The Enzymic Conversion of 13-Hydroperoxylinoleic Acid into 13-Hydroxy-12-oxooctadec-9(*Z*)-enoic Acid and 9-Hydroxy-12-oxooctadec-10(*E*)-enoic Acid: Isotopic Evidence for an Allene Epoxide Intermediate

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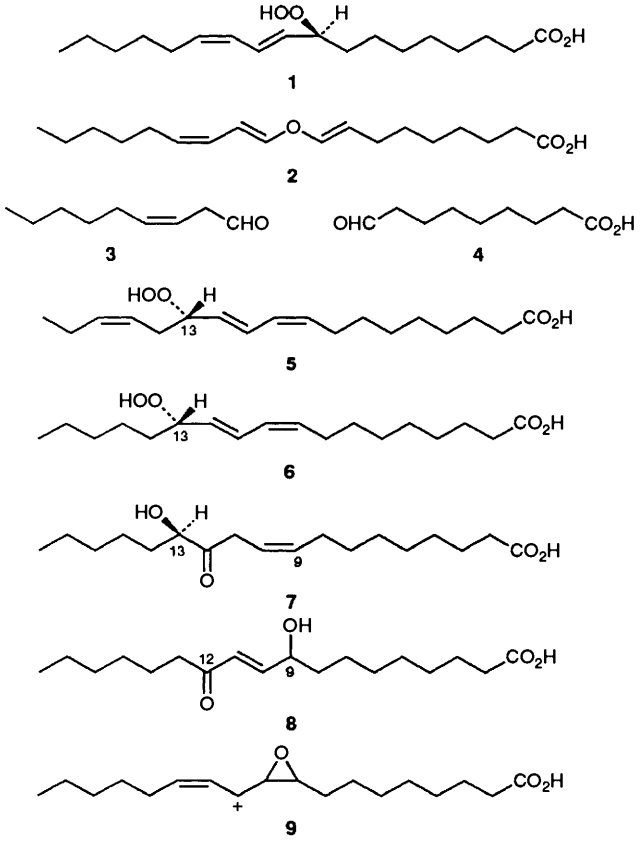
Using synthetic [9,10,12,13-$^2H_4$]linoleic acid as substrate it is shown that flax enzyme preparation forms [9,10,13-$^2H_3$]-13-hydroxy-12-oxooctadec-9(*Z*)-enoic acid and [9,10,13-$^2H_3$]-9-hydroxy-12-oxooctadec-10(*E*)-enoic acid with loss of the deuterium originally at C-12, thus demonstrating that an allene epoxide is involved as an intermediate and that transfer of deuterium from C-12 to C-11 does not occur.

In the preceding paper[1] the mechanism by which the carbon chain of (9*S*)-9-hydroperoxyoctadeca-10(*E*),12(*Z*)-dienoic acid (9-hydroperoxylinoleic acid) **1** is fractured to form colneleic acid **2** by a potato lyase, or non-3(*Z*)-enal **3** and 9-oxononanoic acid **4** by other plant lyases, was discussed in the light of new isotopic evidence. Similarly, (9*S*)-9-hydroperoxyoctadeca-10(*E*),12(*Z*),15(*Z*)-trienoic acid (9-hydroperoxylinolenic acid) gives colnelenic acid by the action of potato lyase, or nona-3(*Z*),6(*Z*)-dienal and 9-oxononanoic acid **4** by other plant lyases. Divinyl ethers are not known to originate from (13*S*)-13-hydroperoxides,[2] though 13-hydroperoxylinoleic acid

