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Origins of Conjugated Triene Fatty Acids. The Biosynthesis of Calendic Acid by *Calendula Officinalis*

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Results from isotopic and incorporation experiments are consistent with formation of calendic acid from linoleic acid *via* a rearranged allyl radical, with loss of a hydrogen atom; since appropriate 9-hydroxy- and 9-hydroperoxy-diene acids were not converted into calendic acid by marigold seed homogenate, an elimination mechanism for formation of the 8(*E*)-double bond is not supported.

Conjugated octadecatrienoic acids of strictly defined geometry occur in a range of higher plant families, octadeca-9(*Z*),11(*E*),13(*E*)-trienoic (α-eleostearic) acid being well known as a component of tung oil, a commercially valuable drying oil from *Aleurites fordii*. Others are octadeca-9(*Z*),11(*E*),13(*Z*)- (punicic), -9(*E*),11(*E*),13(*Z*)- (catalpic), -8(*E*),10(*E*),12(*Z*)- (calendic), and -8(*Z*),10(*E*),12(*Z*)- (jacaric) trienoic acids.[1,2] Apart from direct anabolic processes, such trienes might originate from linolenic acid (**1**) by stereospecific double bond migration (C, Scheme 1) or from 9(*Z*),12(*Z*)-linoleic acid (**2**) *via* epoxidation and stereospecific isomerisation (B) to an hydroxydiene (Scheme 1, Z = OH)[3] which undergoes stereospecific elimination. On the other hand, the fact that the central triene double bond is apparently always (*E*)- makes the operation of a lipoxygenase-like radical process (A), effected on linoleic acid, attractive.[4] The radical could undergo stereospecific loss of a hydrogen atom to give (*Z*),(*E*),(*E*)- or (*Z*),(*E*),(*Z*)-systems (path a) or be trapped (path b, Z = ·OH or ·OOH), stereospecific elimination then ensuing.[5] Despite speculation, experimental information is not available for these triene pathways.

We have now studied the biosynthesis of calendic acid (**3**)[6] using an homogenate of maturing marigold seeds (*Calendula officinalis*). Administration of [1-^{14}C]-linolenic acid showed negligible incorporation, in contrast to [1-^{14}C]-linoleic acid which showed modest incorporation (experiments 1 and 2 of Table 1: for comparison, incorporations of [1-^{14}C]-acetate were 0.54—0.81%). That the incorporation of linoleic acid did not result from complete degradation to labelled acetate, followed by re-synthesis, was shown by hydrogenation of the calendic acid formed to stearic acid, and electrolytic decarboxylation: 83.5% of the [1-^{14}C]-label remained on C-1 with 14.1% distributed in the chain. Thus although some label randomisation occurs, it is restricted. A similar conclusion can

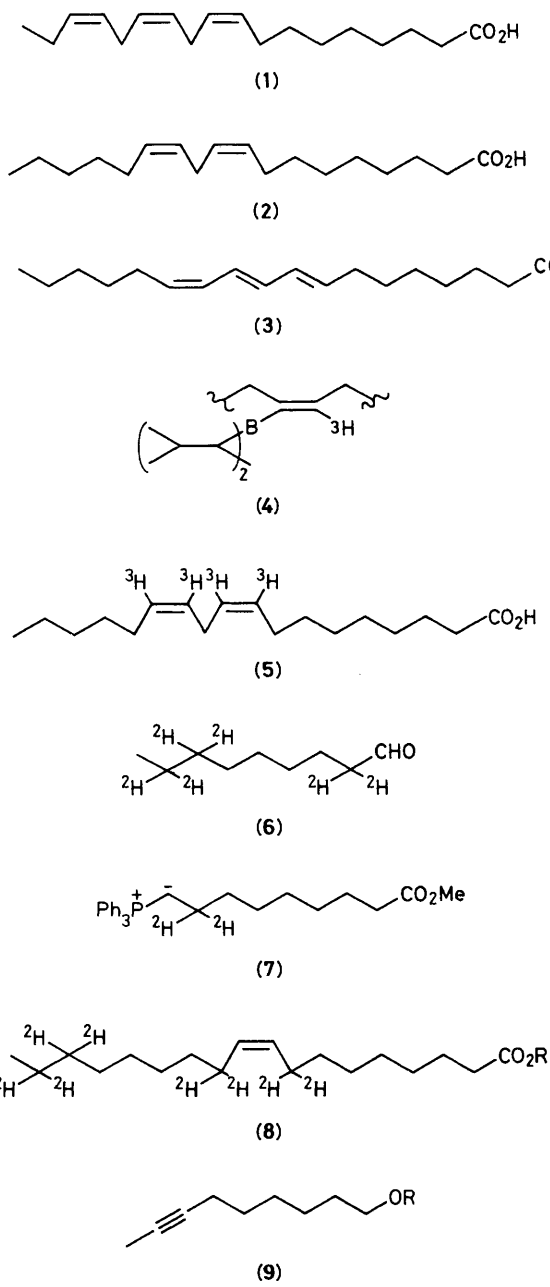
Table 1. Conversion of candidate precursors into calendic acid by marigold seed homogenate.[a]

