Synthesis of [14,14-$^2H_2$]-Linolenic Acid and its Use to Confirm the Pathway to 12-Oxophytodienoic acid (12-oxoPDA) in Plants: A Conspectus of the Epoxy-carbonium Ion Derived Family of Metabolites from Linoleic and Linolenic Acid Hydroperoxides

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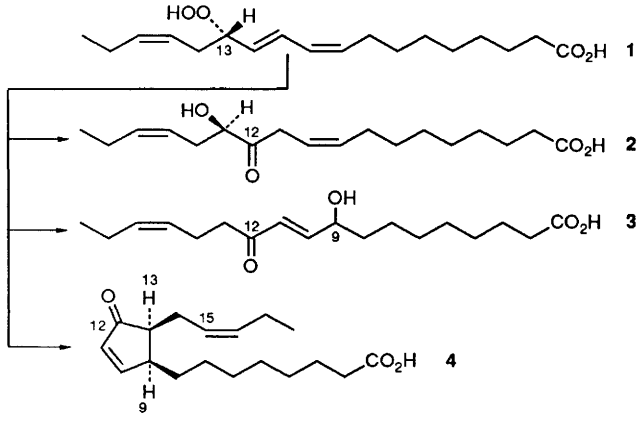
Using acetylene methodology, a synthesis of [14,14-$^2H_2$]-octadeca-9(*Z*), 12(*Z*), 15(*Z*)-trienoic acid is described. The isotopically marked acid is used to distinguish decisively between two possible pathways for the formation of 12-oxophytodienoic acid (12-oxoPDA) by flax enzyme preparation in plants: (*a*) a route *via* antarafacial ring closure of a zwitterion derived from an allene epoxide (no loss of 14,14-$^2H_2$) and (*b*) a pathway resembling the accepted mammalian prostaglandin biosynthesis (loss of one 14-^2H). Pathways to metabolic products formed in Nature from linoleic and linolenic acid are summarised in Scheme 3. The entry species is considered to be the epoxy-carbonium ion. Loss of a proton leads to an allene-epoxide from which are formed the α-ketol, the γ-ketol and 12-oxoPDA. A second group of products (colneleic and colnelenic acid, a hemiacetal, its corresponding aldehydes and an epoxy alcohol) are not formed *via* the allene-epoxide. The epoxy-carbonium ion can be trapped as an epoxy alcohol or rearranged *via* a vinyl-oxonium ion with capture by water to form a hemiacetal. Alternatively, loss of a proton from the epoxy-carbonium ion or vinyl-oxonium ion leads to colneleic and colnelenic acid.

During their important work on the utilisation of linoleic and linolenic hydroperoxides by plant enzymes, Zimmerman and Vick described an enzyme preparation from flax seeds which was capable of converting the (13*S*)-13-hydroperoxide of linolenic acid **1** into the α-ketol **2**, the γ-ketol **3** and 12-oxophytodienoic acid (12-oxoPDA) **4**.[1–3] The enzyme involved



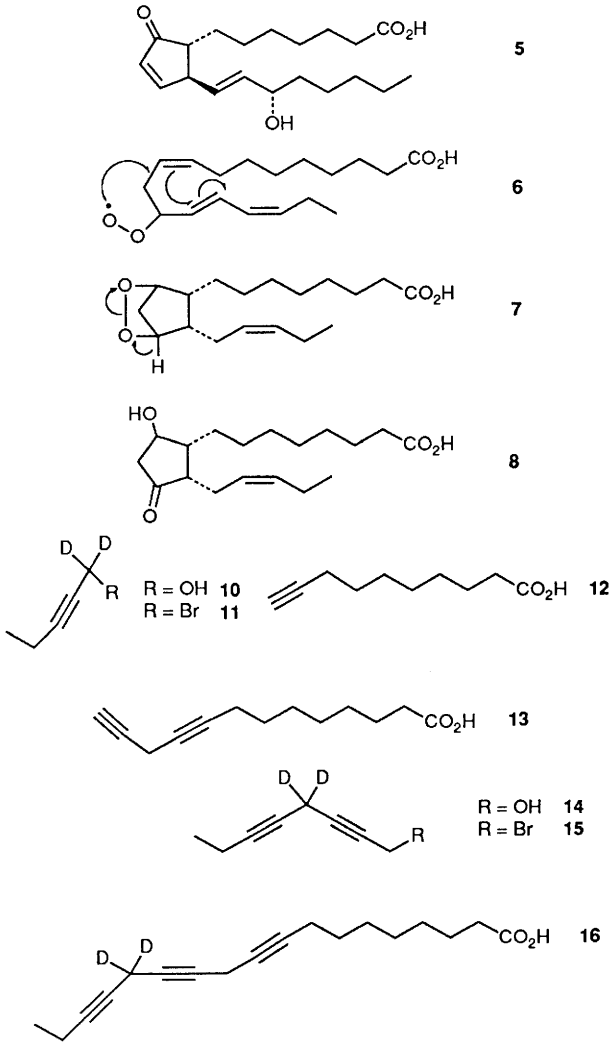
Scheme 1 Products from the treatment of linolenic acid 13-hydroperoxide with flax enzyme preparation

has wide distribution among plants[4] and degradation of 12-oxoPDA leads eventually to the plant-growth regulator jasmonic acid.[5,6] The structure of 12-oxoPDA resembles in some ways a prostaglandin [*e.g.* (PGA_1) **5**][7] though it is built on an 18 rather than a 20 carbon frame, has *cis*- rather than *trans*-attachment of side-chains about the cyclopentane ring, and there are obvious differences in the side chains. As determined for the linoleic system, the origins and mechanisms of formation of the α- and γ-ketols were discussed in the preceding paper[8] and there is little doubt that the same mechanisms apply to linolenic acid. Were the biosynthesis of 12-oxoPDA to follow an hypothetical mechanistic pattern based on that of mammalian prostaglandins, 12-hydroperoxidation would be needed, **6**, followed by reduction of the radical at C-14 formed on cyclisation, and elimination as in **7**, to give **4** *via* **8**. Earlier work on 12-oxoPDA had used the 13-hydroperoxide of linolenic acid as generated by soya-bean peroxidase without rigorous purification,[9] and there was some evidence that the 12-hydroperoxide may be formed by flax seed lipoxygenase.[10] In addition, we had some difficulty in early experiments in incorporating HPLC pure [1-^{14}C]-13-hydroperoxyoctadeca-9(*Z*), 11(*E*), 15(*Z*)-trienioc acid into 12-oxoPDA using flax seed enzyme preparation,[1–3] though this difficulty was later resolved (see below). These uncertainties induced us to carry out the experiment to be described[11] which demonstrates with certainty that the precursor to 12-oxoPDA results from initial oxygen attack at C-13 on linolenic acid.

