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STEREOSPECIFICITY OF BIOSYNTHESIS OF TRITERPENE ALCOHOLS IN *CALENDULA OFFICINALIS* FLOWERS

JACEK ŚLIWOWSKI and ZOFIA KASPRZYK

Department of Biochemistry, University, 02–089 Warszawa, Al Żwirki i Wigury 93, Poland

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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 2*R*, 2*S*, 4*R* or 5*R*,*S* and the [$^{3}H/^{14}C$] ratios determined in squalene and pentacyclic mono- and dihydroxy-triterpene alcohols and also in some derivatives prepared from the triterpene alcohols ^{3}H atoms were located in positions 3, 12, 16, 21, 29, 30 of the ursane skeleton, positions 3, 12, 29, 30 of the lupane skeleton and positions 3, 11, 12, 18 of the oleanane skeleton Stabilization of α- and β-Amyrins, ψ-taraxasterol and lupeol occurs with the elimination of a proton from positions 12, 21 and 29 (or 30) respectively In addition, during hydroxylation of triterpene monols to the corresponding diols a proton is substituted by the hydroxyl group

INTRODUCTION

THE PROPOSALS of Rozicka and co-workers[1–4] on the cyclization of squalene to pentacyclic triterpenes such as β-amyrin have been substantiated with experimental evidence.[5–10] However, no experimental evidence is available on the biosynthesis of other pentacyclic triterpene alcohols with different carbon skeletons or on the mechanism of hydroxylation of these compounds by plants.

In earlier work on *Calendula officinalis*[11–13] the presence of the following triterpene alcohols was established: ursane mono-ols-α-amyrin, ψ-taraxasterol, taraxasterol; ursane diols brein, ursadiol, faradiol, arnidiol; lupane mono-ol-lupeol; lupane diol-calenduladiol; oleanane mono-ol-β-amyrin; oleanane diol-erythrodiol. The present paper describes a study of the stereospecificity of the biosynthesis of these triterpene alcohols using four doubly labelled mevalonic acid (MVA) preparations [2-^{14}C,2*R*,2-^{3}H], [2-^{14}C,2*S*,2-^{3}H], [2-^{14}C,4*R*,4-^{3}H] and [2-^{14}C,5*R*,*S*,5-$^{3}H_2$].

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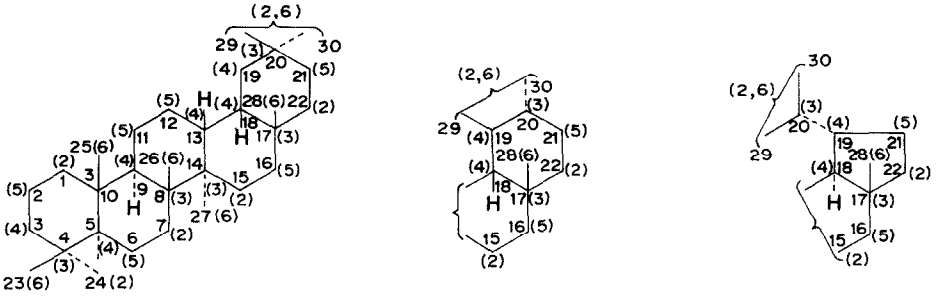
RESULTS AND DISCUSSION

The triterpene alcohols of *C. officinalis* were isolated in the experiments used to study sterol biosynthesis (see preceding paper[14]) and [$^3H/^{14}C$] ratios determined. The free alcohols were then oxidized to the corresponding ketones and the acetate derivatives of the alcohols oxidized with SeO_2 to the corresponding diene (β-amyrenyl acetate) or to the exocyclic aldehyde (ψ-taraxasteryl, taraxasteryl and lupeyl acetates)[13,15] and the isotope ratios again determined

Stereospecificity of the biosynthesis of pentacyclic triterpene mono-ols

Tables 1 and 2 show the [$^3H/^{14}C$] ratios for squalene and the mono-ols isolated after feeding the labelled preparations and also for the various oxidation products derived from the mono-ols. To facilitate the interpretation of the results the distribution of carbon atoms derived from MVA in oleanane, ursane and lupane triterpenes predicted on the basis of the Ruzicka–Eschenmoser schemes is shown in the Structure



