The Biosynthesis of Calendic Acid, Octadeca-(8*E*,10*E*, 12*Z*)-trienoic Acid, by Developing Marigold Seeds: Origins of (*E*,*E*,*Z*) and (*Z*,*E*,*Z*) Conjugated Triene Acids in Higher Plants

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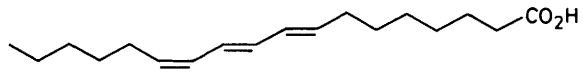
Using an homogenate of marigold seeds, gathered 15 days after flower-drop, [1-^{14}C]linoleic acid and [1-^{14}C]oleic acid were incorporated into calendic acid with little randomisation of the label. [1-^{14}C]Linolenic acid was not incorporated. Despite requiring 12,13-dehydrogenation to form linoleic acid, the putative precursor, oleic acid was better incorporated than administered linoleic acid. Stearic acid, requiring both 12,13- and 9,10-dehydrogenation, was a poor precursor. The results of a series of double-labelling experiments support and supplement these conclusions.

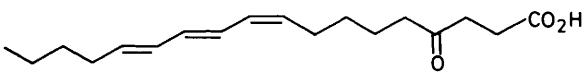
[8,8,11,11,16,16,17,17-$^{2}H_{8}$]Octadec-9-enoic acid was synthesised and employed in a mass-spectral experiment to show that conversion into calendic acid involves loss of two deuterium and two hydrogen atoms (deuterium at C-16 and C-17 was introduced for loading purposes only, in order to increase the sensitivity of the experiment). Taken with [^{3}H]-labelling work, the experiment indicates that during conversion of linoleic acid into calendic acid, there is no loss of the labelled hydrogens at C-9, -10, -12, or -13, but loss of hydrogen from each of C-8 and C-11.

(9*S*)-Hydroxyoctadeca-(10*E*,12*Z*)-dienoic acid (α-dimorphecolic acid) was isolated and converted into (*R*/*S*)-hydroxy- and -hydroperoxy-[9-^{3}H]octadeca-(10*E*,12*Z*)-dienoic acids. Neither labelled specimen was converted into calendic acid by marigold seed homogenate. Abstraction of a hydrogen atom from C-11 of linoleic acid is viewed as giving an (*E*)-allylic radical which, as in lipoxygenase reactions, can be trapped by oxygen at C-9, thus providing a source of α-dimorphecolic acid, a minor component of marigold seed oil. However, this hydroxyacid is apparently a terminus rather than an intermediate for calendic acid. Formation of the latter seems best accounted for by formal loss of a hydrogen atom from C-8 of the (*E*)-allylic radical. The general position relating to the formation of (*E*,*E*,*Z*) and (*Z*,*E*,*Z*)-trienes is summarised.

Glycerides from the seeds of higher plants provide an easily accessible source of pure conjugated triene fatty acids having (*Z*,*E*,*E*) or (*Z*,*E*,*Z*) geometries.[1,2] Thus, acids of the (*Z*,*E*,*E*) group are represented by α-eleostearic acid (**26**) from *Aleurites fordii* (Euphorbiaceae), which produces tung oil used in the paints and coatings industries, catalpic acid (**29**) from *Catalpa ovata* or *C. bignonioides* (Bignoniaceae), and calendic acid (**1**) from *Calendula officinalis* (Compositae). Oxygenated variants such as the keto triene licanic acid (**2**) occur in seed oils of



(1)



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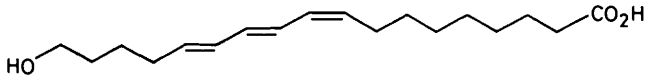
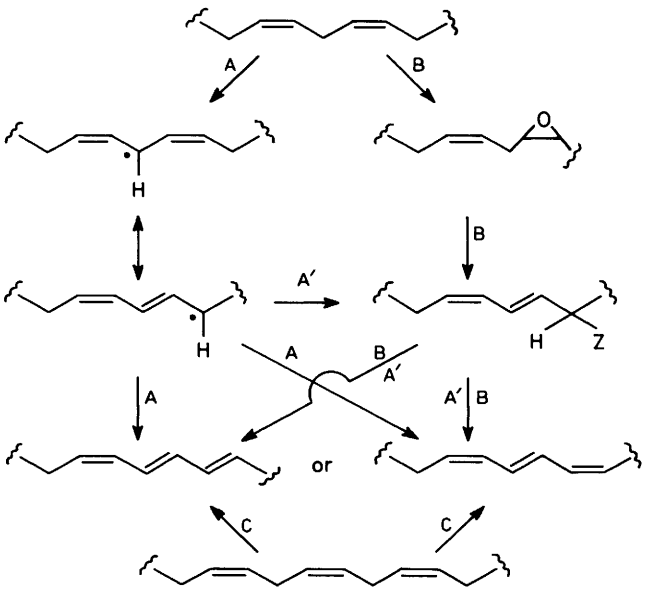


(3)

Licania and *Parinarium spp.* (Rosaceae) (Oiticia oil from *Licania* is also employed in the paint industries), and 18-hydroxyeleostearic acid (**3**) is represented as α-kamlolenic acid from the Kamala tree, *Mallotus philippinensis* (Euphorbiaceae). Representing the (*Z*,*E*,*Z*) group are punicic acid (**25**) from the pomegranate, *Punica granatum* (Punicaceae), and jacaric acid (**27**) from *Jacaranda mimosifolia* (Bignoniaceae). The geometry of (*Z*,*E*,*E*) and (*Z*,*E*,*Z*) fatty acids, as exemplified by α-eleostearic and punicic acids, was first established synthetically in our laboratory[3] and since that time two other synthetic approaches have been reported.[4] Our early synthetic interest has kindled the present investigation on the biosynthetic origins of such trienes.

Useful speculation on the biogenesis of conjugated fatty acids appeared in the literature some 20 years ago,[5,6] but in the intervening period there has been little development of these views or experimentation to establish their validity. Gunstone[7] proposed the intermediacy of a monoepoxide of linoleic acid (Scheme 1, Path B) which undergoes eliminative opening with stereospecificity to give an (*E*)-olefin, resulting in a (*Z*,*E*)-hydroxy diene (Z = OH). Stereospecific dehydration, under enzymic control, is envisaged as forming a new (*Z*)- or (*E*)-olefin according to the enzymes of the plant involved, thus giving the (*Z*,*E*,*Z*) or (*Z*,*E*,*E*) sequence. Morris[8] on the other hand suggested that linoleic acid could be a precursor without the intervention of oxygenated intermediates. Following Path A, generation of a radical as postulated in lipoxygenase-type reactions[9] can then lead by stereospecific formal loss of a hydrogen atom to the (*Z*,*E*,*Z*) or (*Z*,*E*,*E*) combinations. Alternatively (A′), such a radical could be trapped by oxygen to give a hydroperoxide: stereospecific elimination of this or the derived alcohol, as in Gunstone's proposal, would give the two combinations of geometry. A different route to the conjugated trienes might involve linolenic acid as precursor (Route C) and involve stereospecific migration of appropriate double bonds. The present work[10] was initiated to obtain experimental evidence bearing on these various possibilities.



Scheme 1. Stereospecific formation of natural conjugated triene fatty acids: hypotheses

Initial work was directed to the establishment of a suitable biosynthetic system. A germinating seed system would be ideal if turnover were adequate to give reasonable incorporations into the conjugated triene, as work could be carried on without regard to season. Unfortunately, tests using sodium [1-^{14}C] acetate showed very low incorporations into the germinating seeds of *Calendula officinalis* or *Punica granatum* (Table 1: incorporations are maximal as crystallisation to constant count was not achieved). Further experiments involving the tetraene acid, α-parinaric acid (**30**), found in *Impatiens edgworthii*, were no more successful. It was concluded that there was little turnover of conjugated trienes or tetraenes in germinating seeds.

Attention was therefore turned to developing seeds in which trienes are being actively formed and laid down. For us, the marigold (*C. officinalis*)[11] was the easiest triene-bearing plant to grow, and the formation of fatty acids subsequent to flower-drop was studied. Maximal formation of oil was observed at *ca.* 15 days. G.l.c. estimation showed that the 9-day acids (as methyl esters) contained 3% of methyl calendate whilst the 15-day oil contained 34% and this percentage was increasing. Administration of tracers was normally carried out at the latter time. Various methods of administration of radioactive precursors were tried.[12] Administration of sodium [1-^{14}C]acetate to whole seeds or dissected seeds, by flotation on solutions, gave incorporations of ~0.01%, but the use of chopped seed and vacuum infiltration gave still poorer results, presumably because of waterlogging. Wick-feeding to intact seeds on the flower-head gave poor results (0.005% incorporation), but a crude homogenate of 15-day seeds in buffer gave good incorporations (~0.80%) and was used in subsequent work.

