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Regio- and enantioselective oxidation of thiaoleic acids by an algal Δ12-desaturase

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Abstract

In a non aerated incubation medium, the oxidation of the tested thiaoleic acids by *C. vulgaris* was regioselective. Moreover the non oxidised 14-thiaoleic acid had no influence on the endogenous fatty acid pattern whereas the 13-thiaoleic acid, readily oxidised by the microalgae, caused a significant 18:1 accumulation in phospholipids. Examination of the 13-thiaoleic acid metabolism revealed that up to 15% of the substrate was specifically oxidised in the phospholipids before being transferred into the neutral lipids class of *C. vulgaris*. Confirmation of the monooxygenase-like Δ12-desaturase intervention in this biotransformation was obtained by the stereochemical analysis of the optically active sulfoxide isolated from the endoplasmic phospholipids.

Keywords: *Chlorella vulgaris*; Chlorophyceae; Microalga; Oleoyl desaturase; Biotransformation; Stereoselective sulfoxidation; Thialoleic acids; Sulfoxide

1. Introduction

Fatty acid desaturases have been studied for some 40 yrs now, but very little is known regarding the detailed mechanism of the desaturation. Both soluble and membranous desaturases have been described in Eukaryotes. In the first category, the soluble stearoyl acyl carrier protein Δ9 desaturase (Δ9D) from *Ricinus communis* has been shown to be a non-heme diiron-oxo protein by spectroscopic and X-ray crystallographic studies (Fox, Shanklin, Somerville & Münck, 1993; Lindqvist, Huang, Schneider & Shanklin, 1996). Binuclear oxo- or hydroxo-bridged non-heme ferric centers have been identified in this soluble Δ9 desaturase as well as in methane monooxygenase (MMOH) and in ribonucleotide reductase (RNR R2) (Fox, Shanklin, Ai, Loehr & Sanders-Loehr, 1994). These high valent iron-oxo structures have been proposed as catalytic intermediates. Moreover, the primary sequence of these three enzymes revealed conserved aminoacid (Asp/Glu)-X-X-His alignments which act as iron center ligands and are potentially involved in oxygen binding and activation. The structural similarities among the diiron sites of MMOH, Δ9D and RNR R2 have led Que Jr. (1997) to propose a common mechanism for oxygen activation to carry out the respective oxidation reactions catalysed by these three enzymes. Membrane-bound desaturases, such as the plant oleate Δ12-desaturase, have not been characterized so precisely. The *Arabidopsis fad*2 gene, which encodes for this protein, has been identified by mutation experiments (Okuley, Lightner, Feldmann, Yadav, Lark & Browse, 1994) and expressed in *Saccharomyces cerevisiae* in order to provide a powerful system for the study of structure-function relationships of plant membrane-bound desaturases (Covello & Reed, 1996). Despite their structural differences, the membrane-bound and the soluble desaturases have some significant similarities such as the necessary presence of the

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iron in the catalytic site, the inhibition by iron chelates (Ferrante & Kates, 1986) and the stereospecificity of the desaturation reaction. Moreover, the sequence analysis of the membrane-bound desaturases from mammals, fungi, insects, higher plants and cyanobacteria has revealed some regions of conserved histidine residues which could act as ligands for the catalytic center (Shanklin, Whittle & Fox, 1994). Very recent Mössbauer studies have shown that the closely related alkane ω-hydroxylase possesses a catalytically active diiron site as well as the highly conserved multi-histidine domain in its primary sequence (Shanklin, Achim, Schmidt, Fox & Münck, 1997). On the basis of primary sequences comparison, other workers strongly suggested that the oleate hydroxylase from *Ricinus communis* L. was closely related to the microsomal oleate desaturase from *Arabidopsis* (van de Loo, Broun, Turner & Somerville, 1995). Nowadays, there are a growing number of studies (Feig & Lippard, 1994; Lange & Que Jr., 1998; Shanklin & Cahoon, 1998; Shteinman, 1997) suggesting similar reaction mechanisms of oxygen activating non-heme iron enzymes involving iron-oxo intermediates, such as monooxygenase or soluble desaturases. Que Jr. and coworkers (Kim, Dong & Que Jr., 1997) recently demonstrated that a high-valent non-heme iron (IV) oxo complex could convert cumene to methyl styrene and cumyl alcohol. As suggested by Shanklin et al. (1997) dioxygen-dependent enzymatic modifications of hydrocarbon substrates would concern the carboxylate-rich soluble enzymes such as MMOH or Δ9D as well as the histidine-rich membrane-bound enzymes such as oleate desaturase and alkane ω-hydroxylase. A possible mechanistic scheme relating hydroxylation and desaturation has been proposed by Buist & Behrouzian (1996). Their deuterium kinetic isotope effects approach allowed them to demonstrate that by using an in vivo *S. cerevisiae* Δ9 desaturase system and thiastearate analogs, the regioselectivity of sulfoxidation matches that of initial hydrogen abstraction at C-9 of the parent fatty acid substrate. Very recently, these authors used the same strategy to determine the intermolecular primary deuterium isotope effects involved in oleate desaturation using a transformed strain of *S. cerevisiae* containing a functional Δ12 desaturase from *Arabidopsis thaliana* (Buist & Behrouzian, 1998). Their results were consistent with the notion that this process was initiated at C-12 by a slow isotopically-sensitive step and with van de Loo's (1995) suggestion that ricinoleic acid was produced by an altered Δ12 plant desaturase.