SCHEME 1 THE DISTRIBUTION OF THE ATOMS DERIVED FROM MVA IN THE DIFFERENT SKELETONS OF PENTACYCLIC TRITERPENE (MVA ATOMS IN BRACKETS)

The normalized atomic ratios for most of the triterpene mono-ols were close to 1:1 indicating the lack of proton elimination in the cyclization process from positions labelled with ^3H atoms The deviations obtained with some MVA precursors in the biosynthesis of lupeol, α- and β-amyrins and ψ-taraxasterol in which the normalized atomic ratio was lower than 1.1 or 11.6 will be discussed later

Lupeyl acetate isolated after feeding with [2-^{14}C,4R,4-^{3}H] MVA and [2-^{14}C,5R,S.5-$^3H_2$] MVA, as well as the oxidation products with SeO_2 had normalized atomic ratios $^3H/^{14}C$ approx equal to 1 1 or 11:6 confirming the Ruzicka–Eschenmoser scheme for squalene cyclization to lupeol However in the case of lupeol obtained from [2-^{14}C,2R,2-^{3}H] MVA and [2-^{14}C,2S,2-^{3}H] MVA the [$^3H/^{14}C$] ratios were *ca* 5 7 6 indicating a partial elimination of one of the ^3H atoms in these precursors in the cyclization of squalene The Ruzicka–Eschenmoser scheme predicts that during the cyclization of squalene to lupeol the isopropylidene group is formed by the elimination of a proton from position 29 or 30 The carbon atoms of the group (29, 30 and 20) are derived from position 2, 3 and 6 in MVA (Scheme 1), of which only position 2 (labelled with ^{14}C and ^3H) were accessible to analysis in the present investigation The theoretically possible mechanisms of formation

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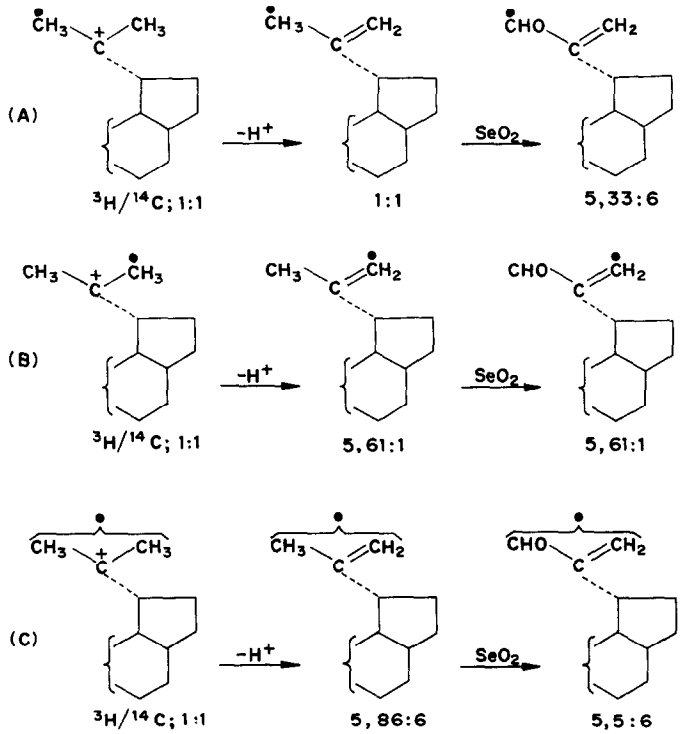
of the isopropylidene group of lupeol during biosynthesis from squalene and its oxidation product, 30-oxo-lup-20(29)-en-3β-yl acetate are shown in Scheme 2. Theoretical values for the [$^3H/^{14}C$] ratio in lupeol formed from MVA labelled with ^3H in position 2 and for the oxidation product of lupeyl acetate with SeO_2 are also given.



