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THE STEREOCHEMISTRY OF HYDROXYLATION OF THE CAROTENOID LUTEIN IN *CALENDULA OFFICINALIS*

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Key Word Index—*Calendula officinalis*; Compositae; marigold; stereochemistry; biosynthesis; carotenoids; lutein.

Abstract—Excised, opening inflorescences of *Calendula officinalis* incorporated (3*RS*, 5*R*)- and (3*RS*, 5*S*)-[2-^{14}C,5-^{3}H$_{1}$]mevalonates into the carotenoid fraction. The ^{14}C:^{3}H ratios of lutein isolated from these tissues showed the hydrogen atom at C-3 of the β-ring is derived from the 5-*pro-S* position of mevalonate, while that at C-3 of the ϵ-ring is derived from the 5-*pro-R* position of mevalonate. Oxidation of lutein to monoketolutein showed that both hydrogen atoms at the C-15,15′ central double bond are derived from the 5-*pro-R* position of mevalonate.

INTRODUCTION

The carbon skeleton and oxygenation pattern of the phytohormone abscisic acid (ABA, **1**) are so similar to those of some carotenoids that it has been suggested that ABA is formed by cleavage of a carotenoid [1]. Violaxanthin (**2**) can be photolysed to give xanthoxin (**3**) which is metabolized to ABA by plants [2]. By this route the C-5 double bond of the β-ring of **2** is isomerized to the C-4, ϵ-position of **1**. Alternatively, cyclization could form the ϵ-ring directly. The stereochemistry of hydroxylation of the β,ϵ-xanthophyll, lutein (**4**), may provide a means for discriminating between the β or ϵ cyclizations during the formation of ABA.

The absolute configuration of the 3′-hydroxyl group of the ϵ-ring of **4** is *R* whereas the 3-hydroxyl group of the β-ring of lutein, and also of other β,β-carotenoids is *S* [3, 4]. If the 3- and 3′-hydroxyl groups on the β- and ϵ-rings, respectively, were inserted with opposite stereochemistry and with retention of configuration of the hydrogen atoms derived from mevalonic acid at C-3 and C-3′, then the ϵ-ring would be expected to lose a 3-*pro-S* and the β-ring a 3-*pro-R* hydrogen atom. Early experiments [5] with [5*R*-5-^{3}H$_{1}$]- and [5*RS*-5-^{3}H$_{2}$]mevalonates indicated that two atoms derived from the 5-*pro-R* position of mevalonate were lost during the formation of lutein as they were from zeaxanthin in etiolated maize seedlings, *Physalis alkekengi* plants and in a *Flavobacterium* species.

This suggested that either the original determinations of ^{14}C:^{3}H ratios were in error or that the hydroxylation occurred with inversion of configuration. The stereochemistry of the hydrogen atoms at C-3′ of lutein has now been re-examined using (3*R*, 5*R*)-[2-^{14}C,5-^{3}H$_{1}$]- and (3*R*, 5*S*)-[2-^{14}C,5-^{3}H$_{1}$]mevalonic acid samples.

RESULTS AND DISCUSSION

The experiments were carried out as before [5] by feeding the stereospecifically tritiated and [2-^{14}C]mevalonic acid samples to excised, opening inflorescences of *Calendula officinalis*. The ray florets absorb and incorporate a considerable proportion of the mevalonate into carotenoids (Table 1). [5*R*-5-^{3}H$_{1}$]- and [5*S*-5-^{3}H$_{1}$]mevalonates were available for these experiments, whereas Walton *et al.* [5] had to compare the ^{14}C:^{3}H ratios of lutein samples biosynthesized from 5*R* and 5*RS* mevalonates.

The lutein samples, derived from the different mevalonates, can be expected to show different ^{14}C:^{3}H ratios depending on which hydrogen atom is lost from C-3′ and which are retained on the central double bond (Table 2). Although there is a possible ambiguity with certain pairs of ratios these were not observed and consequently the central double bond can be deduced to carry two hydrogen atoms derived from the 5-*pro-R* position of mevalonate. The ^{14}C:^{3}H ratios of the lutein samples establish that the 5-*pro-S* hydrogen atom of mevalonate was lost from the ϵ-ring of lutein (Scheme 1), reversing the previous attribution. The established pathway of terpenoid biosynthesis places the 5-*pro-S* hydrogen atom of mevalonate in the 3′-*pro-R* position of the ϵ-ring. Thus the loss of this hydrogen atom during lutein formation, but not during zeaxanthin formation, is now in accord with the absolute configuration at C-3′ of lutein.