We have explored the mechanism of the oleate Δ12 desaturation by studying substrate-activity relationships. We had previously studied the influence of thiaoleic acids on Δ12 desaturation by *Chlorella vulgaris* and shown that 13-thiaoleic acid was a good inhibitor of the in vivo [1-^{14}C] oleic acid desaturation (Poulain, Noiret, Nugier-Chauvin & Patin, 1997). This synthetic analogue of oleic acid appeared to be readily oxidised to the corresponding S-oxide by the microalgae. Examination of its metabolism, especially in phospholipids, as well as the modification of the endogenous fatty acid distribution in this class of lipids, strongly suggested a specific but minor Δ12 desaturase–13-thiaoleic acid interaction (Fauconnot, Nugier-Chauvin, Noiret, Poulain & Patin, 1998). Actually, in aerated incubation media, other oxidative pathways could compete with desaturase-mediated sulfoxidation. Therefore, in this paper, we carefully study the incorporation and biotransformation of 13-thiaoleic acid and its biotransformed product into the cells and into the lipid classes in a different incubation-type experiment that prevents these non selective oxidative processes. Moreover, in order to further explore the influence of sulphur atom position and the regiospecificity of the likely oleoyl desaturase substrate interaction, we report here on the metabolism of the 14-thiaoleic acid which had a very weak effect on the in vivo [1-^{14}C]-oleic acid desaturation (Poulain et al., 1997). Examination of the stereochemistry of the sulfoxide metabolite strengthens the results of this study and is consistent with the highly specific sulfoxidation of 13-thiaoleic acid by the oleate Δ12 desaturase, as previously suggested.

2. Results and discussion

2.1. Influence of the thiaoleic acids on endogenous fatty acid distribution in phospholipids

Cells in the mid-logarithmic phase were incubated in a pH 7.4 phosphate buffer with 6 mg of thiaoleic acid per 30 ml of cellular suspension in non aerated conditions (closed tubes) at 25°. After a 9 h incubation, crude homogenate (cellular fraction) was separated from soluble fraction (supernatant) by centrifugation. The lipids were extracted from the bulk and separated on TLC and the fatty acid composition of phospholipids (PL) of *C. vulgaris* was examined (Fig. 1). The endogenous fatty acid pattern in PL remained unchanged when 14-thiaoleic acid was incubated for 9 h, compared to the control experiment (without the exogenous fatty acid). On the contrary, after a 24 h incubation of 13-thiaoleic acid, 18:1 concentration was increased almost threefold (from 6% to 17%) in 24 h and the endogenous 18:2 was significantly reduced (from 34% to 30%) compared to the control experiment where 18:2 increased. These results are consistent with the effect of sulphur position of thiafatty acids on [1-^{14}C] oleic acid desaturation, as previously reported (Fauconnot et al., 1998). The desaturation