SCHEME 2 ALTERNATIVE MECHANISMS FOR THE FORMATION OF THE *iso*-PROPYLIDENE GROUP OF LUPEOL IN BIOSYNTHESIS FROM SQUALENE AND ITS OXIDATION WITH SeO_2 TO 30-OXO-LUP-20(29)-EN-3-β-OL ●—^{14}C AND ^3H ATOMS DERIVED FROM [2-^{14}C-2R,2-^{3}H] MVA AND [2-^{14}C-2S,2-^{3}H] MVA

Comparison of theoretical ratios with experimental values seems to rule out mechanism A Scheme 2, since the [$^3H/^{14}C$] ratios found for lupeol isolated from MVA preparations labelled with ^3H in position 2 (notwithstanding the stereochemistry) were lower than 1:1 (Table 1). Of the mechanisms B and C, C is more plausible involving a partial or complete randomization of labelling from [2-^{14}C,2-^{3}H] MVA between C-29 and C-30 of the isopropylidene group. This mechanism is supported by the isotope ratio found in 30-oxo-lup-20(30)-3-β-yl acetate (5·6:6) prepared from lupeyl acetate isolated after feeding with [2-^{14}C,2-^{3}H] MVA.

In α- and β-amyrin biosynthesized from [2-^{14}C,5R,S,5-$^3H_2$] MVA the normalized atomic ratios were also lower than 11:6 (10·3:6). The theoretical scheme of Ruzicka–Eschenmoser postulates that in the cyclization of squalene to α- and β-amyrins, a proton is eliminated from position 12 derived from position 5 of MVA (Scheme 1). The investigation of Goodwin *et al.*,[6] who succeeded in locating five out of the six ^3H atoms derived from [2-^{14}C,4R,4-^{3}H] MVA in β-amyrin confirmed to a large extent the postulated mechanism of squalene cyclization to this compound but they did not supply experimental evidence concerning proton elimination from position 12 in the process. Our ratios, however, for

α- and β-amyrin biosynthesized from [2-^{14}C,5R,S,5-^{3}H] MVA indicate that this elimination does occur although it was not possible to establish the stereospecificity of the process. Thus during condensation to squalene one proton derived from position 5 of MVA is removed,[14] leaving three ^{3}H atoms in positions corresponding to 11 and 12 of α- and β-amyrins (statistically 1·5 of ^{3}H in each position). During subsequent introduction of the Δ^{12} double bond one proton is eliminated from position 12 leaving about 0·7 of ^{3}H in this position. This explains the experimentally obtained [^{3}H/^{14}C] ratios of 10·3.6 (not 10·6) for α- and β-amyrins and 9·5.6 (not 9·6) for the oxidation product of β-amyrenyl acetate in which one proton is eliminated from position 11. The isotope ratio of *ca* 5·6 for oleana-11,13(18)-diene-3β-yl acetate prepared from β-amyrenyl acetate after feeding with [2-^{14}C,4R,4-^{3}H] MVA indicate the stereospecific elimination of ^{3}H atoms from position 18 of the oleanane skeleton in agreement with Goodwin *et al.*[6] The ratio of 1·1 for the same compound obtained after feeding with [2-^{14}C,2S,2-^{3}H] MVA provides further experimental evidence for the Ruzicka–Eschenmoser proposals for the cyclization of squalene to β-amyrin.

In ψ-taraxasterol, according to the Ruzicka–Eschenmoser postulates, the elimination of a proton from position 21 occurs during cyclization of squalene. Position 21 corresponds to position 5 in MVA (Scheme 1). The loss of one ^{3}H atom noted for ψ-taraxasterol isolated after feeding with [2-^{14}C,5R,S,5-$^{3}H_2$] MVA and also from 30-oxo-urs-20-en-3β-yl acetate derivatives prepared from both ψ-taraxasteryl and taraxasteryl acetates, confirms this suggestion. Additional confirmation of the presence of a ^{3}H atom in position 21 of the ursane skeleton was obtained from the [^{3}H/^{14}C] ratio of 1·1 found in taraxasterol isolated after feeding [2-^{14}C,5R,S,5-$^{3}H_2$] MVA. However, oxidation of ψ-taraxasteryl and taraxasteryl acetates after feeding with [2-^{14}C,4R,4-^{3}H] MVA did not result in the elimination of ^{3}H atoms (^{3}H/^{14}C *ca* 1·1) further confirming the theoretical predictions concerning ^{14}C and ^{3}H atom distribution in the ursane skeleton.