Our test (Scheme 2) required [14,14-$^2H_2$]linolenic acid **9** and this was synthesised as follows. A Grignard reagent from but-1-yne was converted into [1,1-$^2H_2$]pent-2-ynol **10** by treatment with [$^2H_2$]formaldehyde, the latter being made by deuteriation of dibromomethane, conversion into [$^2H_2$]methylene diacetate and hydrolysis.[12] As an alternative, [1,1-$^2H_2$]pent-2-ynol was also made by reducing ethyl pent-2-ynoate with lithium aluminium deuteride in ether at 5 °C. The alcohol was converted into its bromide **11** with phosphorus tribromide. Dec-9-ynoic acid **12**, as its bis-Grignard, was coupled under copper(I) cyanide catalysis with prop-2-ynyl bromide to give trideca-9,12-diynoic acid **13** which was isolated as an unstable crystalline solid. The di-Grignard was prepared from the latter acid, but attempts to couple this in the presence of copper(I) cyanide with [1,1-$^2H_2$]pent-2-ynyl bromide **11** failed, even on prolonged refluxing. As an alternative therefore, prop-2-ynyl alcohol was protected by tetrahydropyranylation[13] and converted into its Grignard derivative. Addition of [1,1-$^2H_2$]pent-2-ynyl bromide **11** then gave, after removal of the pyranyl protecting group, the deuteriated diacetylenic alcohol **14** in high yield: it was converted into its bromide **15**. Dec-9-ynoic acid **12** was treated with ethylmagnesium bromide (2 mol) and the bis-Grignard reagent was treated with the bromide **15** and a catalytic quantity of copper(I) cyanide to give the desired [14,14-$^2H_2$]octdeca-9,12,15-triynoic acid **16**. The reaction did not go to completion even after prolonged

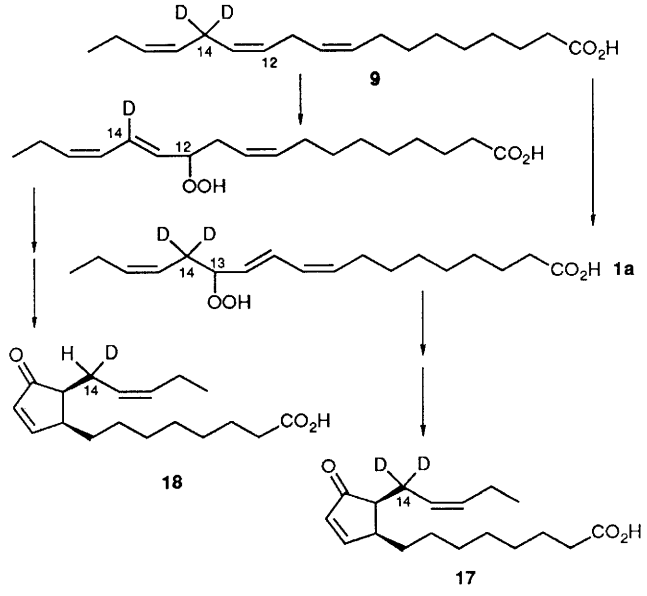
Table 1 ^{1}H NMR (400 MHz; $CDCl_3$) data for methyl 12-oxo-10*Z*,15*Z*)-phytodienoate **4**

Chemical shift (δ)	Multiplicity	Number of protons	Coupling constant in Hz	Assignment
7.74	dd	1	5.8, 2.8	10-H
6.18	dd	1	5.8, 1.8	11-H
5.40	m	2		15-H, 16-H
3.67	s	3		OCH_3
2.98	m	1		9-H
2.51	m	1		14-H
2.44	ddd	1	10.1, 6.2, 4.7	13-H
2.31	t	2	7.5	2-H_2
2.15	ddd	1	15.8, 10.1, 8.1	14-H
2.06	quintet	2	7.5	17-H_2
1.73	m	1		8-H
1.62	m	4		3-H_2, 4-H_2
1.31	m	6		5-H_2,6-H_2,7-H_2
1.15	m	1		8-H
0.97	t	3	7.5	18-H_3



refluxing, though the unchanged bromide was readily recovered. The starting dec-9-ynoic acid and the triynoic acid **16** were not completely separable by column chromatography but a complete separation could be achieved by crystallisation from light petroleum. The triynoic acid was esterified (CH_2N_2) and reduced to methyl[14,14-$^2H_2$]linolenate by di-isoamylborane (generated *in situ* from sodium borohydride, boron trifluoride–diethyl ether and 2-methylbut-2-ene).[14] Careful hydrolysis then gave [14,14-$^2H_2$]linolenic acid **9**.

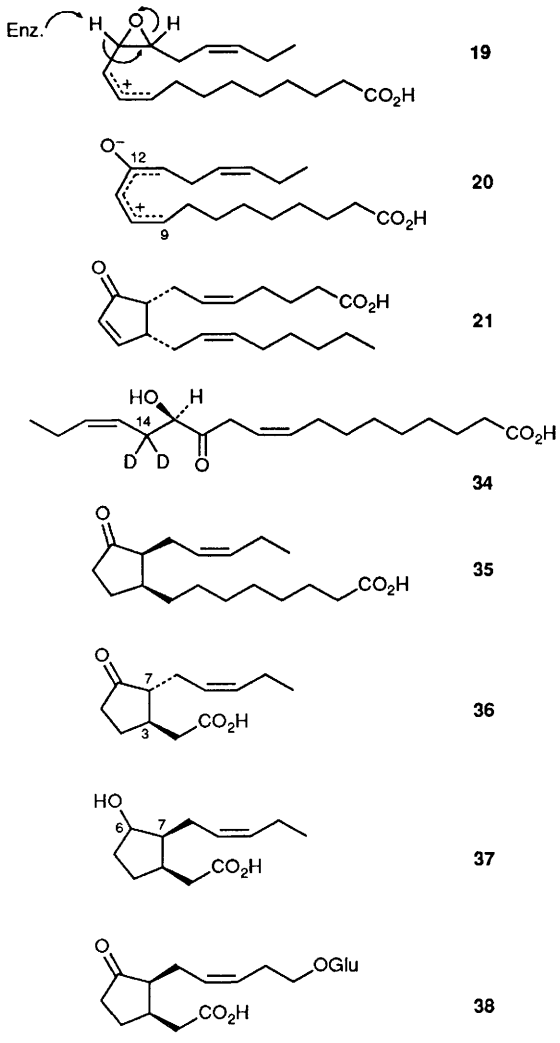
Incubation of the synthetic [14,14-$^2H_2$]linolenic acid with an aqueous extract of flax seed acetone powder gave, after isolation by thin layer chromatography and esterification, deuteriated methyl 12-oxophytodienoate. Mass spectral analysis showed the product to contain two deuterium atoms [$M^{+\bullet}$ 306 + 2, $M^{+\bullet}$ − OMe 275 + 2, $M^{+\bullet}$ − $C_5H_6D_2$ 238 + 0, $M^{+\bullet}$ − $C_5H_6D_2$ − MeOH 206 + 0, $M^{+\bullet}$ − $(CH_2)_6CO_2Me$ 163 + 2, $M^{+\bullet}$ − $(CH_2)_7CO_2Me$ 149 + 2]. Comparison of the ^{1}H NMR spectrum of the sample with that of authentic methyl12-oxo-(10*Z*,15*Z*)-phytodienoate (Table 1) showed the absence of a multiplet at δ 2.51 (14-H) and a ddd at 2.15 (second 14 H) in the former. The ddd at 2.44 (13 H) in the latter appears as a doublet in the former and the olefinic multiplet at 5.40 (15-H, 16-H) is less complex. Thus the two deuterium atoms in the product are both at position C-14 as required by **17** but not by **18**. This clearly establishes that 12-oxoPDA is derived from the 13-hydroperoxide of linolenic acid (**1a**, Scheme 2) and not through