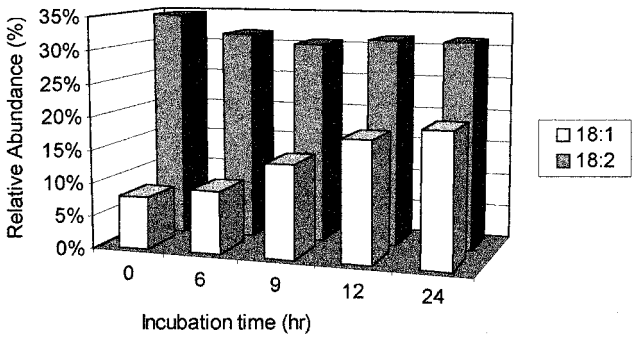


Fig. 1. 18:1 and 18:2 composition of phospholipids of *C. vulgaris* in presence of 13-thiaoleic acid. Incubations were performed in closed flasks with 30 ml of cell suspension and 6 mg of 13-thiaoleic acid for 24 hr at 25° under 15000 lux. Lipids were separated by TLC. After hydrolysis, PL were methylated and fatty acids analysed by GC.

rate was indeed particularly low (10%) in the presence of 13-thiaoleic acid while it was even higher than the 50% desaturation rate observed with exogenous unlabelled oleic acid, when 14-thiaoleic acid was incubated. The strong influence of the 13-thiaoleic acid on the distribution of fatty acids in PL, which was not observed with the 14-thiaoleic acid, suggested different types of metabolism of these two thiafatty acids.

2.2. Whole-cell 13-thiaoleic acid oxidation and 14-thiaoleic incorporation

In whole-cell time course experiments, 6 mg of thiaoleic acid were incubated with 30 ml of cellular suspension of *C. vulgaris*. The incorporation of the sulfide into the cells and its biotransformation were studied during 24 h. After centrifugation, endogenous and exogenous fatty acids were extracted, methylated and separated by GC and HPLC analysis for both cellular and supernative fractions. Both 13- and 14-thiaoleic acids were rapidly incorporated into the cells. After 3 h incubation, no trace of thiafatty acids could be detected in the extracellular fractions. After 24 h incubation, 67% of the 13-thiaoleic acid were oxidised to the corresponding sulfoxide (Fig. 2). The biotransformation is chemoselective: the formation of the corresponding sulfone was not observed and the starting sulfide and the resulting sulfoxide were extracted with quantitative yields. The methyl-S-oxide 13-thiaoleate, isolated from the bulk, was purified by HPLC and fully characterised on the basis of ^{1}H NMR and mass spectral data, as well as by comparison with an authentic synthetic standard (Scheme 1). Contrary to the 13-thiaoleic acid, the 14-thiaoleic acid was not oxidised by *C. vulgaris* (less than 5% after 24 h) (Fig. 2). After 3 h incubation, 80% of the 14-thiaoleate, exclusively extracted from the bulk, was recovered intact in the cell fraction. However, the percentage of recoveries was not quantitative and significantly decreased during the incubation whereas the 14-thiaoleic acid was quantitatively extracted from a blank. This suggests that this thiafatty acid may be undergoing another kind of biotransformation. The sulphur atom which occurs at the 14-position would allow the removal of the hydrogens H_{12} and H_{13} by the Δ12-desaturase, but in that case, the desaturation of the 14-thiaoleate would lead to the unstable 14-thialinoleate which could be rapidly cleaved into the corresponding butanethiol **1** and oxoester **2** (Scheme 2).

