Experimental Procedures

Enzymes and Chemicals

All chemicals were purchased from Sigma-Aldrich (Poole, United Kingdom). Cytochrome $P450_{BM3}$ was kindly provided by Dr Tobias Ost (University of Edinburgh, Edinburgh). The $P450_{BM3}$ concentrations were measured by the method of Omura and Sato[1] using an absorption coefficient of $\varepsilon = 91\ mM^{-1}cm^{-1}$ at 450 nm for the reduced P450-carbon monoxide (P450-CO) complex. Protein purity was confirmed by SDS/PAGE, and by assessments of the ratio A_{418}/A_{280}, where a high level of purity is represented by a ratio of 0.7 or above

Kinetic experiments were carried out in sodium potassium phosphate buffer, pH 8.0, (containing 10 % DMSO) at 25 °C, using a 1 cm path length quartz cell. The reaction mixtures contained $P450_{BM3}$ (20 nM), NADPH (0.2 mM) and linolenic acid. The oxidation of linolenic acid was determined spectrophotometrically by monitoring NADPH consumption at 340 nm (ε=6.22 mM^{-1} cm^{-1}) with the background wavelength at 250 nm over 20 seconds using a Uv-vis spectrophotometer (Shimadzu, Duisburg, Germany).[2] The rates were calculated from the linear part of the graph. Activities were corrected for the noncatalytic disappearance of NADPH. The K_m and V_{max} values were obtained by fitting the date to Michaelis-Menten equation using *Origin 5* software.

Instrumentation

^1H NMR and ^{13}C NMR spectra were recorded on BRUKER AC250. Chemical shifts (δ_H, δ_C) are reported in ppm and coupling constant (*J*) are in Hertz (Hz).

HPLC

Reversed phase HPLC analysis was run on a Phenomenex RP column (4.6 mm x 250 mm) at 25°C, using acetonitrile:water (5:95-95:5 over 30 min) at flow rate of 0.5 ml/min. Chiral HPLC analysis was performed on a Chiralcel OJ-H column (4.6 mm x 250 mm) at 5°C, using hexane:isopropanol (97:3) at a flow rate of 0.5 ml/min.

GC-MS

GC-MS analysis was performed using a temperature program of 120 ºC for 2 minutes, 10 ºC/min up to 260 ºC and hold for 10 minutes, on HP GC-MS system (6890GC and 5973 mass spectrum analyzer with EI ionization) with a HP-5MS (30 m x 25mm) capillary column.

Procedure 1: small scale reactions

To a solution of NADPH (10.4 mg, 0.0125 mmol, 1.25 equiv.) in MOPS buffer (3-(N-morpholino)-propanesulphonic acid, 5 ml, 50mM, pH 7.4) was added linolenic acid (2.8 mg, 0.01 mmol, 1.0 equiv.) in DMSO (100 μl). To this continuously stirred solution was added cytochrome $P450_{BM3}$ giving a final concentration of 0.77 μM. The reaction mixture was stirred at room temperature for 25 minutes in an open flask. The reaction was stopped after the given time by addition of oxalic acid (0.5 M) solution. In order to determine the time dependent product profile, aliquots were taken from the reaction mixture at 1, 10 and 20 minutes intervals and analyzed.