The ratios obtained from ψ-taraxasteryl acetate after feeding with [2-^{14}C,2R,2-^{3}H] MVA and [2-^{14}C,2S,2-^{3}H] MVA (Table 1), *ca* 1·1, irrespective of the configuration of ^{3}H in position 2 of MVA, and for its oxidation product *ca* 5·6:6 for ^{3}H in position 2R and 5·3·6 for ^{3}H in position 2S, showed that in the oxidation of the C-30 methyl group to an aldehyde "1/3" and "2/3" of ^{3}H atom, respectively, are eliminated. Since oxidation causes loss of two of the three H atoms of the C-30 methyl group, the "2/3" result is obvious if a lack of randomization of labelling between C-29 and C-30 is assumed. The "1/3" result is probably evidence for the stereospecificity of the oxidation. The C-30 methyl group in ψ-taraxasterol is most probably derived from C-2 of MVA and as the result, C-29 is derived from C-6 of MVA.

For taraxasteryl acetates isolated after feeding with [2-^{14}C,2R,2-^{3}H] MVA and [2-^{14}C,2S,2-^{3}H] MVA and also for its oxidation product the ratios obtained are more difficult to interpret. In all cases the normalized atomic ratio was close to 1:1 (Table 1). The only possible interpretation of these results is to assume the existence of a difference in the stereochemistry of the shift of one of the twin methyl groups from position 20 to 19 during the biosynthesis of taraxasterol and ψ-taraxasterol. The C-30 methylene group of taraxasterol would thus derive from C-6 of MVA and C-29 from C-2 of MVA. Neither elimination of a proton in the process of squalene cyclization to the alcohol, nor the oxidation of the acetate isolated after feeding with [2-^{14}C,2R,2-^{3}H] MVA and [2-^{14}C,2S,2-^{3}H] MVA could change the [^{3}H/^{14}C] ratio. The [^{3}H/^{14}C] ratio found in taraxasterol isolated after incubation with [2-^{14}C,2R,2-^{3}H] MVA (6·2·6) is probably an indication of con-

TABLE 1. [$^3H/^{14}C$] RATIOS IN SQUALENE AND IN TRITERPENE MONO-OLS ISOLATED FROM *Calendula officinalis* FLOWERS AFTER INCUBATION WITH [2-^{14}C,2R-^{3}H] MVA (1) AND [2-^{14}C,2S,2-^{3}H] MVA (2)

MVA (Compound)	Radioactivity [(dpm) × 10^{-3}] ^3H	^{14}C	[$^3H/^{14}C$] ratio	Normalized [$^3H/^{14}C$] atomic ratio	Theoretical [$^3H/^{14}C$] atomic ratio
(1) Squalene	36	16	2 37	—	6.6
α- and β-amyrenyl acetate	1440	603	2 39	6 06 6	6.6
ψ-Taraxasteryl acetate	1008	428	2 36	5·98:6	6·6
30-Oxo-urs-20-en-3-β-yl acetate	193	88	2 19	5 36 6 5 58 6*	5 6.6
Taraxasteryl acetate	922	379	2 43	6 16 6	6.6
30-Oxo-urs-20-en-3-β-yl acetate	119	49	2 44	6 18.6 6·01 6*	6 6
Lupeyl acetate	310	136	2·28	5 78·6	5·8·6
30-Oxo-lup-20(29)-en-3β-yl acetate	82	39	2 13	5·40·6 5 60.6*	5·5 6
(2) Squalene	336	151	2 22	—	6.6
α- and β-Amyrenyl acetate	4901	2234	2 19	5 94 6	6.6
α-Amyrenyl acetate	2660	1217	2·19	5 92 6 5 98 6*	6.6
Oleana-11,13/18/diene-3β-yl acetate	850	385	2 21	5 97 6 6 03.6*	6·6
ψ-Taraxasteryl acetate	1479	665	2 23	6·02.6	6.6
30-Oxo-urs-20-en-3β-yl acetate	285	146	1 95	5·29 6 5 27 6*	5 3:6
Taraxasteryl acetate	1685	757	2 23	6·02 6	6.6
30-Oxo-urs-20-en-3β-yl acetate	1253	114	2 21	5·98·6 5 96·6*	6.6
Lupeyl acetate	1003	474	2 12	5 72 6	5 8 6
30-Oxo-lup-20/29/-en-β-yl acetate	224	112	2 01	5 44·6 5 70 6*	5 5 6

