversion into the crystalline thiourea adduct [33]

Sterols acetylated in the usual way were purified by TLC in hexane–$CHCl_3$–MeOH (40 20 1). The steryl acetates were separated into individual compounds by $AgNO_3$-silica gel TLC in EtOH-free $CHCl_3$ Under these conditions the steryl acetates gave 4 fractions (i) stigmastan-3-β-ol, (ii) stigmast-7-en-3β-ol and 24-methyl-cholest-7-en-3β-ol, (iii) β-sitosterol and campesterol, (iv) stigmasterol 2 to 3 mg of nonlabelled carrier was added to the isolated steryl acetates and TLC was run once more under the same conditions Purity was checked by autoradiography, 3-fold crystallization after addition of 10 to 30 mg of carrier was the final purity control Δ^7-sterols and stigmastan-3β-ol were not crystallized because an insufficient amount of the carrier was available The ketone derivatives of sterols were obtained by oxidation with Na_2CrO_7 [9] The ketones were purified by TLC on silica gel in hexane–$CHCl_3$–MeOH (40.20 1)

Radioactivity measurement The individual compounds isolated and their chemical derivatives were measured in toluene containing PPO (5 g/l.) and POPOP (0 5 g/l) in a Mark I spectrometer (Nuclear Chicago Corp) equipped with an external standard Quenching was determined by the method of "channel ratio" using results obtained for ^{14}C and ^{3}H standards with known dpm quenched to various degrees [34] The time of measurement was chosen so that the error in radioactivity measurement would not exceed 0 5%

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