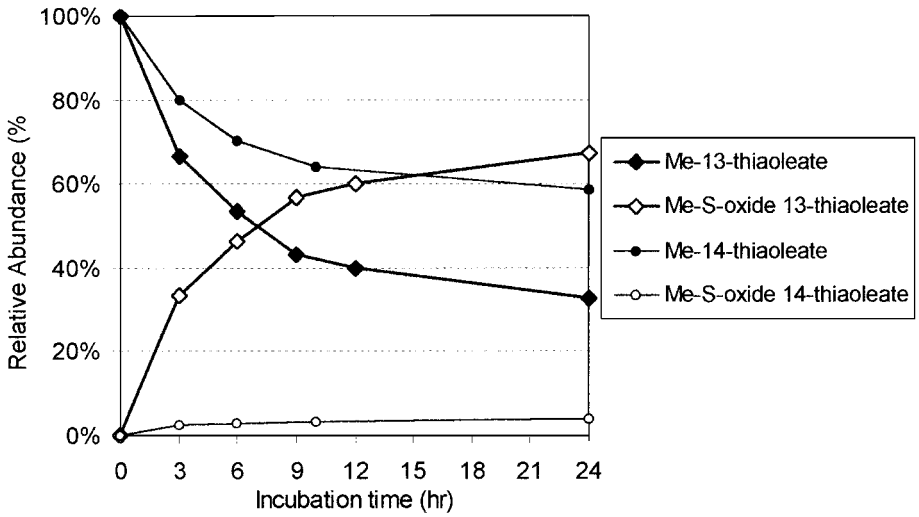
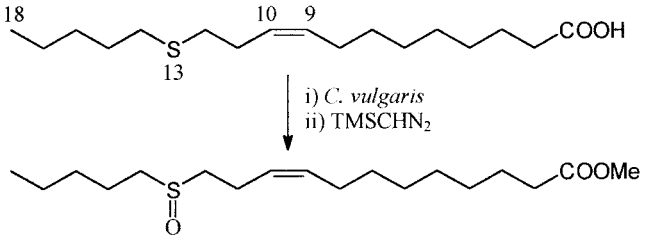
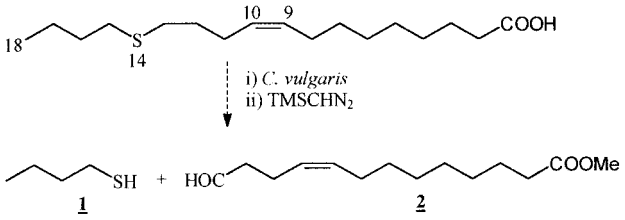


Fig. 2. Time-course of thiaoleic acids biotransformation. Data are mean value from four experiments. Time-course experiments were performed in closed flasks with 30 ml of cell suspension and 6 mg of thiaoleic acids for 24 hr at 25° under 15000 lux. After appropriate treatment, exogenous fatty acids were analysed by GC and HPLC (see experimental).



Scheme 1. Whole-cells oxidation of the 13-thiaoleic acid.



Scheme 2. Cleavage of the supposed desaturated metabolite of the 14-thiaoleic acid.

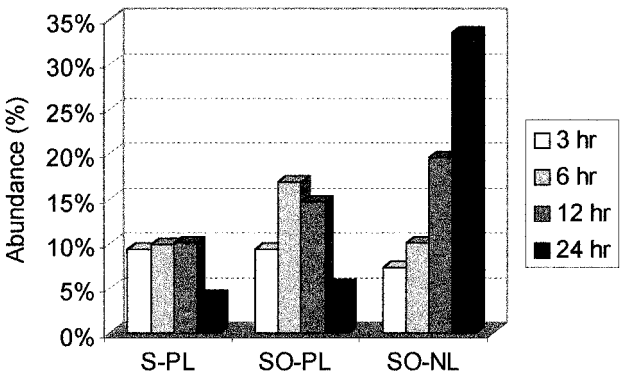


Fig. 3. Time-course of incorporation and oxidation of the 13-thiaoleic acid in lipids of *C. vulgaris*. Timed–course experiments were performed under the same conditions and samples were treated according to the same experimental procedure as described in Fig. 1. Exogenous Me esters were then analysed by GC and HPLC. S-PL is methyl 13-thiaoleate isolated from phospholipids (PL), SO-PL is methyl S-oxide 13-thiaoleate from phospholipids and SO-NL is methyl S-oxide 13-thiaoleate from neutral lipids (NL).