The ratios were normalized by assuming a 1 1 atomic ratio in squalene except (*) which were normalized by assuming a 1 1 atomic ratio in the corresponding triterpene mono-ol

tamination with a radioactive substance with a higher [$^3H/^{14}C$] ratio, which, in spite of the extensive purification procedure used, could not be removed.

According to theoretical predictions, one of ^3H atoms in triterpene mono-ols isolated after feeding with [2-^{14}C,4R,4-^{3}H] MVA should be in the 3 position. Oxidation of the mono-ols to ketone derivatives should result in elimination of this ^3H atom as demonstrated by Goodwin *et al.*[6] for β-amyrin. In order to verify this suggestion all the mono-ols isolated from *C. officinalis* flowers after feeding with this precursor were oxidized to ketones. The normalized atomic ratios amounted to 5:6 (Table 2) for all the ketone derivatives, thus proving elimination of the ^3H atom from the 3α position in ursane, lupane and oleanane mono-ols. However, it should be noted that corresponding [$^3H/^{14}C$] ratios of 5 1–5·3:6 and not 5:6 were found. These deviations of the normalized [$^3H/^{14}C$] ratio from the theoretical values are probably connected with the isotopic effect induced by the presence of ^3H atoms which has been discussed in the preceding paper[14] and which may be also a symptom of a certain nonuniformity in the distribution of radioactivity in the triterpene molecule.

Thus, the presence of ^3H atoms in positions 3, 12, 21 and 29 (for taraxasterol) and 3, 12, 21 and 30 (for ψ-taraxasterol) of the ursane skeleton, in positions 3, 29 and 30 of the

lupane skeleton and 3, 11, 12 and 18 of the oleanane skeleton was proved. It was also demonstrated that stabilization of α- and β-amyrins, as well as of ψ-taraxasterol, is associated with the stereospecific elimination of protons from positions 12 and 21, respectively, whilst stabilization of lupeol involves elimination of the proton from position 29 or 30 These results indicate that biosynthesis of triterpene mono-ols with ursane, lupane and

TABLE 2 [$^3H/^{14}C$] RATIOS IN SQUALENE AND IN PENTACYCLIC TRITERPENE MONO-OLS ISOLATED FROM *Calendula officinalis* FLOWERS AFTER INCUBATION WITH [2-^{14}C,5R,S,5-$^3H_2$] MVA (3) AND [2-^{14}C,4R,4-^3H] MVA (4)