Results from incorporation experiments with four [1-^{14}C]-labelled fatty acids are give in Table 2. The methylene-interrupted triene linolenic acid showed negligible incorporation, whilst a modest incorporation (0.05%) was obtained with linoleic acid. Plant enzymes can dehydrogenate *via* the sequence oleic ⟶ linoleic ⟶ linolenic acid[5,6] and oleic acid was therefore administered. It proved to be an excellent precursor of calendic acid (0.52% incorporation). Compared with linoleic acid this is at first sight surprising, though oleic acid and its CoA derivative are reported to be incorporated into parinaric

Table 1. Test incorporations of sodium [1-^{14}C]acetate into conjugated fatty acids of germinated seed systems

Germinated seeds[a]	$CH_3{}^{14}CO_2H$ administered[b]	Acid isolated	Incorporation (%)
Calendula officinalis	25 μCi	Calendic (**1**)	$<5.52 \times 10^{-3}$
Punica granatum	25 μCi	Punicic (**25**)	$<1.3 \times 10^{-3}$
Impatiens edgworthii	25 μCi	α-Parinaric (**30**)	$<1.3 \times 10^{-4}$

[a] 200 Germinated seeds. [b] Administered in phosphate buffer (pH 6.87) and grown for 6 days in laboratory light.

Table 2. Conversion of [^{14}C]-labelled fatty acids into calendic acid by developing marigold seed homogenate

Expt.	Radiochemicals administered[a]	Calendic acid (mg)[b]	Incorporation (%)
1	[1-^{14}C]Linolenic acid	12.6	negligible
2	[1-^{14}C]Linoleic acid	14.1	0.05
3	[1-^{14}C]Oleic acid	9.0	0.52
4	[1-^{14}C]Stearic acid	14.1	0.03

[a] As sodium salts to homogenate from seeds (50 g) harvested 15 days after flower-drop. [b] Crystallised to constant count.

acid more efficiently than are linoleic acid or linolenic acid and their CoA derivatives.[13] Stearic acid, however, was rather poorly transformed into calendic acid (Table 2).

A danger to be guarded against in experiments of this type is degradation to acetate and resynthesis of calendic acid from the labelled acetate formed. The extent to which this had happened in Expt. 2 of Table 2 was therefore assessed by hydrogenation of the calendic acid product to stearic acid and electrolytic removal of C-1 as $^{14}CO_2$,[14] counting both the carbon dioxide and the residue. This experiment indicated that 83.5% of the label remained at C-1, with 14.1% being distributed in the chain. Some label randomisation apparently does occur but it is restricted, and the experiment leaves no doubt that intact linoleic acid is a precursor of calendic acid. A similar assessment was made for [1-^{14}C]oleic acid (Expt. 3 of Table 2) and showed that 87.5% of the label remained with C-1 of calendic acid, 11.3% being found in the chain.

These conclusions were supported by [^{14}C]/[^{3}H] double-labelling and competition experiments (Table 3). Experiment 5 involving [^{3}H]- and [^{14}C]-labelled linoleic acid confirms that this acid is incorporated largely intact into calendic acid as there is only a small shift in the [^{3}H]/[^{14}C] ratio as between precursor and product. Incorporation *via* degradation to acetate would have brought about large losses of tritium. The position with regard to oleic acid is similar (Expt. 6). Competition experiments (7 and 8) reaffirm that oleic acid is much better incorporated into calendic acid than is linoleic acid, and that stearic acid is also a much less efficient precursor. However, Expt. 9 indicates that stearic acid is dehydrogenated and incorporated essentially undegraded into calendic acid. Thus only a little more than half the tritium is lost from [U-9,10-^{3}H]stearic acid relative to the [1-^{14}C]-standard. Processes of catabolism to acetate, followed by anabolism, would reduce the [^{3}H]/[^{14}C] ratio much below this figure if the pathways are considered.

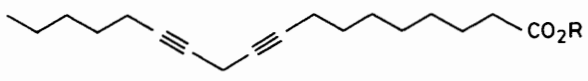
[9,10,12,13-$^{3}H_4$]-Linoleic acid employed in Expt. 5 was made from synthetic methyl octadeca-9,12-diynoate (**4**; R = Me).[15] Following the method of Sgoutas *et al.*,[16] tritiated di-isopentyl)borane was added to the acetylene linkages [*cf.*

Table 3. Double labelling and [^{14}C]/[^{3}H] competitive experiments on formation of calendic acid by developing marigold seed homogenate

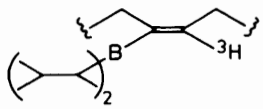
Expt.	Radiolabelled systems administered[a]	Precursor [^{3}H]/[^{14}C] ratio	Calendic acid (mg)[b]	Calendic [^{3}H]/[^{14}C] ratio	Incoporation (%)
5	[9,10,12,13-^{3}H]Linoleic acid	6.74:1	13.8	6.16:1	0.03
	[1-^{14}C]Linoleic acid				0.03
6	[9,10-^{3}H]Oleic acid	6.04:1	18.9	6.41:1	0.34
	[1-^{14}C]Oleic acid				0.32
7	[9,10-^{3}H]Oleic acid	7.00:1	14.2	23.7:1	0.29
	[1-^{14}C]Linoleic acid				0.09
8	[9,10-^{3}H]Oleic acid	6.90:1	19.1	93.5:1	0.30
	[1-^{14}C]Stearic acid				0.02
9	[U-9,10-^{3}H]Stearic acid	6.08:1	22.1	2.41:1	0.01
	[1-^{14}C]Stearic acid				0.03

[a] As Table 2. [b] Purified by h.p.l.c. and crystallised to constant count after preliminary argentation chromatography of methyl ester.

(5)] and the reaction product was decomposed by addition of tritiated acetic acid to give compound (**6**; R = Me), which was hydrolysed to the acid (**6**; R = H). A further deduction from Expt. 5 is that there is little loss of tritium from positions 9, 10, 12, and 13 of linoleic acid during its conversion into calendic acid. Nonetheless, a methylene-interrupted diene has to be converted into a conjugated triene with loss of two hydrogens in the biosynthesis, and confirmation that the two hydrogens come from the flanking 8,11-methylenes was obtained as follows.



(**4**)



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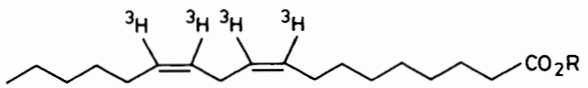
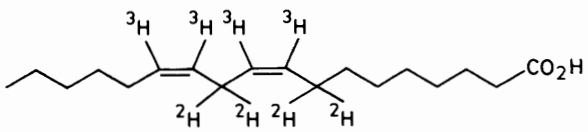


(**6**)



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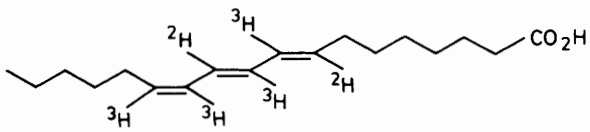
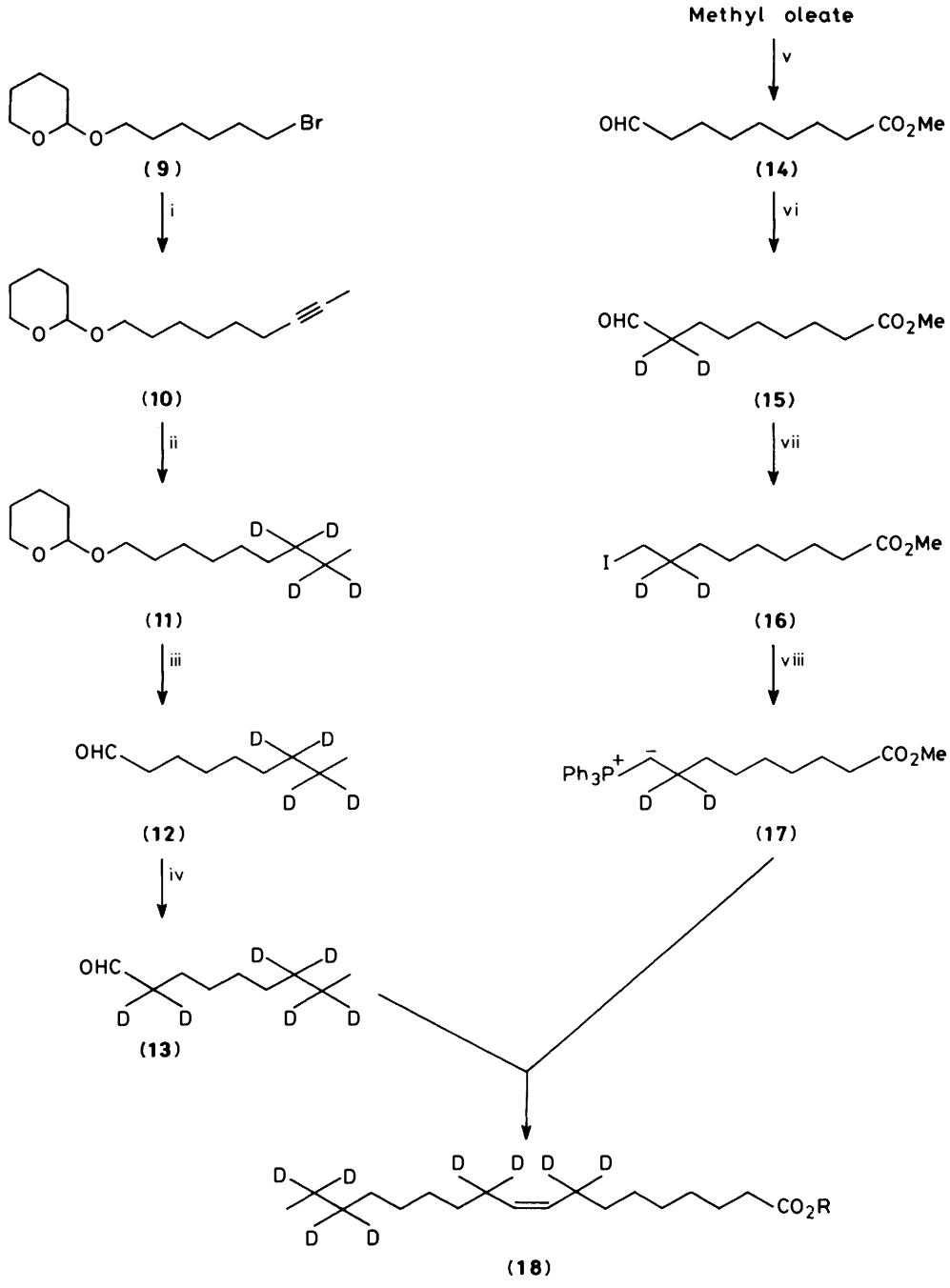