The stereochemistry of the hydroxyl group at C-3′ could not have arisen by hydroxylation and inversion; such reactions could have given the correct absolute configuration but the 5-*pro-R* hydrogen atom of mevalonate would have been lost.

Monoketolutein

Although the ^{14}C:^{3}H ratios in Table 1 are in accord with the loss of the 5-*pro-S* hydrogen atom of mevalonate from C-3′ of lutein, we sought to establish that a 5*R* tritium atom of mevalonate was present at C-3′.

Table 1. ^{14}C:^{3}H ratios of carotenoids isolated from flowers of *Calendula officinalis* that had been fed with (3*RS*,5*R*)-[2-^{14}C,5-^{3}H$_1$]- or (3*RS*,5*S*)-[2-^{14}C,5-^{3}H$_1$]mevalonic acid

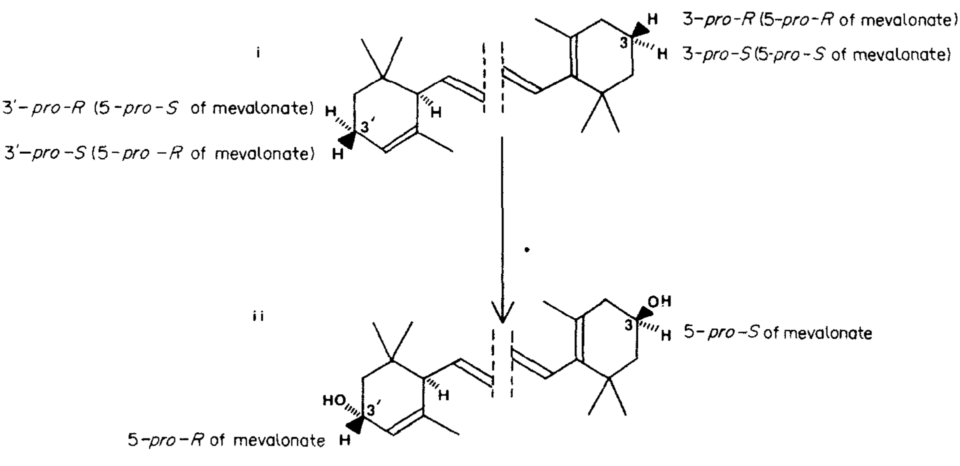
Sample	^{14}C(dpm)	^{3}H(dpm)	^{14}C:^{3}H	^{14}C:^{3}H normalized to MVA	^{14}C:^{3}H*
Expt 1: (3*RS*,5*R*)-[2-^{14}C,5-^{3}H$_1$]mevalonate					
MVA	5421	38869	1:7.17	(1:1)	—
Phytoene	677	4812	1:7.11	8:7.93	—
Neurosporene	1204	5297	1:4.40	8:4.91	(8:5)
β-Carotene	8500	30175	1:3.55	8:3.96	8:4.03
Lutein	9707	26210	1:2.70	8:3.01	8:3.07
Zeaxanthin	5890	10543	1:1.79	8:1.99	8:2.03
Violaxanthin	4741	9292	1:1.96	8:2.19	8:2.23
Expt 2: (3*RS*,5*S*)-[2-^{14}C,5-^{3}H$_1$]mevalonate					
MVA	13619	83076	1:6.10	(1:1)	—
Phytoene	2108	9296	1:4.41	(8:5.78)	(8:6)
β-Carotene	12096	52132	1:4.31	8:5.65	8:5.87
Lutein	6262	23170	1:3.70	8:4.85	8:5.03
Zeaxanthin	3977	17857	1:4.49	8:5.89	8:6.11
Violaxanthin	2273	10160	1:4.47	8:5.86	8:6.08

For expt 1, 0.92 μCi of (3*RS*)-[2-^{14}C]mevalonolactone was mixed with 7.3 μCi of (3*R*,5*R*)-[5-^{3}H$_1$]mevalonolactone, and for expt 2, 7.7 × 10^{4} dpm of (3*RS*)-[2-^{14}C]mevalonolactone was mixed with 4.5 × 10^{5} dpm of (3*R*,5*S*)-[5-^{3}H$_1$]mevalonolactone.

*Expt 1 normalized to neurosporene. Expt 2 normalized to phytoene.