MVA (Compound)	Radioactivity [(dpm) × 10^{-3}] ^3H	^{14}C	[$^3H/^{14}C$] ratio	Normalized [$^3H/^{14}C$] atomic ratio	Theoretical [$^3H/^{14}C$] atomic ratio
(3) Squalene	556	131	4 11	–	11 6
α- and β-Amyrenyl acetate	4750	1230	3·86	10 34 6	10 3 6
α-Amyrenyl acetate	1598	413	3 86	10 34 6	10 3 6
Oleana-11,13 (18)-dien-3β-yl acetate	262	74	3 54	9 47 6	9 6 6
ψ-Taraxasteryl acetate	968	258	3 75	10 05 6	10 6
30-Oxo-urs-20-en-3β-yl acetate	321	85	3 80	10 18 6	10 6
Taraxasteryl acetate	1188	289	4 12	11 02 6	11 6
30-Oxo-urs-20-en-3β-yl acetate	375	97	3 87	10 33 6	10 6
Lupeyl acetate	937	227	4 13	11 05 6	11 6
30-Oxo-lup-20 (29)-en-3β-yl acetate	264	64	4 11	11 01 6	11 6
(4) Squalene	306	115	2 66	—	6 6
α- and β-Amyrenyl acetate	4785	1816	2 64	5 94 6	6 6
α-Amyrin acetate	1477	560	2 64	5 95 6	6 6
				6 01 6*	
Oleana-11,13 (18)-diene-3β-yl acetate	297	140	2 12	4 78 6	5 6
				4 83 6*	
α- and β-Amyrin	1110	431	2 57	5 80 6	6 6
α- and β-Amyrone	480	220	2 19	4 92 6	5 6
				5 09 6*	
ψ-Taraxasteryl acetate	1437	537	2 68	6 03 6	6.6
30-Oxo-urs-20-en-3β-yl acetate	239	89	2 68	6 04 6	6 6
				6 01 6*	
ψ-Taraxasterol	260	96	2 70	6 08 6	6 6
ψ-Taraxasterone	107	46	2 35	5 28 6	5 6
				5 21 6*	
Taraxasteryl acetate	1164	433	2 69	6 06 6	6 6
30-Oxo-urs-20-en-3β-yl acetate	182	68	2 68	6 04 6	6 6
				5 98 6*	
Taraxasterol	153	56	2 73	6 14.6	6 6
Taraxasterone	65	27	2 35	5 29.6	6 6
				5 17 6*	
Lupeyl acetate	545	203	2 68	6 04 6	6 6
				6 09 6*	
30-Oxo-lup-20 (29)-en-3β-yl acetate	107	39	2 72	6 13 6	6 6
				6 09 6*	
Lupeol	308	115	2 67	6 02 6	6 6
Lupenone	96	41	2 33	5 25 6	5 6
				5 23 6*	

The ratios were normalized by assuming 11 6 or 1 1 atomic ratio in squalene except (*) which were normalized by assuming 11 6 or 1 1 atomic ratio in triterpene mono-ols

oleanane skeletons occurs in *C. officinalis* according to the theoretical schemes of Buzicka–Eschenmoser. A supplement to these schemes is the suggestion of the different origin of C-29 and C-30 in ψ-taraxasterol and taraxasterol. Randomization of C-29 and C-30 in the isopropylidene group of lupeol was also noted.

Stereospecificity of the biosynthesis of pentacyclic triterpene diols

The results obtained from the diols isolated after feeding with labelled MVA are given in Tables 3 and 4; faradiol and arnidiol were unresolved by TLC and were estimated together. Erythrodiol, a minor constituent of the flowers, was not labelled confirming the suggestion of Kasprzyk and Wojciechowski,[16] that biological oxidation of β-amyrin to oleanolic acid is a "non-stop" reaction. Erythrodiol and oleanolic aldehyde, the intermediates in this oxidation, were only present as compounds bound to the enzyme surface.

The triterpene diols were labelled much less efficiently than the corresponding mono-ols. However, about 10 times more radioactivity was incorporated into faradiol and arnidiol than into remaining triterpene diols. This is in agreement with the fact that faradiol and arnidiol together constitute about 70% by wt of the triterpene diol fraction isolated from dry *C. officinalis* flowers.[12]

The data obtained from labelled triterpene diols were useful in explaining the mechanism of hydroxylation and for locating ^3H atoms in them after feeding labelled MVA. In *C. officinalis* flowers the OH group is introduced into positions 16 and 21 of the ursane mono-ols and into position 12 of lupane mono-ol. All these positions derive from position 5 of MVA (Scheme 1). The most probable mechanism of triterpene hydroxylation

TABLE 3 [$^3H/^{14}C$] RATIOS IN SQUALENE AND IN PENTACYCLIC TRITERPENE DIOLS ISOLATED FROM *Calendula officinalis* FLOWERS AFTER INCUBATION WITH [2-^{14}C-2R,2-^{3}H] MVA (1) AND [2-^{14}C,2S,^{3}H] MVA (2)