(**8**)

The required test substance was methyl [8,8,11,11,16,16,17,17-$^{2}H_8$]octadec-(9*Z*)-enoate (**18**), the 16,16,17,17-group of deuterium atoms being inserted merely as loading atoms intended to bring the labelled molecules well clear of unlabelled ones in the mass spectrum, thereby allowing sensitive tests to be made. The compound was prepared by Wittig reaction from hexadeuterio aldehyde (**13**) and the dideuterio ylide (**17**) (Scheme 2). Aldehyde (**13**) was made *via* reduction, with $^{2}H_2$ of the acetylene (**10**) using Wilkinson's catalyst. After removal of the pyranyl group with pyridinium toluene-*p*-sulphonate (PPTS),[17] the resulting alcohol was oxidised to the aldehyde by pyridinium chlorochromate (PCC) in dry dichloromethane. Deuteriation at the methylene adjacent to the aldehyde was then carried out according to the procedure of Tucker *et al.*,[18] by heating with dry pyridine and 10 mol equiv. of D_2O, and repeating this process three times. Hexadeuterio aldehyde (**13**) was obtained, after chromatography, in 22% overall yield from the acetylene (**10**).

The other nine-carbon fragment required for the synthesis was made *via* ozonolysis of methyl oleate, using dimethyl sulphide (DMS) for ozonide reductions, following the method of Pappas *et al.*[19] This gave the aldehyde ester (**14**) (57%) which could be selectively deuteriated by the pyridine–D_2O multiple-exchange method[20] to give compound (**15**) (42% after chromatography). Reduction of the aldehyde ester (**15**) with sodium borohydride (94% yield) was followed by conversion into the bromide corresponding to structure (**16**) (67%). A report that the phosphonium salt derived from the bromide is non-crystalline, whereas that from the iodide is,[20] induced us to effect halogen exchange (81%) but in our hands the phosphonium iodide did not crystallise. Using the hindered base t-butyl-lithium, recommended for the avoidance of label-scrambling in similar situations,[20,21] the ylide (**17**) was prepared and allowed to react with the hexadeuterio aldehyde (**13**), after which the reaction was quenched with D_2O. After removal of triphenylphosphine oxide and chromatography on silver nitrate-impregnated silica gel,[22] methyl [8,8,11,11,16,16,17,17-$^{2}H_8$]-octadec-(9*Z*)-enoate was obtained in 34% yield.

The latter ester was analysed mass-spectrometrically[23] for deuterium content, and consisted principally of D_8 and D_7 species, the D_8/D_7 ratio being 7.58. The ester was hydrolysed and administered to marigold homogenate from developing seed (50 g), alongside a separate but similar sodium [1-^{14}C]acetate control experiment which showed a 0.57% conversion into calendic acid (23.0 mg recovered). Calendic acid was isolated and purified from the deuteriated acid feed (18.2 mg), converted into the methyl ester (CH_2N_2), and analysed mass-spectrometrically. The main molecular ion was now at *m/z* 298 [*i.e.* methyl D_8-oleate (**18**; R = Me) (*m/z* 304) minus two hydrogen atoms and minus two deuterium atoms]. The D_6/D_5 ratio in this product (7.53) agreed satisfactorily with the D_8/D_7 ratio above. Thus, in the conversion of oleic acid labelled as in

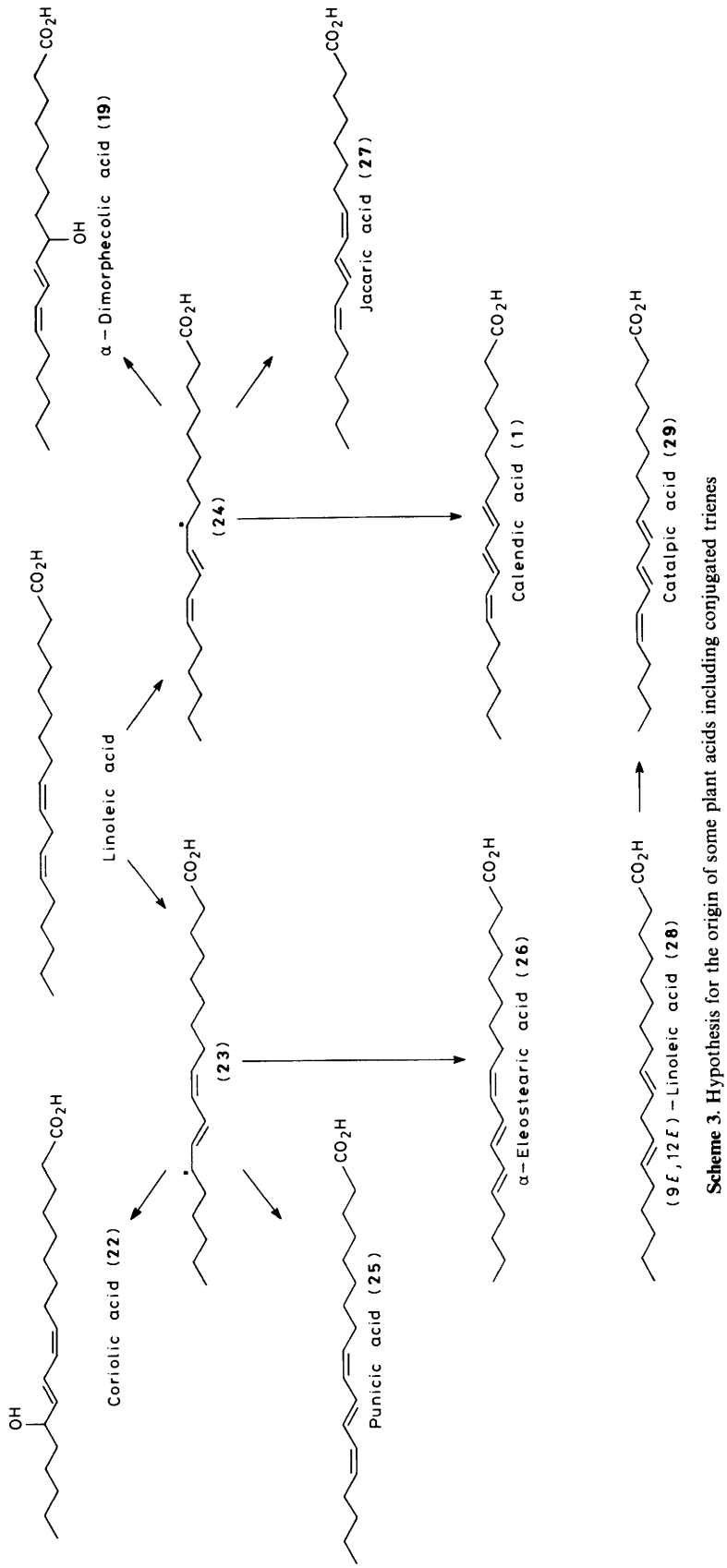


Scheme 2. Synthesis of [8,8,11,11,16,16,17,17-$^2H_8$]octadec-(9*Z*)-enoate. *Reagents and conditions:* i, MeC≡CH, Bu^{n}Li, THF–HMPA, 0 °C; ii D_2–$Rh(PPh_3)_3Cl$–C_6D_6; iii, PPTS–EtOH, then PCC–CH_2Cl_2; iv, D_2O–pyridine, heat; v, O_3–MeOH–0 °C, DMS; vi, D_2O–pyridine, heat; vii, $NaBH_4$–MeOH, then PBr_3–pyridine–0 °C, then NaI–Me_2CO; viii, Ph_3P, then Bu^{t}Li–THF–Et_2O, 0 °C

(**18**; R = H) into calendic acid, two hydrogen and two deuterium atoms are lost, whilst the corresponding loss for similarly labelled linoleic acid as a precursor would be two deuterium atoms. Taking this and Expt. 5 (Table 3) into account, the fate of hydrogen atoms between labelled linoleic acid as represented in structure (**7**) and labelled calendic acid can be shown as in structure (**8**). The stereochemistry of the removal of the two hydrogens at the prochiral 8,11-methylenes has not yet been investigated.