Liaaen-Jensen and Hertzberg [6] have shown that lutein can be oxidized to yield 3′-monoketolutein. They obtained yields of 20% at the 20 mg scale but we seldom obtained yields over 2% working at the 20 μg scale under a variety of conditions in some 40 experiments. *Ca* 30% of the lutein was recovered unchanged and a compound tentatively identified as 3,3′-diketolutein was obtained in *ca* 0.5% yield. In the experiment with the ^{14}C,^{3}H-labelled samples too little diketolutein was obtained to give meaningful ^{14}C:^{3}H ratios.

The results in Table 3 show that the ^{14}C:^{3}H ratio of lutein derived from [5*S*-5-^{3}H$_1$]mevalonate was virtually unchanged on oxidation to monoketolutein whereas the lutein derived from [5*R*-5-^{3}H$_1$]mevalonate showed the loss of one tritium per molecule. This confirmed that the hydrogen atom at C-3′ of lutein was derived from the 5-*pro-R* hydrogen atom of mevalonate because the hydroxyl at C-3′ is allylic and is oxidized to an α,β-unsaturated ketone while the hydroxyl at C-3 is not oxidized as readily.

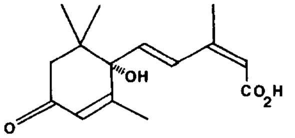


Scheme 1. Hydroxylation of lutein. (i) β,ϵ-Carotene showing the configuration of the hydrogen atoms, derived from C-5 of mevalonate, at C-3 and C-3′. (ii) Lutein showing the 3*R*,3′*S* configuration.

Table 2. Possible $^{14}C:^{3}H$ ratios for lutein derived from 2-^{14}C, 5*R* or 5*S* tritiated mevalonate

	Original normalized $^{14}C:^{3}H$ ratio	Normalized $^{14}C:^{3}H$ ratio in lutein if the hydroxyl group at C-3′ were inserted with retention of configuration	Normalized $^{14}C:^{3}H$ ratio if the hydroxyl group at C-3′ were inserted with inversion of configuration	Observed $^{14}C:^{3}H$ ratio
Ratios if the central double bond were formed *cis* with the retention of two 5-*pro-R* hydrogen atoms of mevalonate				
$2^{14}C,5R\text{-}5\text{-}^{3}H_1$	1:1	8:3	8:2*	8:3
$2^{14}C,5S\text{-}5\text{-}^{3}H_1$	1:1	8:5	8:6*	8:5
Ratios if the central double bond were formed *trans* with the retention of one 5-*pro-R* and one 5-*pro-S* hydrogen atom				
$2^{14}C,5R\text{-}5\text{-}^{3}H_1$	1:1	8:2†	8:1	8:3
$2^{14}C,5S\text{-}5\text{-}^{3}H_1$	1:1	8:6†	8:7	8:5

*,†Possibility of ambiguity with these pairs of ratios.



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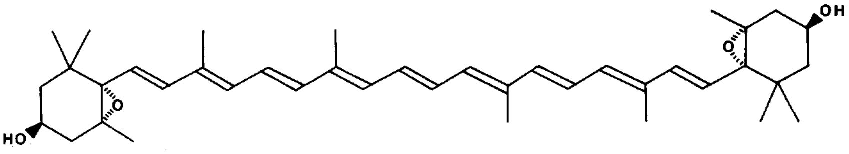

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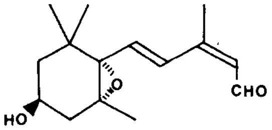

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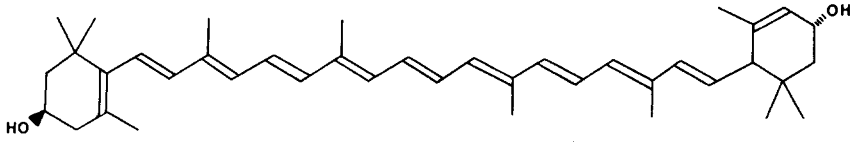

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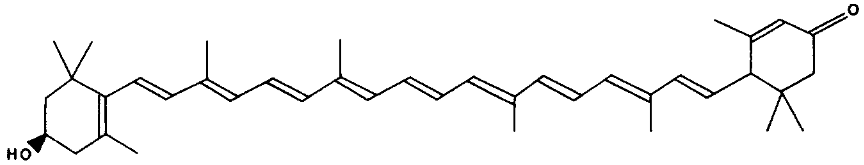

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Fig. 1. Carotenoid structures.