2.3. Incorporation and biotransformation of the thiafatty acids in lipids of C. vulgaris

C. vulgaris cells rapidly converted 13-thiaoleic acid into the corresponding acyl-CoA. During the first 6 h incubation, the 13-thiaoleate remained mainly in the soluble methanol/water phase (55% after 3 h) during the lipids extraction procedure (as described in the experimental section), confirming our first results (Fauconnot et al., 1998) that thiafatty acids compete with oleic acid for long chain acyl-CoA synthetases. A small part of the 13-thiaoleoyl-CoA was slowly oxidised to the S-oxide-thiaoleoyl-CoA during the incubation (12% after 24 h). Then, acyl-CoA transferase(s) seemed to be able to transfer the thioesters into various lipids of *C. vulgaris*. The lipids separation by TLC (Fig. 3) indicated that the 13-thiaoleate was incorporated into neutral lipids (NL i.e. essentially the TAG), phospholipids (PC and PE) and the free fatty acids pool (FFA). The corresponding sulfoxide was detected in these same lipid classes and was mainly located after 24 h in the NL fraction (32%). From 3 h to 24 h, when whole cells were incubated with the 14-thiaoleic acid, the nonoxidised sulfide was entirely recovered in the NL. A constant rate of 2% of the corresponding sulfoxide remained in the FFA pool and a very small proportion of the 14-S-oxide (increasing from 0.5% to 2% between 3 h and 24 h) was also detected in the NL. These results suggest that the S-oxide 13-thiaoleate was probably not produced in this class but came from a transfer from other lipid classes (more likely the PL) or from another cellular compartment (soluble cytosolic compartment for example). In these conditions, it clearly appeared that the storage pathway was favoured, with a limited sulfoxidation of the carboxylic acids in the FFA pool (less than 15%). These results could be related to our previous work (Fauconnot et al., 1998) showing that in other conditions (more aerated incubation media) the oxidation of the 13-thiaoleic acid occurring in the FFA pool was the major oxidative pathway and further analysis (data not shown) confirmed that this nonselective process leads to a racemic sulfoxide. Moreover, under these new conditions (closed flasks incubations), it should be emphasised that a great part of the S-oxide 13-thiaoleate appeared in the PL (15% after 6 h) and suggested that this sulfoxidation occurring in particular in the PC could involve the Δ12-desaturase activity. Nevertheless, during the first 9 h, the 13-S-oxide appeared in the PC more rapidly and to a higher extent compared to the 13-thiaoleate PC decrease. This observation showed that the sulfoxide could also be transferred probably from the soluble fraction into the PL. In order to confirm these results, we purified the S-oxide 13-thiaoleate at each time and from each lipid class and analysed it by HPLC, on a chiral column. Compared to the corresponding racemic synthetic sulfoxide, the biological sulfoxides were isolated from PC, PE and NL respectively with 30, 25 and 20% ee after 3 h, whereas the sulfoxides from the FFA and thioester-CoA pools were racemic. These ee rapidly decreased during the incubation and the sulfoxides were found almost racemic after 24 h. These results confirm those previously obtained for the time course of 13-thiaoleic acid metabolism and suggest that the sulfoxide can be transferred from a cellular compartment to another one: actually, the presence of a nonracemic sulfoxide in NL could be explained by the transfer of the chiral sulfoxide from PL to this storage lipids class. Moreover, after only 3 h, the 30% ee sulf-

oxide in the PC suggests an early major transfer of a racemic sulfoxide (from the acyl-CoA or FFA pools) to the PL.

Since desaturase-mediated sulfoxidation could occur not only with high regioselectivity but also with high enantioselectivity, we conclude that the sulfoxidation of 13-thiaoleate in the PC (and very likely in the PE too) involved the Δ12-desaturase monooxygenase-like activity and gave an optically active sulfoxide in PL produced via at least another non selective oxidative process resulting in the isolation of the methyl-S-oxide 13-thiaoleate with a 30% ee after 3 h.

In order to obtain sulfoxides with higher ee by more specifically targeting the activity of the Δ12-desaturase, studies of metabolism of thiafatty acids (in particular with a sulphur atom at the 12-position) in optimised culture conditions are in progress.

3. Experimental

3.1. Culture conditions

C. vulgaris (strain 211/8 K) was purchased from CCAP (Cambridge, UK). Microalgae were maintained on nutrient agar at 20°. Cells were then used to inoculate 40 ml autoclaved medium (Sorokin & Krauss, 1960) added to (g l^{-1}): glucose 5, $MgSO_4$, 7 H_2O 1, peptone 0.1 and yeast extract 0.1.

3.2. Cultivation of alga

Cells were stirred for one week at 20° in a 100 ml flask. Then, they were transferred to a 2 l incubator containing 1 l of autoclaved culture medium, enriched with 20 ml of a nutrient solution (g l^{-1}): glucose 5 and $MgSO_4$, 7 H_2O 1. Cultures were grown exponentially for at least 24 h at 30°, stirred, aerated and illuminated from above at 15,000 lx. Growth was monitored by A at 550 nm. In the mid-logarithmic phase (A = 4), cells were harvested by centrifugation at 1500 rpm for 15 min. The supernatant was discarded and the cellular fraction was washed (×3) with 0.5 M Pi buffer (pH 7.4). The algal biomass was resuspended in the same buffer to obtain a suspension of 0.05 g ml^{-1}.