Attention was then turned to the possibility that oxygenated intermediates intervene in the conversion of linoleic into calendic acid. (9*S*)-Hydroxyoctadeca-(10*E*,12*Z*)-dienoic acid (α-dimorphecolic acid) (**19**; R = H) occurs in marigold seed oil as a minor component[24] and was isolated by preparative h.p.l.c., esterified, and oxidised to the keto ester (**20**; R = Me) (61%) with manganese dioxide in pentane. The keto ester was reduced by sodium borotritide in methanol at 0 °C and hydrolysed to the tritiated (±)-9-hydroxydienoic acid (**21**; R^1 = H, R^2 = OH). This was purified by h.p.l.c. (74% yield; 16.3 μCi). Test incorporation using marigold seed homogenate, as in earlier experiments, gave no indication of incorporation into calendic acid.

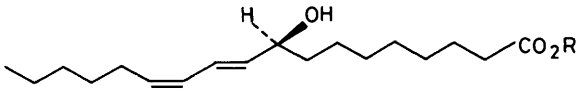
An alternative possibility for an oxygenated intermediate is the hydroperoxy acid (**21**; R^1 = H, R^2 = OOH). This was



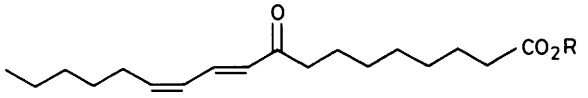
Scheme 3. Hypothesis for the origin of some plant acids including conjugated trienes

prepared using Corey's procedure,[25] from (±)-9-hydroxy[9-^{3}H]octadeca-(10*E*,12*Z*)-dienoate. Treatment with methanesulphonyl chloride and trimethylamine at −65 °C gave the 9-mesylate which was cooled to −110 °C and treated with an excess of 98% ethereal hydrogen peroxide. The crude hydroperoxy methyl ester (38%) was still contaminated with two impurities after preparative t.l.c. (p.l.c.). Owing to its sensitive nature, further purification was not attempted. The ester was saponified with lithium hydroxide in 1,2-dimethoxyethane–water[25] to give the acid having a total activity of 21.4 μCi. Administration of the tritiated hydroperoxy acid preparation gave no incorporation into calendic acid when administered to our marigold seed preparation. We have therefore been unable to provide any evidence for the intervention of a 9-oxygenated intermediate, hydroxide or hydroperoxide, lying betwen linoleic and calendic acid.

Our experimental work appears consistent with the hypothesis of Morris[8] on the origins of higher-plant conjugated triene acids, and does not fully support any of the other hypotheses considered at the outset of this paper. Linoleic acid is viewed as the progenitor of two key radicals (**23**) and (**24**) (Scheme 3), and which radical is formed is viewed as being determined by the enzymes contained in the particular type of plant. One may hypothesise that a delocalised radical of type (**23**) has a geometry (at the enzyme) resembling (**23a**), the 9,10-double bond being held at right angles to the plane containing C-11, -12, and -13, and unable to delocalise the electron. Similarly the radical (**24**) may be represented as having structure (**24a**) at the enzyme. Trapping as a hydroxy diene, probably *via* a hydroperoxide, accounts for coriolic and α-dimorphicolic acids (**22**) and (**19**), respectively. However, in the case of α-dimorphecolic acid we have no evidence that it or its hydroperoxide is an intermediate in triene formation. A somewhat similar situation is the occurrence of ricinoleic acid along with linoleic acid in castor oil seeds: dehydration of the former is not the source of the latter.[5,6]



(**19**)



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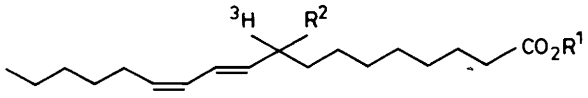
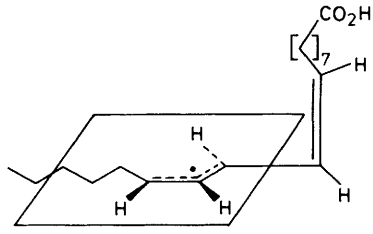
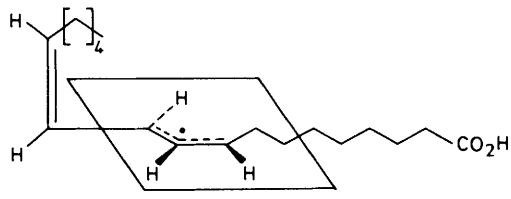


(**21**)

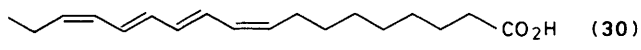
Radical (**23**) can be formally dehydrogenated enzymically at C-14 with (*Z*)-specificity to give punicic acid (**25**), or with (*E*)-specificity to give α-eleostearic acid (**26**). Similarly, radical (**24**) can be dehydrogenated enzymically at C-8 with (*Z*)-specificity to give jacaric acid and with (*E*)-specificity to give calendic acid. Radical (**23**) is the source of natural conjugated trienes with unsaturation beginning at C-9: radical (**24**) is responsible for those commencing at C-8. Catalpic acid presents an apparent irregularity, but it is known that linoleic acid can occur in forms other than the well known (*Z*,*Z*), and the (*E*,*E*)-acid has been found in *Catalpa bignoniodes*.[26] Morris[8] has pointed out that participation of (9*E*,12*E*)-linoleic acid (**28**) could account for catalpic acid: the (9*E*,12*Z*)-form would also be acceptable as a precursor. Operation of such radical processes on linoleic acids with less common geometries (as well as stereomutation during handling) may also account for the minor amounts of geometrically isomeric trienes sometimes found in oils containing one dominant conjugated triene acid.[2,27]



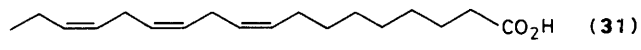
(**23a**)



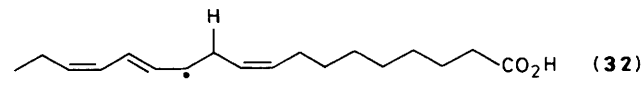
(**24a**)



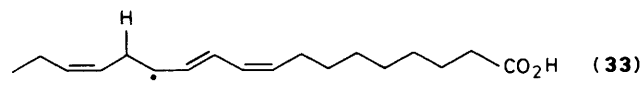
(**30**)



(**31**)



(**32**)



(**33**)

Formation of the conjugated tetraene α-parinaric acid, octadeca-(9*Z*,11*E*,13*E*,15*Z*)-tetraenoic acid (**30**),[28] can be embraced within a similar scheme. Linolenic acid (**31**) is envisaged as the precursor. Stereospecific loss of the 11-hydrogen from radical (**32**) producing an (*E*)-double bond, or similar loss of the 14-hydrogen from compound (**33**), would give conjugated tetraene (**30**) having the required geometry.

Experimental

N.m.r. spectra were determined at 250 MHz in deuteriochloroform unless specified otherwise.

Reference Specimens of Fatty Acids.—These were obtained by saponification of the appropriate seed oil followed by crystallisation from pentane. Calendic acid (**1**) from *Calendula officinalis* seeds had m.p. 39.5—40.5 °C (lit.,[26] 39.0—40.5 °C), punicic acid (**25**) from *Punica granatum* seeds had m.p. 43.0—44.0 °C (lit.,[3] 43.5—44.0 °C), and the tetraene α-parinaric acid (**30**) from *Impatiens edgworthii* seed had m.p. 83.5—84.5 °C (lit.,[29] 85.0—86.0 °C). Octadeca-(8,*E*, 10*E*,12*E*)-trienoic acid

was obtained by stereomutation (I_2, *hν*, pentane) from calendic acid and had m.p. 77.5—78.5 °C (lit.,[11] 77—78 °C). All acids were characterised by u.v., i.r., ^1H n.m.r., and ^{13}C n.m.r. data.

Administration of Sodium [1-^{14}C]*Acetate to* Calendula officinalis.—All seeds were harvested 15—17 days after flower-drop.