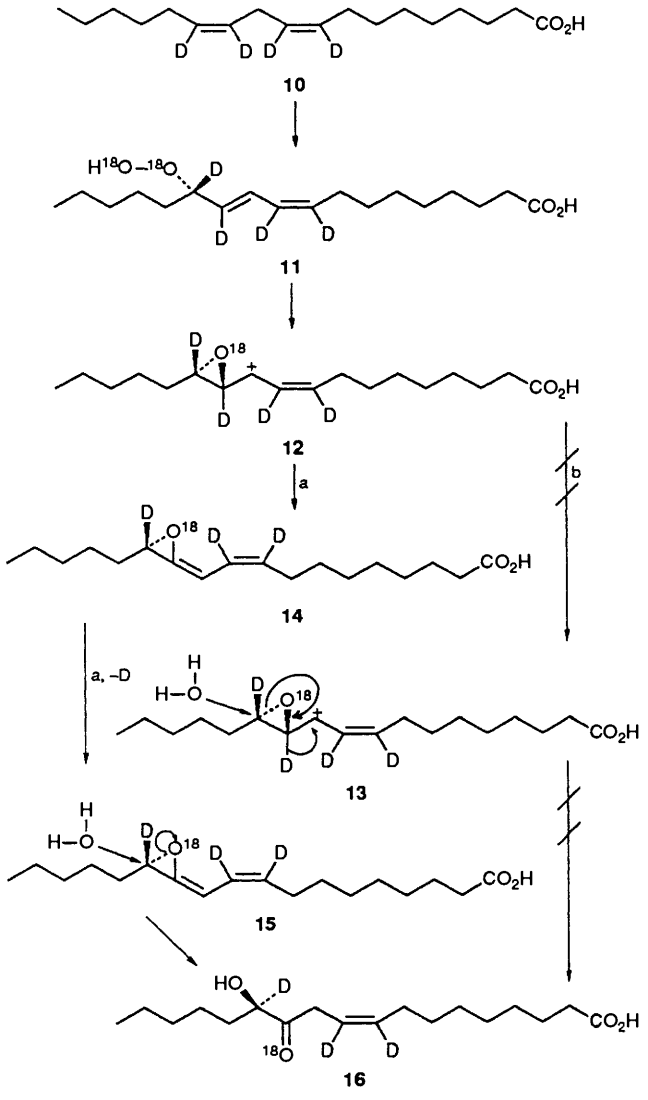


is cleaved enzymically to give hexanal and 11-formylundec-9(*Z*)-enoic acid, and 13-hydroperoxylinolenic acid **5** is cleaved to give the latter and hex-3(*Z*)-enal in many plants. Many plants (flax seed,[3-8] and corn germ[9-12] have been particularly studied), also contain an enzyme (formerly described as an isomerase) which converts both linoleic and linolenic hydroperoxides into α- and γ-ketols, and this paper is devoted to consideration of the mechanism by which the latter arise.

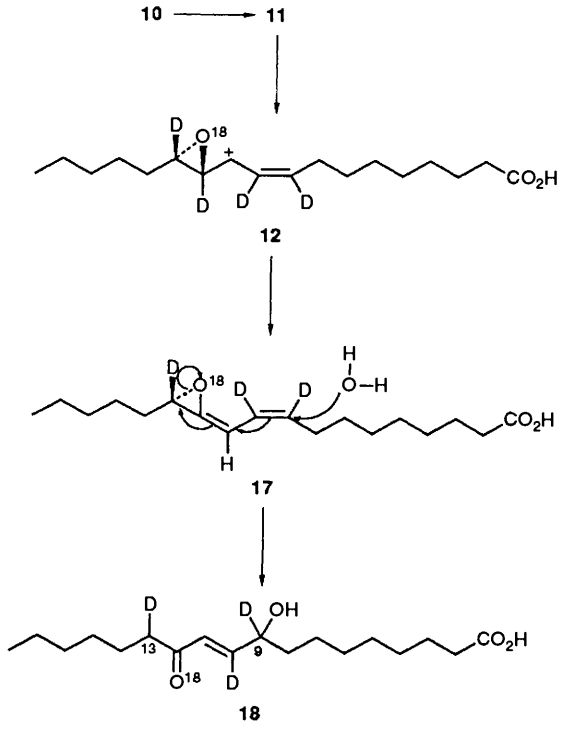
Taking as an example (13*S*)-13-hydroperoxylinoleic acid **6**, which produces 13-hydroxy-12-oxo-octadec-10(*E*)-enoic acid **7** on treatment with a flax enzyme, it is known from the use of the $^{18}O_2$ labelled hydroperoxide that there is an oxygen shift from C-13 to C-12.[6,12] The isotope is incorporated into the 12-carbonyl oxygen whilst the 13-hydroxy comes from water with both inversion and racemisation at the previous hydroperoxy centre. Rather less is known about the γ ketol **8** which is the minor product.[8] Gardner has suggested that the isomerisation to the α-ketol involves the formation of the epoxy cation **9**, followed by nucleophilic attack by water on the epoxide accompanied by hydrogen migration from C-10 to C-11 [*cf.* **12** ⟶ **13**, Scheme 1].[9,13] At the time of our work[14,15] no convincing mechanism for the formation of the γ ketol **8** had been put forward. Since there was no experimental information on the path of hydrogen in these reactions the study reported in this paper was undertaken.