MVA (Compound)	Radioactivity [(dpm) × 10^{-3}] ^3H	^{14}C	[$^3H/^{14}C$] ratio	Normalized [$^3H/^{14}C$] atomic ratio	Theoretical [$^3H/^{14}C$] atomic ratio
(1) Squalene	38	16	2 37	—	6 6
Brein diacetate	139	57	2 41	6 12·6	6.6
Breindione	34	14	2 40	6 09 6 5 97 6*	6 6
Ursadiol diacetate	125	53	2 38	6 03 6	6 6
Faradiol diacetate + Arnidiol diacetate	369	573	2 39	6 06 6	6 6
Calenduladiol diacetate	170	73	2 52	5 88 6	5 8 6
(2) Squalene	335	151	2 22	—	6 6
Brein diacetate	69	31	2 24	6 06 6	6 6
Breindione	13	6	2 23	6 06.6 5 99·6*	6 6
Ursadiol diacetate	100	45	2 23	6·03 6	6·6
Ursadione	18	8	2 51	5 97 6 5 94 6*	6 6
Faradiol diacetate + Arnidiol diacetate	433	1092	2·23	6·03 6	6 6
Calenduladiol diacetate	183	84	2 17	5·87 6	6 6

The ratios were normalized by assuming a 1·1 ratio in squalene, except (*) which were normalized by assuming a 1 1 atomic ratio in the corresponding triterpene diol

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TABLE 4 [$^3H/^{14}C$] RATIOS IN SQUALENE AND IN PENTACYCLIC TRITERPENE DIOLS ISOLATED FROM *Calendula officinalis* FLOWERS AFTER INCUBATION WITH [2-^{14}C,5R,S,5-$^3H_2$] MVA (3) AND [2-^{14}C,4R,4-^3H] MVA (4)

MVA (Compound)	Radioactivity [(dpm) × 10^{-3}] ^3H	^{14}C	[$^3H/^{14}C$] ratio	Normalized [$^3H/^{14}C$] atomic ratio	Theoretical [$^3H/^{14}C$] atomic ratio
(3) Squalene	536	130	4 11	—	11 6
Brein diacetate	97	26	3 71	9 95 6	
				10 58 6*	
Breindione	34	10	3 32	8 89 6	
				9 46 6*	
Ursadiol diacetate	100	27	3 70	9 91 6	
				10 55 6*	
Ursadiane	33	10	3 37	9 03 6	
				9 60 6	
Faradiol diacetate + Arnidiol diacetate	800	221	3 61	9 67 6	
				10 59 6*	
Faradione + Arnidione	195	59	3 30	8 84 6	
				9 68 6*	
Calenduladiol diacetate	117	30	3 92	10 49 6	10 6
				10 45 6*	
Calenduladione	30	9	3 48	9 33 6	9 6
				9 28 6*	
(4) Squalene	306	115	2 66	—	6 6
Brein diacetate	23	9	2 61	5 87 6	6 6
Ursadiol diacetate	26	10	2 60	5 88 6	6 6
Faradiol diacetate + Arnidiol diacetate	550	210	2 61	5 89 6	6 6
Calenduladiol diacetate	52	19	2 69	6 05 6	6 6

The ratios were normalized by assuming a 11 6 or 1 1 atomic ratio in squalene, except (*) which were normalized by assuming a 11 6 or 1 1 atomic ratio in the triterpene mono-ol hydroxylated to corresponding diol

is stereospecific substitution of hydrogen with atmospheric O_2 as demonstrated by Varma and Caspi[17] in gitoxygenin Because a labelled MVA with ^3H not stereospecifically in position 5 (5R,S) was available it could be expected, under the assumption that hydroxylation occurs in *C. officinalis* according to this mechanism, that one ^3H atom would be removed The results in Table 4 showing a distinct depression of the [$^3H/^{14}C$] ratio in the diol fraction and in particular diols isolated after feeding with [2-^{14}C,5R,S,5-$^3H_2$] MVA support this hydroxylation mechanism. The ratios were different for the various diols and probably resulted from two causes (a) the occurrence of the isotopic effect during hydroxylation and (b) the summation of the effects of proton elimination during biosynthesis from squalene e.g proton elimination from position 12 in the biosynthesis of α-amyrin, from position 21 in ψ-taraxasterol, and hydrogen elimination during the hydroxylation of mono-ols to diols viz. α-amyrin to ursadiol or brein and ψ-taraxasterol to faradiol

In the case of calenduladiol formed by the hydroxylation of lupeol summation of these effects was not possible because elimination of a ^3H atom was not noted in the biosynthesis of lupeol from [2-^{14}C,5R,R,5-$^3H_2$] MVA (Table 2) The [$^3H/^{14}C$] ratio (10 4 6) obtained for the compound isolated after feeding with this MVA suggest the occurrence of a small isotopic effect in the hydroxylation process (the ratio should be 10.6).