(a) *Whole seeds.* These (8.6 g) were placed on moist filter-paper in a tray and sodium [1-^{14}C]acetate was added in buffer (pH 6.98; KH_2PO_4–NaOH) solution: the system was kept for 72 h under laboratory light conditions before being worked up.

(b) *Dissected seeds.* As above, seeds split.

(c) *Intact flower heads.* 12 Flower heads with many seeds *ca.* 12 days past flower-drop were arranged with stems in sodium [1-^{14}C]acetate solution for 7 days.

(d) *Vacuum infiltration.* Chopped seed (20 g) was placed in a solution of sodium [1-^{14}C]acetate in phosphate buffer. The pressure in the flask was reduced (water pump) and air was then admitted. This was repeated twice more and the flask was then shaken gently in air (24 h).

Seed material from each experiment was dried and Soxhlet-extracted with light petroleum (b.p. 40—60 °C). The oil obtained was hydrolysed (~2 mol equiv. NaOH in 20% aqueous ethanol) for 1 h (reflux) under nitrogen and the fatty acids were isolated by acidification to pH 1.0. The mushy solid of mixed fatty acids was recrystallised from n-pentane at −10 to −15 °C until pure calendic acid (**1**) of constant radioactive count was obtained.

(e) *Homogenate preparation.* Seeds, freshly harvested, were homogenised in phosphate buffer (200 ml; pH 6.98) using a Waring blender. Sodium [1-^{14}C]acetate in buffer was added and the flask was shaken for 72 h in air under laboratory light. The product was extracted with light petroleum (b.p. 40—60 °C).

Single-isotope Administration to C. officinalis *Homogenate.*—Seed (50 g; 15 days past flower-drop) was homogenised in phosphate buffer (300 ml; pH 6.87) and the appropriate precursor was added as its sodium salt. Flasks were shaken in air for 48 h before work-up. Results are in Table 3. Calendic acid (**1**) specimens usually required 7—9 crystallisations to attain constant count.

Mixed-isotope Administration to C. officinalis *Homogenate.*—Details are as above, but the crude fatty acid mixture obtained during work-up was methylated (CH_2N_2 at 0 °C) and partially separated by chromatography on silver nitrate-impregnated silica gel (17% w/w silver nitrate; elution with ether–hexane, 1:1) before hydrolysis and crystallisation (7—9 times) to constant count. Results are in Table 3.

Hydrogenation and Electrolytic Decarboxylation of Labelled Calendic Acid.—Labelled calendic acid from administration of [1-^{14}C]oleic acid (Table 2, Expt. 3) (8.7 mg) was hydrogenated over 10% palladium–charcol (5 mg) in ethyl acetate (1 ml) to give stearic acid, m.p. 68.0—69.5 °C. This was diluted with 'cold' material to 50 mg (6.44×10^{-2} μCi) and dissolved in a mixture of pyridine (0.3 ml) and water (0.1 ml). The product was transferred to a specially designed electrolytic cell,[14] triethylamine (10 drops) was added, and nitrogen gas rigorously freed from CO_2 was passed through. A constant voltage was applied and the current (65 mA) fell to 12 mA during $1\frac{3}{4}$ h, then remained steady. Throughout the decarboxylation carbon dioxide was swept quantitatively into barium hydroxide solution (3 Dreschel bottles) and carbon dioxide was completely removed from the cell by heating it on a water-bath at 35 °C. Precipitated barium carbonate was collected on a fine sinter-funnel and washed with freshly boiled, distilled water and acetone, all operations being conducted under CO_2-free nitrogen, before the product was dried *in vacuo* and weighed. The dried $BaCO_3$ (16.0 mg) indicated that electrolysis had proceeded to 74.2% of theoretical. The $BaCO_3$ was treated with 2M-hydrochloric acid and the liberated CO_2 was swept into Hyamine solution in a nitrogen stream for counting. The total activity in the $BaCO_3$ was 4.18×10^{-2} μCi. The total activity in the material remaining in the electrolysis cell was 2.18×10^{-2} μCi.

A similar experiment was carried out with calendic acid originating from the linoleic acid experiment (Table 2, Expt. 2). It was hydrogenated to stearic acid (13.9 mg), m.p. 66.5—68 °C, and diluted to 50 mg (6.20×10^{-3} μCi). Electrolytic decarboxylation as above gave $BaCO_3$ (15.3 mg) indicating that the reaction had proceeded to 71.8% of theoretical extent. Carbon dioxide was again counted in Hyamine. The total activity in the $BaCO_3$ was 3.72×10^{-3} μCi. The total activity in the electrolysis residues was 2.33×10^{-3} μCi.

Octadeca-9,12-diynoic Acid (**4**; R = H).—Diethyl malonate (463 mg, 2.90 mmol) in dry tetrahydrofuran (THF) (5 ml) was treated at 0 °C with n-butyl-lithium in hexane (1.43M; 2.1 ml, 2.91 mmol) under nitrogen. A solution of 1-iodohexadeca-7,9-diyne (from the chloride by sodium iodide–acetone exchange) (500 mg, 1.45 mmol) in THF (5 ml) was added dropwise at 20 °C and the mixture was heated under reflux (3 h). Potassium hydroxide (1 g) in a mixture of water (1 ml) and ethanol (10 ml) was added and the mixture was stirred at 20 °C (1 h). It was then acidified to pH 1.0 with hydrochloric acid and evaporated under reduced pressure. Extraction with ether gave heptadeca-8,11-diyne-1,1-dicarboxylic acid which was heated (oven, 140 °C) at 0.2 mmHg pressure for 30 min. Distillation at 160—165 °C/0.1 mmHg gave a light yellow oil which crystallised. Crystallisations from n-pentane at 0 °C under nitrogen gave octadeca-9,12-diynoic acid (**4**; R = H) (128 mg, 32%), m.p. 41—43 °C (lit.,[30] 42—43 °C (M^+, 276.2078. Calc. for $C_{18}H_{28}O_2$: *M*, 276.2089); $\nu_{max.}$ ($CHCl_3$) 1 695 cm^{-1}; δ_H 0.90 (3 H, t, Me), 1.21—1.52 (16 H, br m, 8 × CH_2), 2.18 (4 H, t, 8- and 14-H_2), 2.36 (2 H, t, 2-H_2), 3.07 (2 H, t, *J* 1.1 Hz, 11-H_2), 10.84—11.02 (1 H, br, CO_2H); δ_C 10.0 (C-11), 14.2 (C-18), 19.1 (C-8, -14), 22.4 (C-17), 24.9 (C-3), 28.6—29.0 (C-4, -5, -6, -7, and -15), 31.3 (C-16), 32.4 (C-2), 74.8 (C-9), 75.0 (C-10), 80.6 (C-12), 80.8 (C-13), and 180.2 p.p.m. (C-1).

Methyl octadeca-9,12-diynoate (**4**; R = Me) (109 mg, 82%) was made from the acid (128 mg) by treatment with diazomethane followed by chromatography on silica.

Methyl [9,10,12,13-$^3H_4$]*Octadeca-*(9Z,12Z)*-dienoate* (**6**; R = Me).—Sodium borotritide (150 μCi; Amersham) was diluted with sodium borohydride to 37 mg (1 mmol) and added to stirred diglyme (2 ml) under N_2 for 15 min. A solution of 2-methylbut-2-ene (175 mg, 2.4 mmol) in diglyme (0.5 ml) was then added and the flask was cooled to 0 °C. Freshly distilled boron trifluoride–diethyl ether (0.15 ml, 1.5 mmol) was added during 10 min, and the mixture was then stirred for 2 h at 0 °C, when a solution of methyl octadeca-9,12-diynoate (**4**; R = Me) (100 mg, 0.34 mmol) in diglyme (0.5 ml) was added dropwise. After 30 min at 0 °C the flask was allowed to attain room temperature and the contents were stirred (2 h). Ethylene glycol (0.5 ml) was then added to the mixture (cooled to 0 °C) to destroy excess of hydride; tritiated acetic acid (0.12 ml; 200 μCi) was then added and tritiolysis was completed by stirring the mixture at 20 °C overnight. Acetic acid (1 ml; 50%) was added and protonolysis was completed by further stirring of the mixture at room temperature (8 h). Work-up gave a pale yellow oil, purified first by p.l.c. (ether–hexane, 1:9) and then reversed-phase h.p.l.c. (eluant methanol–water, 9:1). Methyl [9,10,12,13-$^3H_4$]octadeca-(9*Z*,12*Z*)-dienoate (**6**; R = Me) (32.3 mg, 34%;

specific ^3H activity 1 026 μCi mmol^{-1}) had $\nu_{max.}$ 1 725 cm^{-1}; δ_H 0.90 (3 H, t, Me), 1.24—1.43 (14 H, br m, 7 × CH_2), 1.64 (2 H, m, CH_2), 2.01—2.13 (4 H, m, 8- and 14-H_2), 2.32 (2 H, t, 2-H_2), 2.79 (2 H, dd, 11-H_2), 3.67 (3 H, s, OMe), and 5.37 (4 H, m, 9-, 10-, 12-, and 13-H).