Scheme 2 Isotopic labelling test for the origin of 12-oxoPDA from linolenic acid 12- or 13-hydroperoxides

initial 12-oxygenation, and that, as in the cases of the α- and γ-ketols, there is migration of oxygen from C-13 to C-12. It is established that the ketonic oxygen of 12-oxoPDA is derived from the [$^{18}O_2$]-hydroperoxide of linolenic acid.[9] For

completeness, the α-ketol formed in the experiment was also isolated as its methyl ester, and by mass spectrometry and ^1H NMR it was shown to contain two deuterium atoms located at C-14 **34**.



During most of our work we have used the method of Surrey[9] for solubilising fatty acids for enzymic study. The substrate is treated with an equal quantity of Tween 20 and the mixture is dispersed in a small volume of sodium borate buffer (pH 9.0; 5 mmol dm^{-3}). Dilute sodium hydroxide is added to obtain a clear solution and this substrate solution is then diluted with a large volume of the required reaction buffer before being treated with the enzyme source. When purified [1-^{14}C]-13-hydroperoxylinolenic acid **1** was treated in this way no incorporation into 12-oxoPDA was obtained, as mentioned earlier. This difficulty of presentation of the hydroperoxylinolenic acid to the enzyme was overcome by treating the pure 13-hydroperoxide with an equal quantity of Tween 20 and dispersing the mixture directly into the reaction buffer, sodium phosphate buffer (pH 7.0; 50 mmol dm^{-3}). On treatment with the enzyme source conversion to 12-oxoPDA and the α- and γ-ketols proceeded smoothly.

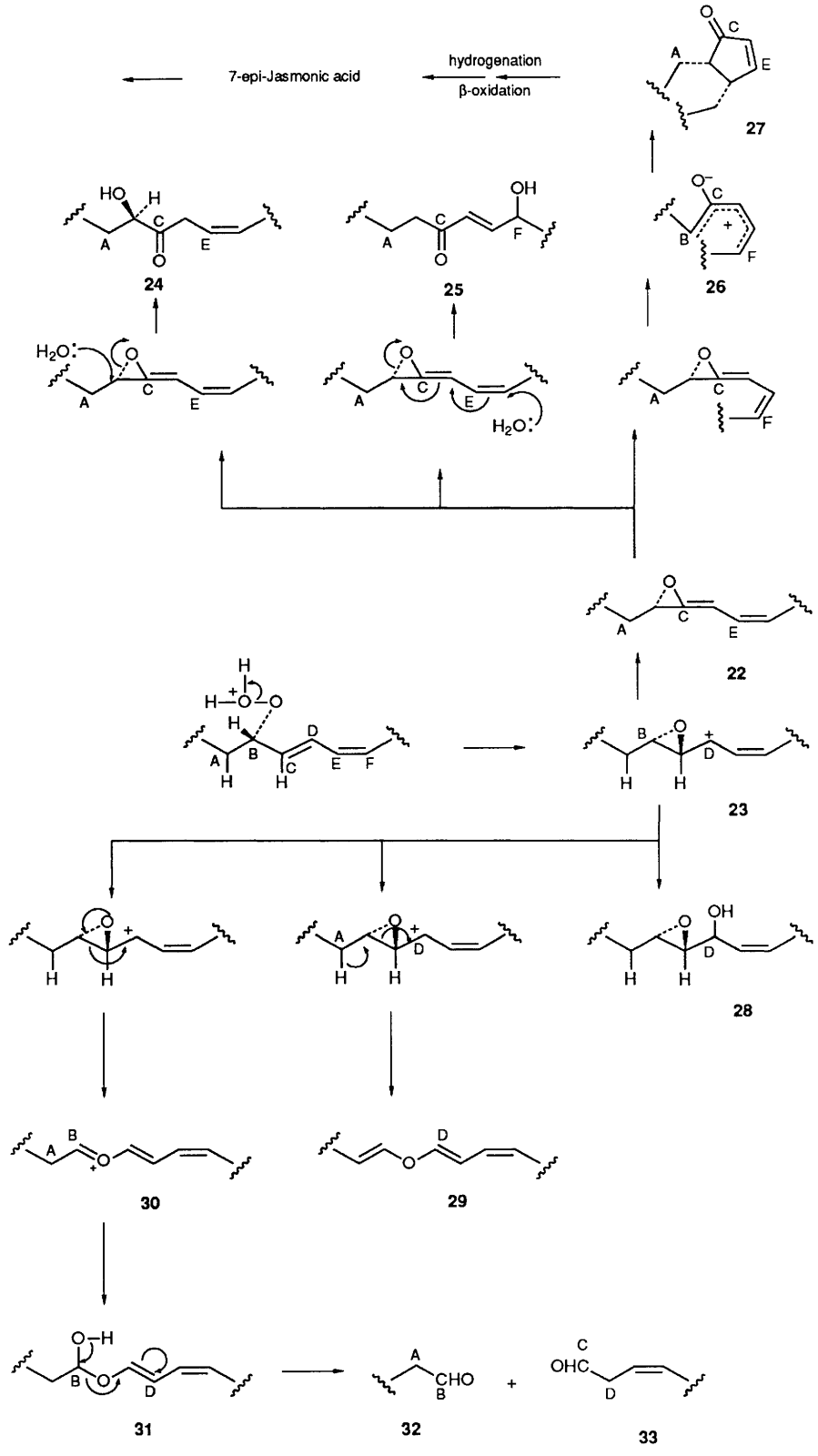
Vick, Feng and Zimmerman[9] have proposed that the flax seed enzyme involved in the conversion of 13-hydroperoxylinolenic acid into 12-oxoPDA proceeds *via* an epoxy-carbonium ion which loses the 12-proton **19** to form a zwitterion **20** which undergoes cyclisation to give 12-oxoPDA. A similar antarafacial cyclisation process has since been invoked by others to explain the formation of preclavulone-A (**21** or enantiomer), which is produced by treating arachidonic acid with an homogenate of certain corals.[16,17] There is ample chemical analogy for such a process.[18] With the recent demonstration[19,20] and isolation[21,22] of the epoxy-allene **22** as a product from the decomposition of the epoxy-carbonium ion (**23**, Scheme 3), it has been shown that treatment of **22** with water rapidly leads not only to the α-ketol **2** discussed in the previous paper,[8] but 12-oxoPDA **4**. As formed in this way, the latter is racemic,[23,24] but Hamberg[24] has also demonstrated that there is an enzymic process catalysed by allene oxide cyclase, present in a number of plants particularly spinach and potato, which leads to a chiral (9*S*, 13*S*, 15*Z*) product.