The flax-seed enzyme catalysed reaction is known to be some 30 times more rapid for the (13-*S*)-hydroperoxide of linoleic acid than for the 9-isomer,[7] and this system was selected for study. First, spectral data were determined for the α-ketol **7**,[4] and more particularly the less worked on γ-ketol **8**;[8,10] ^1H NMR data are given in Tables 1 and 2 and these, ^{13}C NMR, mass spectra and UV data are fully consistent with the structures proposed earlier. Additionally, the α-ketol, after hydrogenation of the double bonds, could be cleaved with periodic acid to give, after methylation (CH_2N_2), hexanal and methyl dodecadioate, thus confirming the carbonyl to be at C-12 and the hydroxy to be at C-13. Treatment of synthetic[1] (9,10,12,13-$^2H_4$)-octadeca-9(*Z*),12(*Z*)-dienoic acid **10**, dissolved in borate buffer in the presence of Tween 20, with flax seed acetone powder extract[4,7,8] in sodium phosphate buffer (pH 7.0), for 90 min, gave, *via* the hydroperoxide **11**, and on work-up and chromatographic purification, deuteriated (13*R*)-13-hydroxy-12-oxo-octadec-9(*Z*)-enoic acid (α-ketol) **16** along with deuteriated 9-hydroxy-12-oxo-octadec-10(*E*)-enoic acid (γ-ketol) **18**. These were separated by careful preparative TLC as their methyl esters.

Mass spectral examination (EI) of the α-ketol showed that it contained not four but three deuterium atoms [M^+ absent, $M^+ - OMe$ 295 + 3, $M^+ - OMe - H_2O$ 277 + 3, $M^+ - OMe - C_5H_{11}$ 227 + 3, $M^+ - CO_2Me - Me[CH_2]_2CH{=}CH$. 195 + 3, $M^+ - OMe - Me[CH_2]_4CD(OH)CO$ 166 + 2 *etc.*]. Comparison of the ^1H NMR spectrum of the deuteriated product with the unlabelled α-ketol showed that the deuterium atoms at the expected 9, 10 and 13 positions



Scheme 1 The path of deuterium in α-ketol formation (with isotopic oxygen added)



Scheme 2 The path of deuterium in γ-ketol formation (with isotopic oxygen labels added)

remained, but that expected to be present at C-11, if Gardner's suggestion of migration **12** ⟶ **13** ⟶ **16**[9,13] were correct, was completely absent.[14] Acceptance of the loss of the deuteron originally at C-12 in the starting linoleic acid leads to an allene epoxide intermediate **14** in place of the migration hypothesis, as pointed out in our preliminary communication.[14] In another connection, such allene epoxides had been synthesised in our laboratory previously and their rearrangement to α-ketols demonstrated.[16] However, the possibility of a post-reaction exchange between a deuterium at position 11, α to the 12 ketone, had to be taken into account.