The ester (32.3 mg) was hydrolysed by being refluxed (N_2) with ethanolic potassium hydroxide. Work-up gave an oil which solidified at −10 °C and which was purified by p.l.c. (eluant ether–hexane, 1:2) to give [9,10,12,13-$^3H_4$]-octadeca-(9*Z*,12*Z*)-dienoic acid (**6**; R = H) (27.5 mg; specific ^3H activity 960 μCi mmol^{-1}); δ_H 0.90 (3 H, t, Me), 1.22—1.46 (14 H, br m, 7 × CH_2), 1.66 (2 H, m, 3-H_2) 1.98—2.16 (4 H, m, 8- and 14-H_2), 2.33 (2 H, t, 2-H_2), 2.80 (2 H, dd, 11-H_2), 5.39 (4 H, m, 9-,10-,12-, and 13-H), and 10.94—11.26 (1 H, br, CO_2H).

9-(*Tetrahydropyran*-2-*yloxy*)*non*-2-*yne* (**10**).—Excess of propyne was passed into a mixture of THF (30 ml) and hexamethylphosphoramide (HMPA) (10 ml) under nitrogen. The solution was cooled (0 °C) and a solution of n-butyl-lithium in hexane (1.4M; 57.5 ml, 0.08 mol) was added dropwise. A solution of 1-bromo-6-(tetrahydropyran-2-yloxy)hexane (**9**) (20 g, 0.075 mol) in HMPA (20 ml) was then added so that the temperature did not exceed 15 °C. The mixture was stirred (30 min) at room temperature and worked up with aqueous ammonium chloride. Distillation gave 9-(*tetrahydropyran*-2-*yloxy*)*non*-2-*yne* (**10**) (11.4 g, 68%), b.p. 112—114 °C/0.4 mmHg (Found: C, 74.7; H, 10.85. $C_{14}H_{24}O_2$ requires C, 75.0; H, 10.8%); δ_H (90 MHz) 1.26—1.73 (14 H, m, 7 × CH_2), 1.75 (3 H, t, *J* 1.2 Hz, C≡CMe), 3.27—4.06 (4 H, m, 2 × CH_2O), 4.58 (1 H, br m, OCHO).

Deuteriation of 9-(*Tetrahydropyran*-2-*yloxy*)*non*-2-*yne* (**10**).—A stirred solution of chlorotris(triphenylphosphine)rhodium (200 mg) in degassed hexadeuteriobenzene was equilibrated under deuterium (99.99%) (2 h) in a hydrogenator. A solution of the tetrahydropyranyloxynon-2-yne (**10**) (2 g, 8.93 mmol) in hexadeuteriobenzene was injected and after 12 h (D_2 absorbed; 371 ml; calc: 400 ml) the catalyst was removed by passage through a short column of alumina several times. The solvent was removed and column chromatography (SiO_2; eluant ether–hexane, 3:2) gave 7,7,8,8-tetradeuterio-1-(tetrahydropyran-2-yloxy)nonane (**11**) (1.86 g), $\delta_{^2H}$ 1.27 (4 D, d, $CH_3CD_2CD_2CH_2$), which was immediately deprotected as described below.

[7,7,8,8-$^2H_4$]*Nonanal* (**12**).—The above tetrahydropyranyl ether (**11**) (1.86 g, 8.02 mmol) was dissolved in ethanol (10 ml) and the solution was stirred with PPTS (0.47 g, 9.0 mmol) for 3 h at 35 °C. Work-up and chromatography (SiO_2; eluant ether–hexane, 3:2) gave [7,7,8,8-$^2H_4$]nonanol (1.1 g, 71% from starting acetylene), *m/z* 130.1643 (M^+ − 18); $\delta_{^2H}$ 1.27 (4 D, d, $CH_3CD_2CD_2CH_2$). The alcohol (1.1 g, 7.43 mmol) was dissolved in dry dichloromethane (5 ml) and the solution was added to a stirred solution of PCC (0.8 g, 10.0 mmol) in dichloromethane (15 ml) under N_2 at 20 °C. The mixture was stirred for 2 h, ether (15 ml) was added, and the dark solution was filtered through a short Florisil column. Evaporation and chromatography (eluant ether–hexane, 1:4) gave [7,7,8,8-^2H]*nonanal* (**12**) (0.74 g, 68%) (M^+, 146.1602. $C_9H_{14}D_4O$ requires *M*, 146.1609); $\nu_{max.}$ 1 725 cm^{-1}; δ_H (90 MHz) 0.89 (3 H, br s, CD_2Me), 1.18—1.79 (8 H, m, 4 × CH_2), 2.42 (2 H, t, CH_2CHO), and 9.80 (1 H, t, CHO).

[2,2,7,7,8,8-$^2H_6$]*Nonanal* (**13**).—Deuterium oxide (99.99%; 2 ml, 100 mmol) was heated at 90—95 °C for 7 h with [7,7,8,8-$^2H_4$]nonanal (**12**) (0.73 g, 5.0 mmol) in stirred, anhydrous pyridine (5 ml) under nitrogen. The solution was poured into ice–water and thoroughly extracted with n-hexane. The hexane extracts were washed successively with aqueous copper sulphate and water and thoroughly dried and evaporated. The deuteriation procedure was then repeated twice more. Silica gel chromatography (eluant ether–hexane, 1:4) gave [2,2,7,7,8,8-$^2H_6$]*nonanal* (**13**) (0.34 g, 46%) (M^+, 148.1726. $C_9H_{12}D_6O$ requires *M*, 148.1734); $\nu_{max.}$ 1 720 cm^{-1}; δ_H (90 MHz) 0.89 (3 H, br s, CD_2CH_3), 1.17—1.79 (8 H, br m, 4 × CH_2), and 9.90 (1 H, s, CD_2CHO); $\delta_{^2H}$ 1.26 (4 D, d, $CH_2CD_2CD_2CH_3$) and 2.41 (2 D, s, CD_2CHO).

Reductive Ozonolysis of Methyl Octadec-(9*Z*)-*enoate* (*Methyl Oleate*).—A solution of methyl octadec-(9*Z*)-enoate (20 g, 0.068 mol) in methanol (100 ml) was stirred at −60 °C and ozonised until a slight excess had been passed. While the mixture was kept at −60 °C, the system was flushed with nitrogen and DMS (5.5 ml, 0.075 ml) was added. The solution was stirred at −10 °C (1 h), 0 °C (1½ h), and 20 °C (1 h), the solvent was removed under reduced pressure, and the residue was extracted with hexane. Evaporation and distillation gave methyl 9-oxononanoate (**14**) (7.2 g, 57%) (M^+, 186.1249. Calc. for $C_{10}H_{18}O_3$: *M*, 186.1256); $\nu_{max.}$ 1 720—1 705br cm^{-1}; δ_H (90 MHz) 1.18—1.86 (10 H, br m, 5 × CH_2), 2.26—2.57 (4 H, m, CH_2CHO and CH_2CO_2Me), 3.74 (3 H, s, OMe), and 9.83 (1 H, t, CHO).

Methyl 9-*Oxo*[8,8-$^2H_2$]*nonanoate* (**15**).—Deuterium oxide (99.99%; 8 ml, 0.4 mol) was added to a solution of methyl 9-oxononanoate (**14**) (7.1 g, 0.038 mol) in anhydrous pyridine (40 ml) the mixture was heated (90—95 °C) under nitrogen for 8 h. After work-up (see above) the procedure was repeated twice more. The product was chromatographed on silica gel (eluant ether–hexane, 2:3) to give methyl 9-oxo[8,8-$^2H_2$]nonanoate (**15**) (3 g, 42%) (M^+, 188.1368. Calc. for $C_{10}H_{16}D_2O_3$: *M*, 188.1381); $\nu_{max.}$ 1 710—1 730 cm^{-1}; δ_H (90 MHz) 1.17—2.81 (10 H, m, 5 × CH_2), 2.31 (2 H, t, CH_2CO_2Me), 3.68 (3 H, s, CO_2Me), and 9.83 (1 H, s, CD_2CHO); $\delta_{^2H}$ 2.29 (2 D, s, CD_2CHO).

Methyl 9-*Hydroxy*[8,8-$^2H_2$]*nonanoate*.—A solution of the aldehyde (**15**) (3.0 g, 16.0 mmol) in dry methanol (10 ml) was added dropwise at 0 °C to sodium borohydride (0.7 g, 16.5 mmol) in methanol (5 ml). After being stirred at 0 °C for 5 min, the mixture was stirred at 20 °C for 30 min and an equal volume of water was added. Extraction with chloroform and chromatography on silica gel (eluant ether–hexane, 1:1) gave the *title compound* (2.86 g, 94%) [*m/z*, 172.1416 (M^+ − 18). $C_{10}H_{18}D_2O_3$ − H_2O requires *m/z*, 172.1432]; $\nu_{max.}$ 1 740 cm^{-1}; δ_H (90 MHz) 1.18—1.78 (10 H, br m, 5 × CH_2), 2.06 (1 H, s, OH), 2.29 (2 H, t, CH_2CO_2Me), 3.51 (2 H, s, CD_2CH_2OH), and 3.67 (3 H, s, OMe); $\delta_{^2H}$ 2.26 (2 D, s, CD_2CH_2OH).