The mechanisms by which linoleic and linolenic 9- and 13-hydroperoxides are decomposed to form α- and γ-ketols, and cyclopentenones under the influence of flax seed enzyme, and are transformed into colneleic and colnelenic acids and epoxy alcohols by potato enzyme, and are chain fractured to give shorter chain aldehydes, can now be collected into a common scheme (Scheme 3). The Scheme shows only the essential five carbon centres and can be read as appropriate depending on whether a 9-or a 13-hydroperoxide is involved. All the product types represented are known, except for some cases involving the 9- and 13-hydroperoxides of linoleic and linolenic acids. The key entry species to all the products is the epoxy-carbonium ion **23**. Initiation of the allene epoxide branch **22**, studied particularly with flax and corn germ, by a hydroperoxide dehydrase enzyme mediated pathway also widely distributed among other plants, involves removal of proton H-C from **23** to give the short lived allene epoxide. By α- and ε-attack the α-**24** and γ-**25** ketols are formed[8] and pericyclic reaction of the zwitterion **26** formed by charge separation about the B–(O) bond gives the 12-oxoPDA type **27**.[9] 12-oxoPDA (which has been isolated from the Composite *Chromolaena morii*, though the prostaglandin-like biogenesis suggested for it is clearly unlikely)[25] is itself the starting point of a cascade of metabolic products.[5,6] Enzymic hydrogenation leads to **35** and successive β-oxidations provide a series of cyclopentanones terminating with 7-*epi*-jasmonic acid (which can be easily epimerised at C-7 to give jasmonic acid). Some of these have known plant regulatory functions, *e.g.* jasmonic acid **36** and cucurbic acid **37** and its relatives[27] are plant growth inhibitors,[28] and the glucoside **38** is the tuber-inducing factor of potato.[29] They are also linked biogenetically with many other natural products such as the *Dicranum* extractives,[30] the pyrethrins,[31] and the algae inhibiting cyclopentenones of the fresh-water plant *Elieocharis microcarpa*.[32]

The second major group of reactions (Scheme 3)[33] does not directly involve an allene epoxide intermediate. In the presence of potato enzyme, at low pH, the epoxy-carbonium ion **23** can be trapped as the epoxy-alcohol **28** but at pH 9.0 colneleic and colnelenic acids **29**[34] are formed through loss of proton H_A in Scheme 3. The pathway of chain fracture by hydroperoxide lyase in most plants, giving two aldehyde components **32** and **33**, appears not to involve this proton loss, but to involve nucleophilic attack by water on the oxonium ion **30** and decomposition of the vinyl-ether acetal **31** rather than the re-formation of such a species through the intermediacy of **29**.[33,35] The fragmented aldehydes, as mentioned previously, become the originators of many other products which modify the taste and odour of fruits and food.

Experimental

Formation of 12-*Oxo*-(10Z,15Z)-*phytodienoic Acid* **4** *and* (13R)-13-*Hydroxy*-12-*oxo-octadeca*-(9Z),15(Z)-*dienoic Acid* **2** *by the Action of an Extract of Acetone Powder of Flax Seed on Linolenic Acid.*—Acetone powder of flax seed (2 g)[8] and sodium phosphate buffer (pH 7.0; 50 mmol dm^{-3}; 20 ml) were stirred with ice cooling (45 min). The mixture was centrifuged



Scheme 3 Summary of reactions resulting from the enzymic decomposition of linoleic and linolenic hydroperoxides

(12 000 × g) at 4 °C (15 min) and the supernatant (10 ml) was added to a mixture of linolenic acid substrate solution (20 ml)[33] and sodium phosphate buffer (pH 7.0; 50 mmol dm^{-3}; 400 ml). After stirring at room temperature in air (90 min), chloroform (200 ml) and methanol (100 ml) were added and the mixture was acidified to pH 3.0 with citric acid (1 mol dm^{-3}), and stirred under nitrogen (30 min). Chloroform (200 ml) was added and the mixture stirred under nitrogen (2 h). The organic phase was separated (separation aided by filtration through a bed of Kieselguhr), dried (Na_2SO_4), and evaporated, to afford the crude reaction mixture (0.22 g) as an oil.

Isolation of methyl 12-*Oxo*-(10Z,15Z)-*phytodienoate* **4**.—The crude reaction mixture was fractionated by preparative thick layer chromatography (silica gel) developing 5 times with chloroform–light petroleum (b.p. 60–80 °C)–acetic acid

(60:25:1) as eluent. The band at R_F 0.48 (UV) was collected. The product was esterified with ethereal diazomethane and further purified by reversed-phase HPLC [eluent methanol–water (4:1)] to give methyl 12-oxo-(10*Z*,15*Z*)-phytodienoate (3.8 mg) (*cf.* ref. 23) as a colourless oil; $\nu_{max}(CHCl_3)/cm^{-1}$ 1735 (ester C=O), 1703 (ketone C=O), 1461, 1353 and 1170 (C–O); δ_H(400 MHz, $CDCl_3$): see Table 1; δ_C(250 MHz; $CDCl_3$) 210.64 (C-12), 174.18 (C-1), 166.88 (C-10), 132.98 (C-11), 132.48 (C-16), 127.01 (C-15), 51.40 (OCH_3), 49.90 (C-13), 44.32 (C-9), 34.05 (C-2), 30.79 (C-8), 29.59 (C-6), 29.11 (C-4), 29.06 (C-5), 27.61 (C-7), 24.91 (C-3), 23.85 (C-14), 20.79 (C-17) and 14.00 (C-18); *m/z* 306 ($M^{+\bullet}$; 28%), 275 ($M^{+\bullet}$ – OMe; 21), 238 ($M^{+\bullet} - C_5H_8$; 35), 206 ($M^{+\bullet} - C_5H_8$ – MeOH; 8), 163 [$M^{+\bullet} - (CH_2)_6Me$; 43] and 149 [$M^{+\bullet} - (CH_2)_7CO_2Me$; 24] (Found: *m/z* 306.2183. $C_{19}H_{30}O_3$ requires *m/z* 306.2171).