Expt. No.	Radiochemicals Administered[b]	Wt. of Calendic Acid (mg)[c]	Linoleic ^{3}H/^{14}C Ratio	Calendic ^{3}H/^{14}C Ratio	Incorporation (%)
1	[1-^{14}C]-Linolenic Acid	12.6	—	—	Negligible
2	[1-^{14}C]-Linoleic Acid	14.1	—	—	0.05
3	[9,10,12,13-^{3}H]-Linoleic Acid [1-^{14}C]-Linoleic Acid	13.8	6.74 : 1	6.16 : 1	0.03 0.03
4	[1-^{14}C]-Oleic Acid	9.0	—	—	0.52
5	[9,10-^{3}H]-Oleic Acid [1-^{14}C]-Oleic Acid	18.9	6.04 : 1	6.41 : 1	0.34 0.32
6	[9,10-^{3}H]-Oleic Acid [1-^{14}C]-Linoleic Acid	14.2	7.00 : 1	23.7 : 1	0.29 0.09
7	[1-^{14}C]-Stearic Acid	14.1	—	—	0.03
8	[9,10-^{3}H]-Oleic Acid [1-^{14}C]-Stearic Acid	19.1	6.90 : 1	93.5 : 1	0.30 0.02

[a] Homogenates from 50 g seed harvested 15 days after flower drop and used immediately. [b] All as sodium salts. [c] Purified by h.p.l.c. and crystallised to constant count after argentation chromatography of the methyl esters.

be drawn from expt. 3 where the $^{3}H/^{14}C$ ratio in the double labelling experiment does not undergo any large alteration between precursor and product.

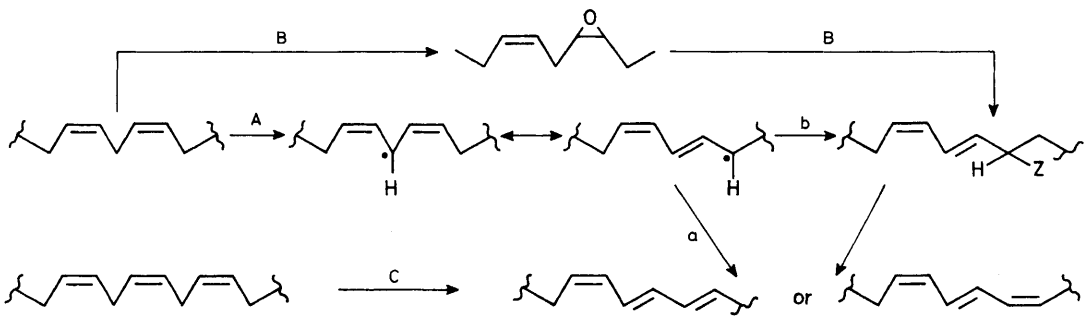
In higher plant work, oleic acid sometimes shows the best fatty acid incorporations.[7,8] Although it has to be 12,13-dehydrogenated in the plant, it is a much better precursor in our system than linoleic acid (expt. 4) and is incorporated with 86.9% of the label remaining on C-1 and 11.4% in the chain. Again the $^{3}H/^{14}C$-ratio test (expt. 5) indicates that most is incorporated without degradation and re-synthesis. Stearic acid incorporations are very modest (expts. 7 and 8).



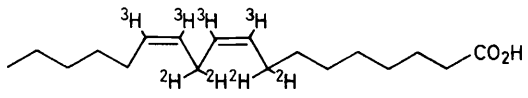
The [9,10,12,13-^{3}H]-linoleic acid (**5**) used in expt. 3 was made from synthetic methyl octadeca-9,12-diynoate[9] by addition of tritiated di-isoamylborane, *cf.* (**4**), followed by tritiolysis with tritiated acetic acid and ester hydrolysis.[10] A further deduction from expt. 3 is that there is little loss of tritium in the 9-, 10-, 12,- and 13-positions during the conversion into calendic acid. However, two hydrogens have to be lost in the conversion of a methylene-interrupted diene into a conjugated triene, and confirmation that those come from the 8,11-methylenes was obtained by synthesising methyl [8,8,11,11,16,16,17,17-^{2}H]-octadec-9(*Z*)-enoate (**8**; R = Me), four extra deuterium atoms being loaded onto C-16 and -17 to bring labelled molecules well clear of unlabelled in the mass spectrum and allow sensitive tests.