Methyl 9-*Iodo*[8,8-$^2H_2$]*nonanoate* (**16**).—The above alcohol (2.7 g, 0.014 mol) was dissolved in ether (10 ml) containing 5 drops of pyridine and the solution was stirred with freshly distilled phosphorus tribromide (2.0 g, 0.0073 mol) at 0 °C for 2 h and then at 20 °C for 5 h. Ice and ether (20 ml) were added. Separation of the ether layer and work-up by chromatography on silica gel (eluant ether–hexane, 1:4) gave methyl 9-*bromo*[8,8-$^2H_2$]*nonanoate* (2.4 g, 67%) (M^+, 254.0675. Calc. for $C_{10}H_{17}BrD_2O_2$: *M*, 254.0694); $\nu_{max.}$ 1 740 cm^{-1}; δ_H (90 MHz) 1.14—1.81 (10 H, br m, 5 × CH_2), 2.28 (2 H, t, CH_2CO_2Me), 3.34 (2 H, s, CD_2CH_2Br), and 3.66 (3 H, s, OMe).

A solution of the bromo compound (2.3 g, 9.1 mmol) in dry ethyl methyl ketone (20 ml) was heated with sodium iodide (1.4 g, 10 mmol) under reflux (8 h), poured into water, and chromatographed to give the straw-coloured iodo compound (**16**) (2.2 g).

Methyl [8,8,11,11,16,16,17,17-$^2H_8$]*Octadec*-(9*Z*)-*enoate* (**18**; R = Me).—The iodide (above) (2.2 g) was refluxed under nitrogen (14 h) with triphenylphosphine (2.1 g, 8 mmol) in dry

benzene and worked up to give the phosphonium iodide (**17**) (3.2 g, 64%) which failed to crystallise satisfactorily. A solution of the phosphonium iodide (0.83 g, 1.6 mmol) in dry THF–ether (1:1; 5 ml) was stirred at 0 °C and t-butyl-lithium in hexane (1.3M; 1.2 ml, 1.6 mmol) was added dropwise. After the mixture had been stirred at 0 °C (5 min), a solution of [2,2,7,7,8,8-$^2H_6$]nonanal (**13**) (0.24 g, 1.6 mmol) in dry THF (2 ml) was added dropwise at 0 °C, and the mixture was stirred and slowly allowed to attain room temperature during 2 h. Deuterium oxide (99.99%; 1 ml) was added and the solvent was evaporated off under reduced pressure. The residue was taken up into ether, the extract was dried and evaporated, and the residue was taken up in n-pentane to leave most of the triphenylphosphine oxide behind. Chromatography on silver nitrate-impregnated silica gel (17% w/w $AgNO_3$; eluant ether–hexane, 1:1) gave methyl [8,8,11,11,16,16,17,17-$^2H_8$]octadec-(9*Z*)-enoate (156 mg, 32%) as an oil having the following deuterium distribution: *m/z* 297, $C_{19}H_{35}DO_2$ (0.55%); *m/z* 298, $C_{19}H_{34}D_2O_2$ (0.63%); *m/z* 299, $C_{19}H_{33}D_3O_2$ (0.97%); *m/z* 300, $C_{19}H_{32}D_4O_2$ (2.0%); *m/z* 301, $C_{19}H_{31}D_5O_2$ (3.5%); *m/z* 302, $C_{19}H_{30}D_6O_2$ (3.2%); *m/z* 303, $C_{19}H_{29}D_7O_2$ (10.41%); *m/z* 304, $C_{19}H_{28}D_8O_2$ (78.87%); and *m/z* 305, $C_{19}H_{27}D_9O_2$ (0.45%); $\nu_{max.}$ 1 730 cm^{-1}; δ_H (90 MHz) 0.90 (3 H, s, CD_2Me), 1.13—1.82 (18 H, br m, 9 × CH_2), 2.28 (2 H, t, CH_2CO_2Me), 3.74 (3 H, s, OMe), and 5.37 (2 H, br s, $CD_2CH{=}CHCD_2$); $\delta_{^2H}$ 1.29 (4 D, d, $CH_3CD_2CD_2CH_2$) and 2.03 (4 D, s, $CD_2CH{=}CHCD_2$).

Administration of [8,8,11,11,16,16,17,17-$^2H_8$]*Octadec-*(9Z)*-enoic Acid* (**18**; R = H) *to* Calendula officinalis *Homogenate.*—The above ester (**18**; R = Me) (150 mg) was hydrolysed with potassium hydroxide in aqueous ethanol. Work-up gave the title acid (**18**; R = H) (126 mg); its R_F value was closely similar to that of authentic oleic acid.

The octadeuterio acid (**18**; R = H) (50 mg) was neutralised with dil. aqueous sodium hydroxide and added to freshly prepared marigold seed (50 g; 15 day after flower-drop) homogenate. The mixture was shaken for 48 h with access to air. Calendic acid (**1**) (18.2 mg) was isolated after 4 recrystallisations, m.p. 39.0—40.0 °C. It was esterified (CH_2N_2) and further purified by reversed-phase h.p.l.c. with methanol–water (9:1) as eluant. The pure methyl ester showed *m/z* 297 [$C_{19}H_{29}D_7O_2$ − (2 H + 2 D)] and *m/z* 298 [$C_{19}H_{29}D_8O_2$ − (2 H + 2 D)] in the ratio 1:7.53.

A control experiment, run consecutively with the above, used the same seed homogenate, with added sodium [1-^{14}C]acetate and used an identical procedure. The calendic acid (**1**) isolated, m.p. 39.5—40.5 °C, $\lambda_{max.}$(EtOH) 261, 272, and 282 nm, showed an incorporation of 0.57%.

Isolation of Methyl (9S)-*Hydroxyoctadeca-*(10E,12Z)*-dienoate* (**19**; R = Me) *from* Calendula officinalis *Seed Oil.*—Mature *C. officinalis* seeds (150 g) were ground and extracted with n-hexane to give seed oil (36 g). Saponification gave fatty acids (29.6 g) which were esterified (CH_2N_2) to give the mixed fatty acid methyl esters (29.1 g). Reversed-phase preparative h.p.l.c. (Waters preparative instrument), with methanol–water (9:1) as eluant, gave methyl (9*S*)-hydroxyoctadeca-(10*E*,12*Z*)-dienoate (**19**; R = Me) (260 mg) as an early eluted band (before methyl calendate). The hydroxy ester was further purified by p.l.c. on silica (eluant ether–hexane, 1:1). The natural hydroxy ester had $[\alpha]_D^{19}$ +3.7° (*c* 1.9 in EtOH) {lit.,[24] $[\alpha]_D^{20}$ +3.6° (*c* 19.4 in $CHCl_3$)}, $\nu_{max.}$(film) 3 630 and 1 730 cm^{-1}; $\lambda_{max.}$(EtOH) 233 nm (ε 26 800); δ_H 0.90 (3 H, t, Me), 1.17—1.71 (16 H, br m, 8 × CH_2), 2.16—2.72 (7 H, m, 14-, 8-, and 2-H_2 and 9-OH) (OH lost on shaking with D_2O), 3.69 (3 H, s, OMe), 4.14 (1 H, m, 9-H), and 5.27—6.68 (4 H, m, $J_{10,11}$ 14.6 Hz, 10-, 11-, 12-, and 13-H). The trimethylsilyl ether was prepared and had M^+, 382.2915 ($C_{22}H_{42}O_3Si$ requires *M*, 382.2903).

Oxidation of Methyl (9S)-*Hydroxyoctadeca-*(10E,12Z)*-dienoate* (**19**; R = Me).—The title hydroxy diene (100 mg) was stirred with activated manganese dioxide (1 g) in pentane under nitrogen for 48 h. Filtration, evaporation, and p.l.c. on silica (eluant ether–hexane, 3:2) gave methyl 9-oxo-octadeca-(10*E*,12*Z*)-dienoate (**20**; R = Me) (60 mg). The dienone had M^+, 308.2348 ($C_{19}H_{32}O_3$ requires *M*, 308.2351), $\lambda_{max.}$(EtOH) 276 nm (ε 17 400); δ_H (90 MHz) 0.90 (3 H, t, Me), 1.14—1.74 (16 H, br m, 8 × CH_2), 2.14—2.66 (6 H, m, 2-, 8-, and 14-H_2), 3.69 (3 H, s, OMe), 5.75—6.32 (3 H, m, 10-, 12-, and 13-H), and 7.38—7.69 (1 H, q, 11 H).