Isolation of methyl (13R)-*Hydroxy*-12-*oxooctadeca*-9(Z),15(Z)-*dienoate*. The crude reaction mixture was fractionated by preparative thick layer chromatography (silica gel), developing 5 times with chloroform–light petroleum (b.p. 60–80 °C)–acetic acid (60:25:1) as eluent. The band at R_F 0.42 (located by strip spraying with *o*-anisaldehyde reagent) was collected. The product was esterified with ethereal diazomethane and further purified by C-18 reversed-phase HPLC (methanol–water, 4:1) to give methyl (13*R*)-hydroxy-12-oxooctadeca-9(*Z*),15(*Z*)-dienoate (9.8 mg) (*cf.* ref. 23) as a colourless oil; $\nu_{max}(CHCl_3)/cm^{-1}$ 3450 (OH) and 1725 br (ester C=O and ketone C=O): δ_H(400 Hz; $CDCl_3$) 5.59 (3 H, m, 9-H, 10-H and 16-H), 5.32 (1 H, m, 15-H), 4.31 (1 H, m, 13-H), 3.67 (3 H, s, OCH_3), 3.42 (1 H, br d, 4.2, OH), 3.27 (2 H, m, 11-H_2), 2.61 (1 H, m, 14-H), 2.46 (1 H, m, 14-H), 2.31 (2 H, t, *J* 7.5, 2-H_2), 2.07 (4 H, m, 17-H_2 and 8-H_2), 1.65 (4 H, m, 3-H_2 and 7-H_2), 1.35 (6 H, m, 4-H_2, 5-H_2 and 6-H_2) and 0.98 (3 H, t, *J* 7.4, 18-H_3); δ_C(250 MHz; $CDCl_3$) 209.64 (C-12), 173.84 (C-1), 135.54 (C-15), 134.36 (C-10), 122.50 (C-16), 119.96 (C-9), 75.99 (C-13), 51.34 (OCH_3), 37.29, 34.17, 31.79, 29.26, 29.14, 27.67, 25.02, 20.88 (10 CH_2 units) and 14.06 (C-18); *m/z* 324 ($M^{+\bullet}$; 1%), 306 ($M^{+\bullet} - H_2O$; 2), 227 ($M^{+\bullet} - CH_3CH_2CH{=}CHCH_2CO$; 38), 195 ($M^{+\bullet}$ – MeOH – $CH_3CH_2CH{=}CHCH_2CO$; 35) and 166 [$M^{+\bullet}$ – MeOH – $CH_3CH_2CH{=}CHCH_2CH(OH)CH$; 37] (Found: *m/z* 324.2297. $C_{19}H_{32}O_4$ requires *m/z* 324.2295).

[$^2H_2$]*Dibromomethane*.—A mixture of dibromomethane (250 g; 1.44 mol) and 10% sodium deuteroxide in deuterium oxide (105 ml) was refluxed with stirring (24 h). The organic layer was separated and refluxed successively with decreasing amounts of 10% sodium deuteroxide (80, 95, 65, 50, 50 50 ml). The organic phase was separated and distilled through a short Vigreux column. An azeotropic mixture passed over first followed by pure [$^2H_2$]dibromomethane. The azeotrope was dried over phosphorus pentoxide and redistilled. The two fractions were combined giving [$^2H_2$]dibromomethane[12] (145.7 g; 58%; 97 atom %D), b.p. 98–100 °C; *m/z* 178 ($CD_2{}^{81}Br_2$; 81%), 177 ($CDH^{81}Br_2$; 6), 176 ($CD_2{}^{81}Br^{79}Br$ and $CH_2{}^{81}Br_2$; 100), 175 ($CDH^{81}Br^{79}Br$; 9), 174 ($CD_2{}^{79}Br_2$ and $CH_2{}^{81}Br^{79}Br$; 88), 173 ($CHD^{79}Br_2$; 4) and 172 ($CH_2{}^{79}Br_2$; 1).

[$^2H_2$]*Methylene Diacetate*.—To a mixture of [$^2H_2$]dibromomethane (70 g, 0.40 mol), glacial acetic acid (240 ml) and acetic anhydride (12 g) was slowly added freshly fused potassium acetate (117.6 g; 1.20 mol), with mechanical stirring. The reaction mixture was refluxed with stirring (25 h), cooled to room temperature and poured into dry ether (600 ml). Precipitated potassium bromide was removed by filtration and washed well with dry ether. The filtrate and washings were combined and the ether and the bulk of the acetic acid removed by distillation. The residue was distilled to give [$^2H_2$]methylene diacetate[12] (30.2 g; 57%; 97 atom %D), b.p. 72–74 °C, 17 mmHg: *m/z* 104 ($M^{+\bullet}$ – Me – Me; 31%) and 75 ($M^{+\bullet}$ – OAc; 100); ^1H NMR indicated the product contained 97 atom %D.

[$^2H_2$]*Paraformaldehyde*.—A mixture of [$^2H_2$]methylene diacetate (15.75 g, 118 mmol), water (5 ml) and concentrated hydrochloric acid (0.5 ml) was gently refluxed (17 h). The reaction mixture was cooled to room temperature and distilled (20 mmHg) to remove the water and acetic acid and leave a residual white solid. A second distillation of the disillate gave a second smaller crop. The combined crops were dried *in vacuo* giving [$^2H_2$]paraformaldehyde[12] (1.86 g, 50%) as a white powder.