Table 3. $^{14}C:^{3}H$ ratios of lutein and monoketolutein formed from (3*RS*,5*R*)-[2-^{14}C,5-$^{3}H_1$]- or (3*RS*,5*S*)-[2-^{14}C,5-$^{3}H_1$]mevalonate

Sample	^{14}C(dpm)	^{3}H(dpm)	$^{14}C:^{3}H$	$^{14}C:^{3}H$ normalized to neurosporene	$^{14}C:^{3}H$ normalized to original lutein sample
Expt 1: (3*RS*,5*R*)-[2-^{14}C,5-$^{3}H_1$]mevalonate					
Lutein	6311	10476	1:1.66	8:2.97	8:3.04
Monoketolutein	932	1081	1:1.16	8:2.10	8:2.12
Expt 2: (3*RS*,5*S*)-[2-^{14}C,5-$^{3}H_1$]mevalonate					
Lutein	5463	14750	1:2.70	8:4.88	8:5.03
Monoketolutein	761	2024	1:2.66	8:4.82	8:4.95

Lutein, synthesized from either (3*RS*,5*R*)-[2-^{14}C,5-$^{3}H_1$]- or (3*RS*,5*S*)-[2-^{14}C,5-$^{3}H_1$]mevalonate by flowering *C. officinalis* heads, was subjected to NiO_2 oxidation. The monoketolutein and lutein fractions were recovered and their radioactivity measured as described in the Experimental.

The central double bond

The origin of the hydrogen atoms and the geometry of the central double bond have both been determined in few carotenoids. In some organisms the central double bond is formed *trans* with the retention of one 5-*pro-R* and one 5-*pro-S* hydrogen atom from mevalonate while in others it is formed *cis* and retains two 5-*pro-R* hydrogen atoms [7]. Higher plants have been found to make 15-*cis*-phytoene and isomerize the double bond at one or more stages during the progressive desaturation to lycopene. A possible source of error in $^{14}C:^{3}H$ ratios, hitherto not considered, is that both isomers of phytoene could be formed with the retention of the hydrogen atoms described. The isomers could be converted into different products so that, for example, β,ϵ-carotene could be made from *trans*-phytoene while β,β-carotene could be derived from the *cis* isomer. If this occurred then lutein derived from [5R-^{3}H]- or [5*S*-3H]mevalonate would have a different $^{14}C:^{3}H$ ratio from β,β-carotene and the use of phytoene or β,β-carotene as a standard for comparison with lutein would be invalid. The nil or unitary change in $^{14}C:^{3}H$ ratios on removal of the ^{3}H at C-3′ of the lutein samples established the stereochemical origin in mevalonate of the C-3′ hydrogen atom and also confirmed the origin of the hydrogen atoms on the central double bond.

EXPERIMENTAL

Materials. (3*RS*)-[2-^{14}C]Mevalonolactone (17.5 Ci/mol) was purchased from The Radiochemical Centre, Amersham, Bucks, U.K., (3*R*, 5*R*)-[5-$^{3}H_1$]mevalonolactone was a gift from Dr. G. T. Phillips, Milstead Laboratory of Chemical Enzymology, Sittingbourne, Kent, U.K. and (3*R*, 5*S*)-[5-$^{3}H_1$]mevalonolactone was a gift from Professor D. Arigoni, Organisch-Chemisches Laboratorium de Eidgenössische Technische Hochschule, Zürich, Switzerland. [U-^{3}H]Toluene (124 μCi/mol) and [U-^{14}C]toluene (23.5 μCi/mol) were obtained from Packard Instruments, Downers Grove, IL., U.S.A. All other reagents and solvents were the best available grade. Unopened inflorescences of *Calendula officinalis* were obtained locally.

Preparation of [^{14}C,^{3}H]*mevalonic acids.* The [^{14}C,^{3}H]mevalonates were prepared by dissolving the required [^{3}H]- and [^{14}C]mevalonolactones, as $CHCl_3$ solns in 0.1 M KOH (0.5 ml) and incubating at 22° for 2 hr to hydrolyse any lactone. The soln was neutralized (0.1 M, HCl, 0.5 ml) and the $CHCl_3$ was evaporated under N_2 and the aq. mevalonic acid soln diluted with Tris–HCl buffer (20 mM, pH 7.8, 2.5 ml).