To study this, undeuteriated α-ketol was treated with buffer solutions exactly as in the original experiment except that the buffers were made up in D_2O, both with and without flax seed extract. The reaction time was 90 min as before and in both experiments an exchange of 1.1 atoms of deuterium was obtained at C-11 after work-up. Using ^1H NMR and irradiating the olefinic protons to remove their coupling to C-11 protons, analysis of the signal at δ 3.25 showed that 22% of the molecules contained both protons at C-11 unexchanged, 14% had one of the protons exchanged (proton A) and 32% the other (proton B) (*i.e.* there was some selectivity of exchange as between protons A and B), and 32% had both protons exchanged. It is clear that, even neglecting the slower breaking of a C–D bond, the *total* loss of deuterium in the original experiment could not be accounted for by post-exchange. Lengthening the exchange time to 5 h resulted in an exchange of 1.4 atoms of deuterium and just over 1.5 atoms after 7 h. As summarised in Scheme 1, although results are otherwise in accord with Gardner's epoxy-carbonium ion suggestion, the proton originally at C-12 is lost rather than transferred to C-11 of the ketol and an epoxy allene intermediate must be involved. When the labelling position of the γ-ketol was examined, further powerful evidence for the epoxy-allene intermediate was obtained.[15]

The deuteriated γ-ketol **18** produced from [9,10,12,13-$^2H_2$]linoleic acid **10** in the flax seed experiment (above) was shown by mass spectrometry to contain three, and not four, deuterium atoms (M^+ absent, $M^+ - OMe$ 295 + 3, $M^+ - C_5H_{10}$ 356 + 3 *etc.*). Using ^1H NMR spectroscopy, it was shown by comparison with the undeuteriated γ-ketol that the broad quartet at δ 4.32 (1 H, 9-H), the double doublet at 6.78 (1 H, 10-H), and one of the pair of hydrogens at 2.56 (1 H, 13-H), all of which were present in unlabelled γ-ketol, were not now present. The 11-H resonance, formerly a double doublet at δ 6.30 (*J* 15.9 and 1.6 Hz) now appeared in singlet form at δ 6.29. Explanation of the loss of the deuterium atom originally at C-12 by exchange with buffer is no longer an acceptable possibility and the C-13 deuterium remains in position, unchanged. A convincing pathway, previously not available, for formation of the γ-ketol becomes clear. Intervention of the epoxy-carbonium ion **12**, with loss of C-12 hydrogen gives again the allene epoxide **17**. The process of forming the γ-ketol is completed by ε-attack on the allene epoxide, just as the α-ketol forms through α-attack on the allene epoxide.

The loss of hydrogen originally at C-12 with the production of a reactive allene epoxide intermediate has since been elegantly confirmed by its actual isolation as a short-lived species by Hamberg using corn-germ enzyme[17] and by Brash and his colleagues[18] using flax enzyme. The methyl ester is stable at −70 °C with a half-life of several hours at 0 °C, though the acid itself has $t_{\frac{1}{2}}$ 15–30 s at 0 °C and pH 7.4.[18] The fatty acid allene epoxide can be stabilised by the presence of serum albumin.[19] It is readily attacked non-enzymically by

Table 1 ^{1}H NMR (250 MHz; $CDCl_3$) for methyl (13*R*)-13-hydroxy-12-oxooctadeca-(9*Z*)-enoate **7**

Chemical shift δ	Multiplicity	Number of protons	Coupling constant (Hz)	Assignment
5.59	m	2		9-H, 10-H
4.26	m	1		13-H
3.67	s	3		OCH_3
3.43	d	1	5.3	OH
3.25	m	2		11-H_2
2.30	t	2	7.5	2-H_2
2.01	m	2		8-H_2
1.90–1.20	m	18		3-H_2 to 7-H_2 14-H_2 to 17-H_2
0.91	t	3	6.9	18-H_3