[1,1-$^2H_2$]*Pent*-2-*yn*-1-*ol* **10**.—(a) *From* [$^2H_2$]-*Paraformaldehyde*. A Grignard reagent was prepared from magnesium turnings (1.88 g, 78 mmol), dry tetrahydrofuran (20 ml) and a crystal of iodine under a nitrogen atmosphere, by addition of bromoethane (9.40 g, 86 mmol) in dry tetrahydrofuran (20 ml). The reaction mixture was cooled to 0 °C and an excess of but-1-yne vapour was bubbled through the stirred reaction mixture (1 h) the temperature being maintained at 0 °C. The reaction mixture was refluxed (30 min) to remove excess of but-1-yne, cooled to room temperature, and dry [$^2H_2$]paraformaldehyde (2.50 g, 78 mmol) was added. The mixture was slowly (30 min) brought to reflux and then refluxed (1.5 h), cooled in ice and cautiously treated with sulphuric acid (2 mol dm^{-3}; 20 ml) and ice–water (20 ml); it was then extracted with ether (3 × 20 ml). The combined ethereal extracts were dried ($MgSO_4$) and the ether was distilled off through a short Vigreux column, and the residue was distilled to give [1,1-$^2H_2$]pent-2-yn-1-ol **10** (4.48 g, 67%; 98 atom %D), b.p. 63–65 °C/11 mmHg; ν_{max}(liquid film)/cm^{-1} 3347br (OH), 2260 (C≡C) and 2220 (C≡C); δ_H(90 MHz; $CDCl_3$) 4.27 (0.04 H, m, 1-H_2), 4.61 (1 H, br s, OH), 2.22 (2 H, q, *J* 7.4, 4-H_2) and 1.18 (3 H, t, *J* 7.4, 5-H_3); *m/z* 86 ($M^{+\bullet}$, 89%) and 57 ($M^{+\bullet}$ – Et, 61).

(b) *From ethyl pent-2-ynoate*. Lithium aluminium deuteride (1 g, 24 mmol) in dry ether (50 ml) was added at 5 °C under nitrogen to a solution of ethyl pent-2-ynoate (5.80 g, 46 mmol) in dry ether (30 ml) (1.5 h). The reaction mixture was stirred at 5 °C (1 h) and then treated with ethyl acetate (20 ml), followed by water (100 ml), and then hydrochloric acid (2 mol dm^{-3}). The combined ethereal extracts were washed with saturated brine (40 ml) and water (3 × 40 ml), dried ($MgSO_4$) and evaporated. Purification was effected by dry column chromatography over silica gel (100 g) with light petroleum (b.p. 40–60 °C)–ether (95:5) as eluent, fractions (20 ml) being collected. Fractions (5–14) were combined and evaporated to give recovered ethyl pent-2-ynoate (0.47 g, 8%). The polarity of the solvent was then increased to afford [1,1-$^2H_2$]pent-2-yn-1-ol (98 atom %D) **10** (2.23 g, 56%), b.p. 79–81 °C at 35 mmHg identical with an authentic sample.

[1,1-$^2H_2$]-1-*Bromopent*-2-*yne* **11**.—A mixture of [1,1-$^2H_2$]-pent-2-yn-1-ol **10** (2.00 g, 23 mmol), dry ether (15 ml) and dry pyridine (0.13 ml) was cooled to −35 °C under a nitrogen atmosphere. Phosphorus tribromide (0.80 ml, 8 mmol) was added with stirring (45 min) at −35 °C. The mixture was stirred at −35 °C (2 h), slowly allowed to warm to room temperature (1.5 h) and then refluxed (30 min). It was cooled to room temperature and treated with saturated brine (30 ml). The ethereal layer was separated and the aqueous phase extracted with ether (3 × 15 ml). The combined ethereal extracts were dried ($MgSO_4$) and the ether was distilled off through a short Vigreux column; distillation of the residue gave [1,1-$^2H_2$]-1-bromopent-2-yne **11** (2.08 g, 60%; 98 atom %D), b.p. 60–62 °C at 23 mmHg; ν_{max}(liquid film)/cm^{-1} 2245(C≡C and 2220 (–C≡C); δ_H(90 MHz; $CDCl_3$) 3.95 (0.04 H, m, 1-H_2), 2.29 (2 H, q, *J* 7.4, 4-H_2) and 1.15 (3 H, t, *J* 7.4, 5-

H_3); *m/z* 150 ($M^{+\bullet}$, ^{81}Br; 45%), 148 ($M^{+\bullet}$, ^{79}Br; 43) and 69 ($M^{+\bullet}$ − Br, 100).

Trideca-9,12-diynoic Acid **13**.—Ethylmagnesium bromide was prepared under nitrogen from magnesium (0.73 g, 30 mmol), a crystal of iodine and bromoethane (3.60 g, 33 mmol) in dry THF (30 ml). Dec-9-ynoic acid **12** (2.50 g, 15 mmol) was added dropwise with stirring at 0 °C (45 min). The reaction mixture was stirred at room temperature (2 h) and then dry copper(I) cyanide (100 mg) was added. The mixture was stirred (10 min) and then prop-2-ynyl bromide (80% solution in toluene; 1.60 ml) in dry THF (10 ml) was added dropwise (15 min). The mixture was refluxed (24 h), cooled and then treated with sulphuric acid (1 mol dm^{-3}; 50 ml) and crushed ice (50 g) and extracted with ether (4 × 20 ml). The combined ethereal extracts were dried ($MgSO_4$) and evaporated and the residue was rapidly (to reduce decomposition) distilled under diminished pressure. Early fractions afforded recovered dec-9-ynoic acid. The highest boiling point fraction afforded trideca-9,12-diynoic acid **12** (0.70 g, 23%) as an unstable white crystalline solid, b.p. 164–168 °C at 0.8 mmHg; $\nu_{max}(CHCl_3)/cm^{-1}$ 3320 (HC≡), 3050 (OH), 2130 (C≡C), 1705 (C=O) and 905 cm^{-1}; δ_H(250 MHz; $CDCl_3$) 10.5 (1 H, v br s, OH), 3.15 (2 H, q, 2.4 Hz, 11-H_2), 2.35 (2 H, t, *J* 7.4, 2-H_2), 2.16 (2 H, m, 8-H_2), 2.07 (1 H, t, *J* 2.7, 13-H), 1.68 (2 H, m, 7-H_2), 1.53 (2 H, m, 3-H_2) and 1.38 (6 H, m, 4-H_2, 5-H_2 and 6-H_2).

Tetrahydro-2-(prop-2′-ynyloxy)pyran.—A mixture of prop-2-ynyl alcohol (5 g, 89 mmol), 2,3-dihydropyran (15 g, 179 mmol), pyridinium toluene-4-sulphonate (2.2 g, 9 mmol) and dry dichloromethane (100 ml) was stirred under a nitrogen atmosphere at room temperature (4.5 h). Work-up gave an oil which was distilled to give tetrahydro-2-(prop-2′-ynyloxy)pyran (11.1 g, 89%), b.p. 80–82 °C at 16 mmHg (lit.,[13] b.p. 78 °C at 25 mmHg); ν_{max}(liquid film)/cm^{-1} 2120 (C≡C): δ_H(90 MHz; $CDCl_3$) 4.87 (1 H, br t, *J* 2, 2-H), 4.30 (2 H, d, *J* 2.1, 1′-H_2), 3.89 (1 H, m, 3-H), 3.59 (1 H, m, 3-H), 2.49 (1 H, t, *J* 2.3, 3′-H) and 2.10–1.40 (6 H, m, 4-H_2, 5-H_2 and 6-H_2); *m/z* 139 ($M^{+\bullet}$ − 1) (Found: *m/z* 139.0755. $C_8H_{11}O_2$ requires *m/z* 139.0751).