The triterpene diols isolated after feeding with [2-^{14}C,5R,S,5-$^3H_2$] MVA were oxidized to ketones The ketone derivatives (Table 4) exhibited [$^3H/^{14}C$] ratios around unity which

[17] VARMA, K R and CASPI, E (1970) *Phytochemistry* **9**, 1539

were lower than the normalized atomic ratios for the corresponding diols, thus confirming the location of ^3H atoms in the hydroxylated positions.

The structure of ursadiol was a separate problem[12,18] Analysis of this compound and its derivatives by NMR, ORD, CD, IR and MS methods suggested the location of OH groups in positions 3 and 21. Physico-chemical analysis did not rule out, however, the possibility of the presence of a second OH group in positions 7 or 22; both these positions, in contrast to position 21, are derived from position 2 of MVA (Scheme 1). The [$^3H/^{14}C$] ratio for ursadiol isolated after feeding [2-^{14}C,2R,2-^{3}H] MVA and [2-^{14}C,2S,2-^{3}H] MVA and also for ketone derivatives of ursadiol was about 1:1, thus excluding the presence of OH group in position 7 or 22. On the other hand, the depressed [$^3H/^{14}C$] ratio for ursadiol isolated after feeding with [2-^{14}C,5R,S,5-^{3}H] MVA and a further decrease of this ratio after oxidation of the compound to the diketone confirmed the location of the OH group in position 21. The data suggest that hydroxylation of mono-ols to diols occurs in *C. officinalis* by the substitution of hydrogen by an OH group. Also in triterpene diols isolated after feeding with [2-^{14}C,5R,S,5-$^3H_2$] MVA ^{3}H atoms are located in positions 12, 16 and 21, which is in agreement with the ^{14}C and ^{3}H atom distribution predicted by the Ruzicka–Eschenmoser schemes for ursane and lupane triterpenes.

EXPERIMENTAL

The material, radioactive precursors, administration of doubly labelled MVA preparations and radioactivity measurement are described in detail in the preceding paper [14]

Fractionation of triterpene alcohols Pentacyclic triterpene mono-ols and diols were isolated by TLC from the unsaponifiable fraction at the same time as the sterol and squalene fractions as described in the preceding paper.[14] The mono-ol fraction was re-chromatographed in hexane–$CHCl_3$–MeOH (20·10:1) for complete separation from 4-methyl-sterols and then separated into individual compounds by $AgNO_3$-silica gel TLC in EtOH-free $CHCl_3$ The separated mono-ols were acetylated and then purified by TLC on silica gel impregnated with Rhodamine 6G in hexane–$CHCl_3$–MeOH (40 20 1) The mono-yl acetates after addition of about 2 mg of carrier were re-chromatographed on $AgNO_3$-silica gel with autoradiographic control of purity Final purity was checked by three-fold crystallization after addition of 10–30 mg of carrier

The diol fraction was acetylated and the acetates were purified by the TLC method used for the mono-yl acetates and the individual diols separated using $AgNO_3$-silica gel TLC and C_6H_6 (2 developments) and EtOH-free $CHCl_3$ The isolated diol acetates, after addition of about 2 mg carrier, were rechromatographed under the same conditions with autoradiographic control of purity and then crystallized three times Oxidation of triterpene mono-ols and diols to ketones and the oxidation of mono-yl acetates with SeO_2 were carried out using published methods [12,13,18]

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[18] ŚLIWOWSKI, J and KASPRZYK, Z (1972) *Tetrahedron* **28**, 991