Table 2 ^{1}H NMR (400 MHz; $CDCl_3$) for methyl 9-hydroxy-12-oxooctadeca-10(*E*)-enoate **8**

Chemical shift δ	Multiplicity	Number of protons	Coupling constant (Hz)	Assignment
6.78	dd	1	15.9, 5.4	10-H
6.30	dd	1	15.9, 1.6	11-H
4.32	br q	1	5.4	9-H
3.67	s	3		OCH_3
2.56	t	2	7.4	13-H_2
2.31	t	2	7.4	2-H_2
1.75–1.20	m	20		3-H_2 to 8-H_2 14-H_2 to 17-H_2
0.88	t	3	7.6	18-H_3

water or other nucleophiles, water leading to the α-ketol (above) and other products (see following paper).[17,18]

Experimental

Acetone Powder of Flax-seed.—Flax seed (*Linum usitatissimum*, linseed) (10 g), ground to a powder, was shaken with ice-cold acetone (3 × 50 ml). It was collected by filtration, washed with cold acetone (20 ml) and with ether (20 ml), dried (6.5 g) and stored at −25 °C.

Formation of (13R)-13-*Hydroxy*-12-*oxooctadec*-9(*Z*)-*enoic Acid* **7** *and* 9-*Hydroxy*-12-*oxooctadec*-10(E)-*enoic Acid* **8** *by the Action of an Extract of Acetone Powder of Flax seed on Linoleic Acid.*—A mixture of an 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 3 °C (15 min). The supernatant (10 ml) was added to a mixture of linoleic acid substrate solution (20 ml) and sodium phosphate buffer (pH 7.0; 50 mmol dm^{-3}; 400 ml). The reaction mixture was stirred in an open vessel at room temperature (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 a nitrogen atmosphere (30 min). Chloroform (200 ml) was added and the mixture was stirred under nitrogen (3 h). The organic phase was separated (separation being aided by filtration through a bed of Kieselguhr), dried (Na_2SO_4) and evaporated to give the crude reaction mixture.

(a) *Isolation of methyl* (13R)-13-*hydroxy*-12-*oxooctadec*-9-(Z)-*enoate* **7**. The crude reaction mixture was esterified with ethereal diazomethane and then fractionated by preparative thick layer chromatography (silica gel) developing 3 times with light petroleum (b.p. 60–80 °C)–ether (3:1) as eluent. The band at R_F 0.43 (2,4-dinitrophenylhydrazine spray) was collected. The product was further purified by C_{18}-reversed-phase HPLC eluting with methanol–water (4:1) to give methyl (13*R*)-13-hydroxy-12-oxooctadec-9(*Z*)-enoate **7** (27 mg) as a colourless oil. It had $\nu_{max}(CHCl_3)/cm^{-1}$ 3475 (OH), 1712br (ester C=O and ketone C=O), 1598 (C=C), and 1106 (C–O); δ_H(250 MHz; $CDCl_3$) see Table 1: δ_C(250 MHz; $CDCl_3$) 210.47 (C-12), 174.30 (C-1), 134.32 (C-10), 119.71 (C-9), 76.13 (C-13), 51.47 (OCH_3), 36.75, 34.06, 33.65, 31.64, 29.71, 29.16, 29.10, 29.06, 27.56, 24.90, 24.49, 22.51 (12 CH_2 units) and 14.00 (C-18): *m/z* 295 ($M^{\cdot+}$ − OMe; 5%), 277 ($M^{\cdot+}$ − OMe − H_2O; 3), 227 ($M^{\cdot+}$ − OMe − C_5H_{11}; 42), 195 [$M^{\cdot+}$ − CO_2Me − $CH_3(CH_2)_2CH{=}CH_2$; 100] and 166 [$M^{\cdot+}$ − OMe − $CH_3(CH_2)_4CH(OH)CO$; 40]: (Found: *m/z* 295.2311. $C_{18}H_{31}O_3$ requires *m/z* 295.2349).