[4,4-$^2H_2$]*Octa-2,5-diyn-1-ol* **14**.—A Grignard reagent was prepared under nitrogen from magnesium (0.68 g, 28 mmol), a crystal of iodine and bromoethane (3.05 g, 28 mmol) in dry tetrahydrofuran (30 ml). Tetrahydro-2-(prop-2′-ynyloxy)pyran (3.92 g, 28 mmol) in dry THF (10 ml) was added to it dropwise with stirring (25 min) and the mixture was refluxed (3 h); it was then cooled and copper(I) chloride (120 mg) added. The mixture was then stirred (15 min) and [1,1-$^2H_2$]-1-bromopent-2-yne **11** (4.02 g, 27 mmol) in dry THF (10 ml) was added dropwise (20 min). It was then further stirred (1 h) and finally refluxed (16 h). After work-up, the residual yellow oil (6.36 g) was dissolved in ethanol (35 ml) and toluene-4-sulphonic acid (300 mg) was added and the mixture refluxed with stirring under nitrogen (6 h). The reaction mixture was concentrated to a small volume, water (40 ml) was added, and the mixture extracted with ether (3 × 20 ml). The combined ethereal extracts were washed with saturated aqueous sodium hydrogen carbonate (20 ml) and water (20 ml), dried ($MgSO_4$) and evaporated. The residual oil was distilled to give [4,4-$^2H_2$]octa-2,5-diyn-1-ol **14** (2.49 g, 74%), b.p. 77–79 °C/0.2 mmHg; ν_{max}(liquid film)/cm^{-1} 3320 (OH) and 2250 (C≡C); δ_H(90 MHz; $CDCl_3$) 4.25 (2 H, s, 1-H_2), 2.98 (1 H, s, OH), 2.18 (2 H, q, 7.6 Hz, 7-H_2) and 1.12 (3 H, t, *J* 7.6, 8-H_3); *m/z* 124 ($M^{+\bullet}$) (Found: *m/z* 124.0853. $C_8H_8D_2O$ requires *m/z* 124.0849).

[4,4-$^2H_2$]-1-*Bromoocta-2,5-diyne* **15**.—A mixture of [4,4-$^2H_2$]octa-2,5-diyn-1-ol **14** (2.20 g, 17.7 mmol), dry ether (20 ml) and dry pyridine (0.15 ml) was cooled to −10 °C under nitrogen and treated with phosphorus tribromide (1.76 g, 6.5 mmol) in dry ether (5 ml) at −10 °C (45 min). After being stirred at −10 °C (30 min), the mixture was worked up and distilled to give [4,4-$^2H_2$]-1-bromoocta-2,5-diyne **15** (2.40 g, 72%) (98 atom %D), b.p. 59–60 °C/0.05 mmHg; ν_{max}(liquid film)/cm^{-1} 2295 (C≡C), 2250 (C≡C): δ_H(90 MHz; $CDCl_3$), 3.95 (2 H, s, Cl-H_2), 2.19 (2 H, q, *J* 7.6, C7-H_2) and 1.13 (3 H, t, 7.6 Hz, C8-H_3): *m/z* 188 ($M^{+\bullet}$, ^{81}Br) and 186 ($M^{+\bullet}$, ^{79}Br).

[14,14-$^2H_2$]*Octadeca-9,12,15-triynoic Acid* **16**.—A Grignard reagent was made from magnesium (0.49 g, 20 mmol), a crystal of iodine, and bromoethane (2.40 g, 22 mmol) in dry THF (30 ml). Dec-9-ynoic acid **12**[33] (1.68 g, 10 mmol) in dry THF (20 ml) was added dropwise at 0 °C (45 min) to the mixture which was then refluxed (2 h). After this copper (I) cyanide (0.30 g) was added to the mixture which was then stirred at 20 °C (15 min); [4,4-$^2H_2$]-1-bromoocta-2,5-diyne **15** (0.94 g, 5 mmol) in dry THF (20 ml) was then added dropwise (20 min). After being stirred at 20 °C (16 h) the mixture was refluxed (31 h). Crushed ice (30 g) and sulphuric acid (1 mol dm^{-3}; 50 ml) were added and the product was extracted with ether (3 × 30 ml). Work-up by silica gel chromatography, with light petroleum (b.p. 60–80 °C)–ether–acetic acid (80:20:1) as eluent gave a series of fractions (10 ml). Fractions (17–20) were combined and evaporated to give recovered [4,4-$^2H_2$]-1-bromo-octa-2,5-diyne **15** (0.54 g, 58%). Fractions (30–68) were combined and evaporated. Recrystallisation of the residue from light petroleum (b.p. 60–80 °C) gave white crystals of [14,14-$^2H_2$]-1-octadeca-9,12,15-triynoic acid **16** (200 mg, 15%), m.p. 78–80 °C; $\nu_{max}(CHCl_3)/cm^{-1}$ 3130 (OH) and 1700 (acid C=O); δ_H(90 MHz; $CDCl_3$) 10.71 (1 H, br s, OH), 3.16 (2 H, t, *J* 2.7, 11-H_2), 2.25 (6 H, m, 2-H_2, 8-H_2 and 17-H_2), 1.80 − 1.20 (10 H, m, 3-H_2 to 7-H_2) and 1.12 (3 H, t, *J* 7.6, 18-H_3); *m/z* 274 ($M^{+\bullet}$ weak) (Found: *m/z* 274.1901. $C_{18}H_{22}D_2O_2$ requires *m/z* 274.1903).

Methyl [14,14-$^2H_2$]*Octadeca-9,12,15-triynoate*.—[14,14-$^2H_2$]Octadeca-9,12,15-triynoic acid (400 mg) was esterified with ethereal diazomethane and chromatographed over silica gel eluting with light petroleum (b.p. 40–60 °C)–ether (97:3), to give methyl [14,14-$^2H_2$]octadeca-9,12,15-triynoate (370 mg, 88%), ν_{max}/cm^{-1} 1743 (ester); *m/z* 273 ($M^{+\bullet}$ − Me) and 257 ($M^{+\bullet}$ − OMe) (Found: *m/z* 273.1833. $C_{18}H_{21}D_2O_2$ requires *m/z* 273.1845).