Procedure 2: large scale reactions

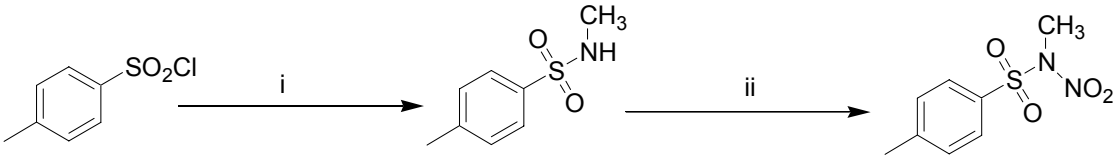
To a solution containing NADP (12 mg, 0.016 mmol, 0.06 equiv.), glucose-6-phosphate (86 mg, 0.305 mmol, 1.2 equiv.) and 175 U of glucose-6-phosphate dehydrogenase in MOPS buffer (3-(N-morpholino)-propanesulphonic acid, 400 ml, 50mM, pH 7.4) was added linolenic acid (70 mg delivered in DMSO, 0.25 mmol, 1.0 equiv.). To this stirred solution was added cytochrome $P450_{BM3}$ giving final concentration of 38.8 nM. After addition of catalytic amount of NADPH, the reaction mixture was stirred at room temperature for 25 minutes. At the end of the biotransformation, oxalic acid solution (0.5 M) was added until desired pH of 4.0 was obtained.

Work up procedure was as follows**;** after acidifying the reaction mixture, extraction was carried out with cold diethyl ether (3 x 200 mL). The organic fraction was combined, washed with water (200mL) and dried over anhydrous sodium sulfate. Solvent was removed under reduced pressure giving a colourless oil (76 mg). Thin-layer chromatography (*n*-hexane: ethyl acetate, 3:2) showed two main and two small spots; linolenic acid (starting material) R*f* =0.45, mono epoxy product , R*f* = 0.40 and closely placed spots R*f* = 0.25.

Chromatographic separation was carried out using a column of 2 x 16 cm packed with BDH silica gel (40-63 μm). Solvent mixture (*n*-hexane: ethyl acetate, 3:2) was used for elution, from which linolenic acid (27 mg, 36 %), mono epoxy product (29 mg, 39%) and a mixture of unknown products with mass of M+32 (20 mg, yellow pale oil, 25 %) were obtained.

Characterisation of the mono-epoxy product

To be able to analyse by GC-MS, the mono-epoxy acid was converted to the mono-epoxy methyl ester by diazomethane procedure. The diazald was prepared by following the scheme to produce the precursor of diazomethane.



i. aq. CH_3NH_2; ii. $NaNO_2$, $HClO_4$ (5M), H_2O, DCM

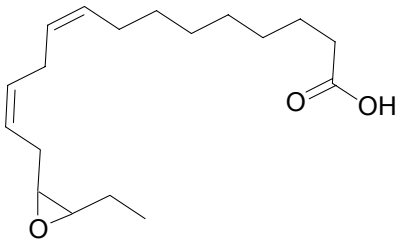
The reaction producing diazomethane was carried out in the Aldrich Diazald Apparatus. To a solution of KOH 11 M (2 mL) and ethanol (3ml) was added drop-wise diazald (*N*-methyl-*N*-nitro-*p*-toluensulfonamide, 210 mg, 0.9 mmol, 10 equiv.) in diethyl ether (5 mL). During addition of ethereal solution of Diazald, the mixture was warmed to 65-70 °C in order to distil to ethereal solution of diazomethane generated. The rate of distillation was the same as the rate of diazald solution addition. The distillate was collected and reacted with the mono-epoxy acid (29 mg, 0.1 mmol, 1.0 equiv.) in a stirred solution of methanol/diethyl ether (1/4) at 0 °C.

Preparation of epoxy standards of linolenic acid

Before carrying out the chemical epoxidation, linolenic acid was converted to the methyl ester in methanol and catalytic hydrochloric acid for analysis by GC-MS. A solution of the methyl ester of linolenic acid (126 mg, 0.43 mmol, 1.0 equiv. in 6 ml of CH_2Cl_2) was stirred for 2 h at room temperature with addition of MCPBA (52 mg, 0.30mmol, 0.7 equiv.). At the end of the reaction, the white precipitate was washed with 8 ml of $NaHCO_3$ (5%) and water. The organic solution was dried over anhydrous sodium sulfate and filtered. After removal of solvent, a crude product which contains unreacted linolenic methyl ester, mono-epoxy product-three regioisomers and a small quantity of diepoxy product was obtained. The mixture was then subjected to a chromatographic separation using n-hexane/ethylacetate (9:1) on a silica gel column. Surprisingly, one of the regio-isomers of epoxy-linolenic acid methyl ester was easily separated and showed quite different ^{1}H NMR signals than that of the remaining regio-isomers. GC-MS analysis of

all three regio-isomers showed three reasonable resolved peaks with retention times of 15.26, 15.30 and 15.34 minutes.