Methyl octadeuterio-oleate (**8**; R = Me) was made by Wittig reaction between the hexadeuterio-aldehyde (**6**) and dideuterio-ylide (**7**). The loading deuterium atoms in (**6**) were inserted by treating acetylene (**9**) with deuterium and tris(triphenylphosphine)rhodium(I) chloride in deuteriobenzene and working from the product. The remaining deuterium atoms in (**6**) and (**7**) were inserted by heating the appropriate aldehyde with dry pyridine and 10 equivalents of D_2O, repeating the process three times. Methyl octadeuterio-oleate was purified by argentation chromatography, mass spectral examination showing D_7 (10.41% of the total D_1—D_9 species) and D_8 (78.87%) species having D_8/D_7 = 7.58. Hydrolysis and administration to marigold seed homogenate gave calendic acid, esterified to methyl calendate, which showed the main deuteriated ion at *m/z* 298 [*i.e.* D_8-methyl oleate (304)−(2H + 2D)]. The ratio of this ion to that due to the product from D_7-methyl oleate [*i.e.* D_7-methyl oleate (303) −(2H + 2D)] was 7.53, comparing well with the precursor D_8/D_7 ratio. Thus on conversion of (**8**; R = H) into calendic acid, two hydrogen atoms and two deuterium atoms are lost and the corresponding loss from linoleic acid would be two deuterium atoms. Taking this and expt. 3 into consideration, the fate of hydrogen atoms between a labelled linoleic acid (**10**) and a labelled calendic acid can reasonably be shown as in (**11**).

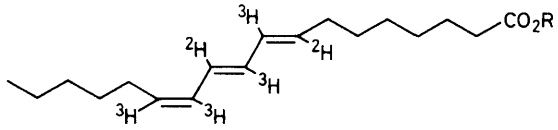
To test the hypothesis that 9-hydroxyoctadeca-10(*E*),12(*Z*)-dienoic acid is intermediate in the formation of calendic acid, this acid (α-dimorphecolic) was isolated from marigold seed oil where it occurs as a minor component in (*S*)-(+)-form (**12**; R = H).[11] It was esterified, oxidised (MnO_2) to the ketone (**13**; R = Me), and reduced with sodium borotritide at 0 °C to give, after hydrolysis, the (±)-[9-^{3}H]-hydroxy-acid (**14**; R^1 = H, R^2 = OH). Administration to marigold seed homogenate



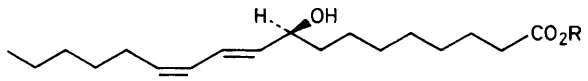
Scheme 1. Hypothesis for stereospecific conjugated triene formation.



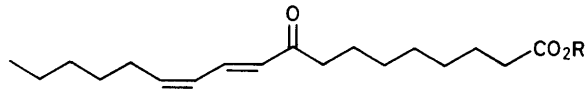
(10)



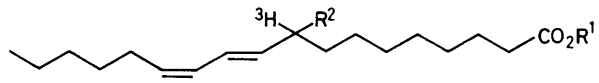
(11)



(12)



(13)



(14)

gave no incorporation into calendic acid. The tritiated hydroxy-ester (**14**; R^1 = Me, R^2 = OH) was converted into its methanesulphonate and then into the hydroperoxy-ester by treatment with ethereal hydrogen peroxide at −110 °C, followed by purification and hydrolysis using lithium hydroxide and hydrogen peroxide in dimethoxyethane and water.[12] This hydroperoxy-acid (**14**; R^1 = H, R^2 = OOH) was also not incorporated into calendic acid.

Thus the present work gives no support to pathways B or C (Scheme 1) to calendic acid, nor to a direct anabolic construction. Linoleic acid is established as the precursor and path A, following sub-pathway a and not b, is consistent with our isotopic experiments concerning the fate of hydrogen atoms on C-8 to C-13, and with successful and unsuccessful incorporation experiments. We would expect this type of route to apply in the case of natural conjugated triene fatty acids of a similar kind. Whether the radical of path A(a) leads to an (*E*)- or a (*Z*)-olefin by loss of a hydrogen atom is presumably enzyme determined.

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