(b) *Isolation of methyl* 9-*Hydroxy*-12-*oxooctadec*-10(E)-*enoate* **8**. Two batches of the crude reaction mixture were combined and fractionated by preparative thick layer chromatography (silica gel) developing 3 times with light petroleum (b.p. 60–80 °C)–ether (1:1 plus a few drops of formic acid) as eluent. The band at R_F 0.23 (UV) was collected. The product was esterified with ethereal diazomethane and further fractionated by preparative thick layer chromatography (silica gel) developing 5 times with light petroleum (b.p. 60–80 °C)–ether (3:2) as eluent. The band at R_F 0.35 (faint UV) was collected. Finally, purification by reverse-phase HPLC [methanol–water (7:3)] gave methyl 9-hydroxy-12-oxo-octadec-10(*E*)-enoate (1.9 mg) as white crystals. It had $\nu_{max}(CHCl_3)/cm^{-1}$ 3420(OH), 1730 (ester C=O), 1695 (α,β-unsaturated ketone C=O), 1634 (C=C) and 918 (*trans*-C=C); δ_H(400 MHz, $CDCl_3$) see Table 2: *m/z* 326 ($M^{\cdot+}$; 3%), 295 ($M^{\cdot+}$ − OMe; 12), 256 ($M^{\cdot+}$ − C_5H_{10}; 8), 187 [$M^{\cdot+}$ − $CH_3(CH_2)COCH{=}CH$; 20], 169 [$M^{\cdot+}$ − $CH_2(CH_2)_6CO_2Me$; 12], and 139 [$M^{\cdot+}$ − $CH(OH)(CH_2)_7CO_2Me$; 42] (Found: *m/z* 326.2450. $C_{19}H_{34}O_4$ requires *m/z* 326.2443).

Formation of [9,10,13-$^2H_3$]-(13R)-13-*Hydroxy*-12-*oxooctadeca*-9(Z)-*enoic Acid* **16** *and* [9,10,13-$^2H_3$]-9-*Hydroxy*-12-*oxooctadeca*-10(E)-*enoic Acid* **18** *by the Action of an Extract of Acetone Powder of Flax seed on* [9,10,12,13-$^2H_4$]*Linoleic Acid* **10**.—A mixture of [9,10,12,13-$^2H_4$]linoleic acid **10** (55 mg) and Tween 20 (50 mg) was dispersed in sodium borate buffer, (pH 9.0; 5 mmol dm^{-3}; 10 ml). Aqueous sodium hydroxide (2 mol dm^{-3}) was added dropwise until a clear solution was obtained. This was diluted with sodium phosphate buffer (pH 7.0; 50 mmol dm^{-3}; 400 ml). A mixture of acetone powder of flax seed (2g) and sodium phosphate buffer (pH 7.0; 20 ml) was stirred with ice-cooling. The mixture was centrifuged (12 000 × g) at 3 °C (15 min). The supernatant (10 ml) was added to the above solution and the mixture stirred in air at room temperature (90 min). The reaction mixture was extracted as described above.

(a) *Isolation of methyl* [9,10,13-$^2H_3$]-(13R)-13-*hydroxy*-12-*oxooctadec*-9(Z)-*enoate* **16**. The product was isolated as described earlier to give the ester (19 mg) as a colourless oil; δ_H(400 MHz; $CDCl_3$) 3.67 (3 H, s, OCH_3), 3.39 (1 H, br s, OH), 3.23 (2 H, AB system, *J* 17.0, 11-H_2), 2.30 (2 H, t, *J* 7.6, 2-H_2), 2.01 (2 H, t, *J* 7.0, 8-H_2), 1.90–1.20 (18 H, m, 3-H_2 to 7-H_2 and 14-H_2 to 17-H_2) and 0.90 (3 H, t, *J* 6.9, 18-H_3); *m/z* 298 ($M^{\cdot+}$ − OMe; 8%), 280 ($M^{\cdot+}$ − OMe − H_2O; 5), 230 ($M^{\cdot+}$ − OMe − C_5H_{11}; 35), 198 [$M^{\cdot+}$ − CO_2Me − $CH_3(CH_2)_2C{=}CH_2$; 87] and 168 [$M^{\cdot+}$ − OMe − $CH_3(CH_2)_4CD(OH)CO$; 85].