Feeding techniques. Unopened inflorescences of *Calendula* were cut with short stems (40 mm) and placed in tubes containing either (3*RS*, 5*R*)-[2-^{14}C,5-$^{3}H_1$]- or (3*RS*,5*S*)-[2-^{14}C,5-$^{3}H_1$]mevalonate (0.5 ml). When the solns had been absorbed the stems and tubes were rinsed with H_2O which was replenished over 4 days. The flowers opened and became coloured under tungsten illumination at 26°.

Isolation of carotenoids. The fully opened flowers were sliced and extracted with Me_2CO (100 ml/flower) containing the antioxidant 2,6-di-*t*-butyl methylphenol (500 ml/l. Me_2CO) in darkness at 26° for 12 hr. The extraction was repeated twice and the Me_2CO extracts were combined. These and all subsequent manipulations were carried out in dull light. The Me_2CO extract was concd. to a vol. of 1.5 l. This was mixed with an equal vol. of Et_2O and sufficient satd. aq. NaCl soln added (500 ml) to achieve phase separation. The Et_2O layer, which contained the chlorophyll and carotenoid fractions, was collected, washed twice with H_2O, dried and evaporated under vacuum at 40°.

The dried Et_2O extracts were chromatographed in EtOAc–toluene (3:5) in an activity I Al_2O_3 column (25 × 2.5 cm). This system gave clear separation of carotenoids and chlorophylls. The carotenoid fractions were combined, evaporated and saponified in EtOH–KOH (10 M) (2:1, 10 ml) 12 hr, 20°. This soln was extracted with Et_2O (2 × 25 ml) which was then dried under N_2. The fraction was chromatographed on an Al_2O_3 column as before to give two carotenoid fractions: (a) the hydrocarbon fraction containing β-carotene, neurosporene and phytoene; (b) the xanthophyll fraction containing lutein, violaxanthin and zeaxanthin. Fraction (b) was then chromatographed on Al_2O_3 (activity I) in EtOAc–CH_2Cl_2–petrol (5:2:7), the three bands containing the lutein, violaxanthin and zeaxanthin were collected individually and dried under N_2. Each was re-dissolved in C_6H_6 (300 μl) and chromatographed (25 μl injection) on a Waters HPLC semi-prep. μPorosil column (Water Associates, Milford, MA., U.S.A.) in hexane–*iso*-PrOH (19:1). The hydrocarbon fraction (a) was treated similarly except that the hexane–*iso*-PrOH solvent was 99:1. The flow rate was 4.0 ml/min, 100 kg/cm^2 and the $A_{436\,nm}$ was monitored. Trace amounts of various *cis* isomers of each xanthophyll were separated and the central zone only of each compound was collected for further analysis. All solvents were redistilled, filtered and degassed before use.

Identification of carotenoids. Phytoene, neurosporene, β-carotene, lutein, violaxanthin and zeaxanthin were identified by their chromatographic behaviour, visible absorption spectra and chemical ionization (CH_4) MS [8–14]. Professor R. Buchecker, of the Organisch-Chemisches Institut, Universitat Zurich-Irchel, subjected a subsample of the lutein from *Calendula* to HPLC with authentic lutein (hexane, 65; CH_2Cl_2, 35; MeOH 0.1; ethyl diisopropylamine, 0.1 on a Spherisorb S5–CN 250 × 4.5 mm column). The *Calendula* lutein co-chromatographed with the authentic material while it was completely separated from an authentic sample of 3′-*epi*-lutein (calthaxanthin). Thus the lutein from *C. officinalis* is (3R,3′R,6′R)-3,3′- dihydroxy-β,ϵ-carotene.

Oxidation of lutein. Monoketolutein (3-hydroxy-3′keto-ϵ-carotene, **5**) was prepared from [^{14}C,^3H]lutein according to the method of ref. [6]. Lutein (500 μg) was dissolved in dry Et_2O (250 μl) and C_6H_6 (250 μl). Nickel peroxide (4.6 mg), corresponding to an available oxygen content of 2.87×10^{-3} g atom/g nickel, was added. The mixture was magnetically stirred at 30° for 30 min, after which the mixture was filtered to terminate the reaction. The monoketolutein and unreacted lutein were separated by HPLC as described above.

Scintillation spectrometry and mass spectrometry. The methods described previously [15] were used.

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