Methyl (±)-9-*Hydroxy*[9-3H]*octadeca-*(10E,12Z)*-dienoate* (**21**; R^1 = Me, R^2 = OH).—A solution of the dienone (**20**; R = Me) (32 mg) in dry methanol (3 ml) was stirred at 0 °C (5 min) with a mixture of sodium borotritide and sodium borohydride (7 mg; ^3H activity 100 μCi). Work-up gave an oil, which was purified by t.l.c. (eluant ether–hexane, 1:1). The methyl ester (**21**; R^1 = Me, R^2 = OH) (23 mg) had specific ^3H activity 229.4 μCi $mmol^{-1}$, 1.64 × 10^6 d.p.m. mg^{-1}; δ_H (80 MHz) (*inter alia*) 0.90 (3 H, t, Me), 1.16—1.89 (16 H, br m, 8 × CH_2), 3.68 (3 H, s, OMe), 4.15 (1 H, m, 9-H), 5.27—6.68 (4 H, m, $J_{10,11}$ 14.6 Hz, $J_{12,13}$ 10.3 Hz, 10-, 11-, 12-, and 13-H); $\delta_{^3H}$ 4.15 (s, 9-^3H).

The ester (23 mg) was hydrolysed by being refluxed with potassium hydroxide (200 mg) in 80% ethanol–water (2 ml) for 1 h under nitrogen. Work-up gave the acid (**21**; R^1 = H, R^2 = OH) (20 mg, total ^3H activity 16.3 μCi) which was neutralised with sodium hydroxide and administered to *C. officinalis* homogenate (see below).

Methyl (±)-9-*Hydroperoxy*[9-^{3}H]*octadeca-*(10E,12Z)*-dienoate* (**21**; R^1 = Me, R^2 = OOH).—The hydroxy ester (**21**; R^1 = Me, R^2 = OH) (27 mg; 34.1 μCi total ^3H activity) was treated with freshly distilled methanesulphonyl chloride (22 mg) and triethylamine (22 mg) in dry dichloromethane (3 ml) at −65 °C under nitrogen. The solution was stirred (20 min), cooled to −110 °C, and treated with excess of 98% ethereal hydrogen peroxide (1.5 ml). After being stirred at −110 °C for 15 min the mixture was allowed to warm to 0 °C and water was added. Dichloromethane (10 ml) was added and the solution was quickly dried ($MgSO_4$). After evaporation under reduced pressure the remanent oil (24 mg) was purified by t.l.c. (eluant ether–hexane, 1:1). The purified methyl 9-hydroperoxy[9-^{3}H]octadeca-(10*E*,12*Z*)-dienoate (21 mg; 26.8 μCi total ^3H activity) was re-examined by ^1H n.m.r. spectroscopy and t.l.c., but the latter showed two impurities, suggesting decomposition.

The ester (20 mg) was therefore hydrolysed without delay using lithium hydroxide (150 mg) in 1,2-dimethoxyethane–water (3 ml; 1:1). Aqueous hydrogen peroxide (0.5 ml; 10%) was added and the solution was stirred (1½ h) at 20 °C. Extraction with ether and evaporation of the extract under reduced pressure gave the acid (**21**; R^1 = H, R^2 = OOH) (17 mg; total ^3H activity 21.4 μCi) which was neutralised and administered to *C. officinalis* homogenate without delay.

Administration of (±)-9-*Hydroxy- and* -9-*Hydroperoxy-*[9-^{3}H]*octadeca-*(10E,12Z)*-dienoic Acids to* Calendula officinalis *Homogenate.*—The sodium salt of the hydroxy acid (**21**; R^1 = H, R^2 = OH) was administered to the buffered homogenate from seed (50 g) gathered 15 days after flower-drop in the usual way. Calendic acid was isolated and after 5 crystallisations (14.7 mg; m.p. 39.5—40.5 °C) had ^3H activity of less than twice that of the background.

The sodium salt of the hydroperoxy acid (**21**; R^1 = H, R^2 = OOH) similarly administered gave calendic acid which was first purified by argentation chromatography as the methyl ester. The ester was hydrolysed and after 4 recrystallisations gave

calendic acid (10.4 mg; m.p. 38.5—39.5 °C) having ^{3}H activity of less than twice that of the background.

In each case the activity of the homogenate was verified by a parallel experiment using sodium[1-^{14}C]acetate.

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References

1 C. Y. Hopkins in 'Topics in Lipid Chemistry,' ed. F. D. Gunstone, Logos Press, London, 1972, vol. 3.
2 T. Takagi and Y. Itabashi, *Lipids*, 1981, **16**, 546.
3 L. Crombie and A. G. Jacklin, *J. Chem. Soc.*, 1957, 1632.
4 V. Ratovelomanana and G. Linstrumelle, *Tetrahedron Lett.*, 1984, **25**, 6001; L. D. Bergelson and M. M. Shemyakin, *Angew. Chem., Int. Ed. Engl.*, 1964, **3**, 250.
5 C. Hitchcock and B. W. Nichols, 'Plant Lipid Biochemistry,' Academic Press, London, 1971.
6 'Recent Advances in the Chemistry and Biochemistry of Plant Lipids,' eds. T. Galliard and E. I. Mercer, Academic Press, London, 1975.
7 F. D. Gunstone, *Chem. Ind. (London)*, 1965, 1033; 1966, 1551; H. R. S. Conacher and F. D. Gunstone, *Lipids*, 1970, **5**, 137.
8 L. J. Morris and M. O. Marshall, *Chem. Ind. (London)*, 1966, 1493.
9 For a review see G. A. Veldink, J. F. G. Vliegenthart, and J. Boldingh, *Prog. Chem. Fats Other Lipids*, 1977, **15**, 131; See also O. S. Privett, E. C. Nickell, W. O. Lundberg, and P. D. Boyer, *J. Am. Oil Chem. Soc.*, 1955, **32**, 505; R. T. Holman, P. O. Egwim, and W. W. Christie, *J. Biol. Chem.*, 1969, **244**, 1149; M. Hamberg, *Anal. Biochem.*, 1971, **43**, 515; M. Roza and A. Francke, *Biochim. Biophys. Acta*, 1973, **316**, 76.
10 For a preliminary communication see L. Crombie and S. J. Holloway, *J. Chem. Soc., Chem. Commun.*, 1984, 953.
11 M. J. Chisholm and C. Y. Hopkins, *Can. J. Chem.*, 1960, **38**, 2500; C. Y. Hopkins and M. J. Chisholm, *ibid.*, 1962, **40**, 2078.
12 For a review of techniques see S. A. Brown in 'Biosynthesis,' (Specialist Periodical Reports) The Chemical Society, London, 1972, vol. 1.
13 M. Noda, K. Ohga, Y. Nakagawa, and K. Ichiara in 'Biogenesis and Functions of Plant Lipids,' eds. P. Mazliak, P. Benveniste, C. Costes, and R. Douce, Elsevier/North Holland Biomedical Press, 1980, p. 215.
14 H. Simon and H. G. Floss, 'Bestimmung der Isotopenverteilung in Markierten Verbindungen,' Springer Verlag, Berlin, 1967.
15 W. J. Gensler and G. R. Thomas, *J. Am. Chem. Soc.*, 1951, **73**, 4601.
16 D. S. Sgoutas, H. Sanden, and E. M. Young, *J. Lipid Res.*, 1969, **10**, 642.
17 M. Miyashita, A. Yoshikoshi, and P. A. Greico, *J. Org. Chem.*, 1977, **42**, 3772.
18 W. P. Tucker, S. B. Tove, and C. R. Kepler, *J. Labelled Compd.*, 1971, **7**, 137.
19 J. J. Pappas, W. P. Keaveney, E. Gancher, and M. Berger, *Tetrahedron Lett.*, 1966, 4273.
20 W. J. Delarjais and E. A. Emken, *Lipids*, 1976, **11**, 594.
21 G. W. Buchanan and A. E. Gustafson, *J. Org. Chem.*, 1973, **38**, 2910.
22 For a review see L. J. Morris, *J. Lipid Res.*, 1966, **7**, 717.
23 K. Biemann, 'Mass Spectrometry,' McGraw-Hill, New York, 1962.
24 R. C. Badami and L. J. Morris, *J. Am. Oil Chem. Soc.*, 1965, **42**, 1119.
25 E. J. Corey, J. O. Albright, A. E. Barton, and S. Hashimoto, *J. Am. Chem. Soc.*, 1980, **102**, 1435.
26 M. J. Chisholm and C. Y. Hopkins, *Can. J. Chem.*, 1963, **41**, 1888; 1965, **43**, 2566.
27 A. P. Tulloch, *Lipids*, 1982, **17**, 544.
28 M. O. Bagby, C. R. Smith, and I. A. Wolff, *Lipids*, 1966, **1**, 263.
29 T. Takagi, *J. Am. Oil Chem. Soc.*, 1966, **43**, 249.
30 R. A. Raphael and F. Sondheimer, *J. Chem. Soc.*, 1950, 2100.

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