Spectroscopic data



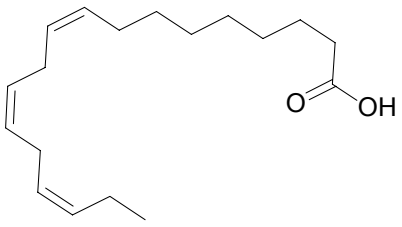
Mono-epoxy linolenic acid

15, 16-epoxyoctadeca-9,12-dienoic acid

^{1}H NMR (250 Hmz, $CDCl_3$, δ/ppm) 5.80-5.38 (m, 4H: H_9, H_{10}, H_{12}, H_{13}), 2.86-3.04 (m, 2H: H_{15}, H_{16}), 2.87 (t, ^{3}J= 6.1 Hz, 2H:H_{11}), 2.55-2.20 (m, 2H: H_{14}, partially hidden by H_2 triplet signal), 2.41 (t, ^{3}J=7.5 Hz, 2H: H_2), 2.10 (bm, 2H:H_8), 1.75-1.65 (m, 2H: H_3, partially overlapping with H_{17} signal), 1.70-1.55 (m, 2H: H_{17}, partially overlapping with H_3 signal), 1.38 (s, 8H: H_4, H_5, H_6, H_7), 1.12 (t, ^{3}J=7.5 Hz, 3H: H_{18}).

^{1}C NMR (63 MHz, δ/ppm) 179.1 (COOH), 130.7 (vinylic C), 130.4 (vinylic C), 127.1 (vinylic C), 124.0 (vinylic C), 58.3 (C of epoxy ring), 56.5 (C of epoxy ring), 33.8, 30.8, 29.4, 28.0, 27.0, 26.0, 25.7, 25.4, 24.5, 20.9, 10.5 (-CH_3).

GC-MS: (methyl ester) Rt = 15.30 min., M^+ = 308.1



Linolenic acid

Octadeca-9,12,15-trienioc acid

^{1}H NMR (250 Hmz, $CDCl_3$, δ/ppm) 5.65-5.30 (m, 6H: H_9, H_{10}, H_{12}, H_{13} H_{15}, H_{16}), 2.82 (t, ^{3}J= 11.0 Hz, 4H:H_{11},H14), 2.36 (t, ^{3}J=7.4 Hz, 2H: H_2), 2.12 (t, ^{3}J=7.4 Hz, 2H: H_8, partial overlapping with H_{17} triplet), 2.06 (t, ^{3}J= 7.5 Hz, 2H: H_{17}, partial overlapping with H_8 triplet), 1.64(bm, 2H: H_3), 1.33 (s, 8H: H_4, H_5, H_6, H_7), 0.99 (t, ^{3}J = 7.5 Hz, 3H: H_{18}).

^{1}C NMR (63 MHz, δ/ppm) 180.3 (COOH), 131.8 (vinylic C), 130.1 (vinylic C), 128.1 (vinylic C x 2), 127.6 (vinylic C), 127.0 (vinilic C), 34.0, 29.4, 29.0, 28.9, (x2), 27.0, 25.5, 25.4, 24.5, 20.4, 14.1 (-CH_3).

GC-MS: (methyl ester) Rt = 13.72 min., M^+ = 292.1.

References

(1) Omura, T.; Sato, R., *J. Biol. Chem.,* **1964***, 239,* 2370.

(2) Matson, R. S.; Hare, R. S.; Fulco, A. J., *Biochim. Biophys. Acta,* **1977***, 487,* 487.