Methyl [14,14-$^2H_2$]*Linolenate*.—Sodium borohydride (114 mg, 3 mmol) in dry diglyme (5 ml) was treated with 2-methylbut-2-ene (530 mg, 7.6 mmol) under nitrogen at 0 °C. Boron trifluoride–ether (0.5 ml, 4 mmol), freshly distilled from calcium hydride, was added dropwise with stirring and the mixture was stirred at 0 °C (30 min) and then at 20 °C. The reaction mixture was cooled to 0 °C and stirred with ethylene glycol (1 ml) and acetic acid (1 ml) at room temperature for 15 h. The product was acidified to pH 1.0 with 2 mol dm^{-3} hydrochloric acid and extracted with hexane (4 × 20 ml). Chromatography on silica gel, eluting with light petroleum (b.p. 60–80 °C)–ether (199:1), gave methyl [14,14-$^2H_2$]linolenate (60 mg, 54%) as a colourless oil; δ_H(90 MHz; $CDCl_3$) 5.40 (6 H, m, 9-H, 10-H, 12-H, 13-H, 15-H and 16-H), 3.67 (3 H, s, OCH_3), 2.82 (2 H, t, *J* 5.5, 11-H_2), 2.31 (2 H, t, *J* 7.5, 2-H_2), 2.08 (4 H, m, 8-H_2 and 17-H_2), 1.80–1.20 (10 H, m, 3-H_2 to 7-H_2) and 0.99 (3 H, t, *J* 7.5, 18-H_3); *m/z* 294 ($M^{+\bullet}$).

[14,14-$^2H_2$]*Linolenic Acid* **9**.—Methyl [14,14-$^2H_2$]linolenate (150 mg), potassium hydroxide (1 g), ethanol (2 ml) and water (8 ml) were gently refluxed with stirring under nitrogen (1 h). After acidification the product was extracted with ether (4 × 10 ml). The combined ethereal extracts were dried ($MgSO_4$) and evaporated. The produce was purified by silica gel dry column

chromatography with light petroleum (b.p. 60–80 °C)–ether–acetic acid (85:15:1) as eluent to give [14,14-$^2H_2$]linolenic acid **9** (136 mg, 95%) as a colourless oil; δ_H(90 MHz; $CDCl_3$) 10.47 (1 H, br s, OH), 5.42 (6 H, m, 9-H, 10-H, 12-H, 13-H, 15-H and 16-H), 2.85 (2 H, t, *J* 5.5, 11-H_2), 2.39 (2 H, t, *J* 7.5, 2-H_2), 2.11 (4 H, m, 8-H_2 and 17-H_2), 1.80–1.20 (10 H, m, 3-H_2 to 7-H_2) and 0.99 (3 H, t, *J* 7.5, 18-H_3): *m/z* 280 ($M^{+\bullet}$).

Formation of Methyl [14,14-$^2H_2$]-12-*Oxo*-(10Z,15Z)-*phytodienoate* **17** *and Methyl* [14,14-$^2H_2$]-(13R)-13-*Hydroxy*-12-*oxo*-*octadeca*-9(Z),15(Z)-*dienoate* **34** *by the Action of an Extract of Acetone Powder of Flax Seed on* [14,14-$^2H_2$]*Linolenic Acid* **9**.—A mixture of [14,14-$^2H_2$]linolenic acid (59 mg) and Tween 20 (50 mg) was dispersed in sodium borate buffer, pH 9.0 (10 ml). Aqueous sodium hydroxide (2 mol dm^{-3}) was added dropwise until a clear solution was obtained. The solution was diluted with sodium phosphate buffer (pH 7.0; 50 mmol dm^{-3}; 400 ml). A mixture of acetone powder of flax seed (2 g) and sodium phosphate buffer (pH 7.0; 50 mmol dm^{-3}; 20 ml) was gently stirred with ice–cooling (45 min). The mixture was centrifuged (12 000 g) at 4 °C (15 min) and the supernatant (10 ml) was added to the above solution and the mixture stirred at room temperature in the air (90 min). The reaction mixture was extracted as described above.

Isolation of methyl [14,14-$^2H_2$]-12-*oxo*-(10Z,15Z)-*phytodienoate* **17**. The product was isolated, esterified and purified as above to give the methyl ester of **17** (2.6 mg) as a colourless oil; δ_H(400 MHz; $CDCl_3$) 7.74 (1 H, dd, *J* 5.8 and 2.8, 10-H), 6.18 (1 H, dd, *J* 5.8 and 1.8, 11-H), 5.40 (2 H, m, 15-H and 16-H), 3.67 (3 H, s, OCH_3), 2.98 (1 H, m, 9-H), 2.44 (1 H, d, *J* 6.3, 13-H), 2.31 (2 H, t, *J* 7.4, 2-H_2), 2.06 (2 H, quintet, *J* 7.4, 17-H_2), 1.73 (1 H, m, 8-H), 1.62 (4 H, m, 3-H_2, 4-H_2), 1.31 (6 H, m, 5-H_2, 6-H_2 and 7-H_2), 1.15 (1 H, m, 8-H) and 0.97 (3 H, t, *J* 7.5, 18-H_3); *m/z* 308 ($M^{+\bullet}$), 277 ($M^{+\bullet}$ – OMe), 238 ($M^{+\bullet}$ – $C_5H_6D_2$), 206 ($M^{+\bullet}$ – $C_5H_6D_2$ – MeOH), 165 ($M^{+\bullet}$ – $(CH_2)_6CO_2Me$) and 151 [$M^{+\bullet}$ – $(CH_2)_7CO_2Me$].

Isolation of methyl[14,14-$^2H_2$]-(13R)-13-*hydroxy*-12-*oxo*-*octadeca*-9(Z),15(Z)-*dienoate* **34**. The product was isolated, esterified and purified as described earlier to give the ketol (6.7 mg); δ_H(400 MHz; $CDCl_3$) 5.59 (3 H, m, 9-H, 10-H and 16-H), 5.32 (1 H, d, *J* 10.8, 15-H), 4.31 (1 H, d, *J* 4.8, 13-H), 3.67 (3 H, s, OCH_3), 3.42 (1 H, d, *J* 5.2, OH), 3.27 (2 H, m, 11-H_2), 2.31 (2 H, t, *J* 7.5, 2-H_2), 2.07 (4 H, m, 17-H_2 and 8-H_2), 1.65 (4 H, m, 3-H_2 and 7-H_2), 1.35 (6 H, m, 4-H_2, 5-H_2 and 6-H_2) and 0.98 (3 H, t, *J* 7.4, 18-H_3); *m/z* 308 ($M^{+\bullet}$ – H_2O), 227 ($M^{+\bullet}$ – CH_3CH_2CH = $CHCD_2CO$), and 166 [$M^{+\bullet}$ – MeOH – CH_3CH_2CH = $CHCD_2CH(OH)CO$].

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