Isolation of methyl [9,10,13-$^2H_3$]-9-*hydroxy*-12-*oxooctadec*-10(E)-*enoate* **18**.—The product was isolated as described earlier to give the ester (0.7 mg); δ_H(400 MHz; $CDCl_3$) 6.29 (1 H, br s, 11-H), 3.67 (3 H, s, OCH_3), 2.54 (1 H, m, 13-H), 2.31 (2 H, t, *J* 7.5, 2-H_2), 1.75–1.22 (20 H, m, 3-H_2 to 8-H_2 and 14-H_2 to 17-H_2) and 0.88 (3 H, t, *J* 7.6, 18-H_3); *m/z* 298 ($M^{\cdot+}$ − OMe; 8%), 259 ($M^{\cdot+}$ − C_5H_{10}: 4), 188 [$M^{\cdot+}$ − $CH_3(CH_2)_2CHD$-

COCH=CD; 16], 172 [$M^{\bullet+}$ – $CH_2(CH_2)_6CO_2Me$; 9] and 141 [$M^{\bullet+}$ – $CD(OH)(CH_2)_7CO_2Me$; 60].

Methyl [11-^{2}H]-(13R)-13-*Hydroxy*-12-*oxooctadec*-9(Z)-*enoate*.—A mixture of acetone powder of flax seed (1 g), deuteriated sodium phosphate buffer (pH 7.0; 50 mmol dm^{-3}; 10 ml) (buffer made up in D_2O) was stirred with ice-cooling. The mixture was centrifuged (12 000 × g) at 10 °C (15 min). The supernatant (3 ml) was added to a mixture of linoleic acid substrate solution (5 ml) and deuteriated sodium phosphate buffer (pH 7.0; 50 mmol dm^{-3}; 90 ml). The reaction mixture was stirred in air at room temperature (90 min). The reaction mixture was extracted as above.

The product was isolated and purified as described earlier to give methyl [11-^{2}H]-(13*R*)-hydroxy-12-oxooctadec-9(*Z*)-enoate (6 mg) as a colourless oil; δ_H(250 MHz; $CDCl_3$) 5.59 (2 H, m, 9-H and 10-H), 4.26 (1 H, m, 13-H), 3.43 (1 H, d, *J* 5.1 OH), 3.25 (0.90 H, m, 11-H), 2.31 (2 H, t, *J* 7.6, 2-H_2), 2.01 (2 H, m, 8-H_2), 1.90–1.20 (18 H, m, 3-H_2 to 7-H_2 and 14-H_2 to 17-H_2) and 0.90 (3 H, t, *J* 7.0, 18-H_3); *m/z* 296 ($M^{\bullet+}$ – OMe; 8%), 278 ($M^{\bullet+}$ – OMe – H_2O; 7), 228 ($M^{\bullet+}$ – OMe – C_5H_{11}; 28), 196 [$M^{\bullet+}$ – CO_2Me – $CH_3(CH_2)_2C{=}CH_2$; 71] and 167 [$M^{\bullet+}$ – OMe – $CH_3(CH_2)_4CH(OH)CO$; 80]. The same product was produced when (13*R*)-13-hydroxy-12-oxooctadec-9(*Z*)-enoic acid **7** was solubilized in deuteriated buffer with and without flax seed extract (90 min).

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