3.3. Whole-cells biotransformation assays

To 30 ml of cellular suspension previously described, an EtOH solution of thiaoleic acid was added to a final concentration of 0.2 mg ml^{-1}. The reaction mixture was stirred at 25° under 15,000 lx illumination in closed flasks. For kinetic studies, we took samples of 6 ml and lipids were fixed in boiling EtOH. Cultures were then centrifugated at 1500 g for 10 min and supernatants and cellular fractions were separated. Cellular fractions containing total lipids were saponified by addition of 6 ml of 12% KOH in EtOH, at 70° for 30 min. The acidification step was then realised by dripping HOAc up to pH = 5. The acidification of the medium must be carefully controlled because chiral sulfoxides are prone to racemisation under acidic conditions (Jones-Hertzog & Jorgensen, 1995). After extraction (×3) by 6 ml of CH_2Cl_2, fatty acids were then methylated under neutral conditions by addition of 500 μl of C_6H_6, 200 μl of MeOH and 200 μl of 0.2 M $TMSCHN_2$ in hexane. Reaction mixtures were stirred under N_2 for 30 min at 20°. After addition of 3 ml of NaCl solution, fatty acids Me esters were extracted (×3) by 2 ml of CH_2Cl_2. The same procedure was used on blank samples: a Pi buffer solution of 13-thiaoleic acid permitted us to evaluate the atmospheric and chemical oxidation rate of the substrate (< 1%) during the incubation and extraction procedures. A Pi buffer solution of 14-thiaoleic acid permitted us to check that the substrate was extracted with quantitative yields. A Pi buffer solution of a chiral dialkylsulfoxide (but-1-enebutylsulfoxide) allowed us to test the non racemisation effect of this method (Gautier, Noiret, Nugier-Chauvin & Patin, 1997).

Cellular and supernatant fatty acid Me esters were analysed by FID capillary GC on a 30 m × 0.25 mm Supelco S-2380 column. The carrier gas was He (70 kPa). Operating conditions used were temp. programmed 70° to 100° at 15° min^{-1} then 100° to 220° at 4° min^{-1} and finally isothermal at 220° for 10 min. Injector and detector heater temperature were 250°. The split ratio used in the injection system was 7 ml min^{-1} and the injection volume for all samples was 0.5 μl. For measuring amounts of fatty acid Me esters, 17:0 was added as internal standard before saponification.

3.4. Lipid extraction

After incubation, algal suspensions were centrifuged at 1500*g* for 5 min. In order to prevent hydrolysis of lipids during the procedure, collected cells were suspended in EtOH and boiled for 5 min. Lipids were extracted by addition of 5 ml of $CHCl_3$–MeOH–H_2O (1:2:1). Once tubes were closed under N_2, the reaction mixture was heated at 70° for 30 min. Then, 1 ml of H_2O and 1 ml of a 2 M solution of NaCl in KPi buffer (pH 7.4) were added. After briskly stirring and decanting, the organic phase was collected. Total lipids were extracted (×3) by addition of 3 ml of $CHCl_3$. The solvent was then evaporated under N_2.

3.5. Lipid separation

Lipids were separated by TLC. The residue was resuspended in 500 μl of $CHCl_3$–MeOH (1:1). The

entire lipid solution was deposited on silica gel plates (Merck G60) which had been previously activated at 60° for 1 h. Polar and neutral lipids were separated with a first development (two-thirds of the plate) in $CHCl_3$–Me_2CO–MeOH–HOAc–H_2O (10:4:1:2:1) and a second development in petrol–Et_2O–HOAC (70:30:1). After developments, plates were dried under N_2 and the separated lipids detected with I_2 vapour and identified using authentic standards. Lipids were removed, hydrolysed and methylated as previously described. Fatty acids Me esters were extracted and analysed by capillary GC as described above.

3.6. Purification of metabolic products

At the end of the incubation period, supernatant and cellular fractions were separated by centrifugation. Lipids were saponified and free fatty acids were then methylated as described above. Fatty acids Me esters were resuspended in hexane and analysed by HPLC. The sulfoxide was separated from endogenous fatty acids by HPLC on a Hypersil BDS C_{18} column (250 × 10 mm). A CH_3CN–H_2O gradient from 80:20 to 95:5 in 10 min, with a flow rate of 4 ml min^{-1}, was used. Esters were detected by UV at 208 nm. The sulfoxide was collected and identified by ^1H NMR and MS data.

3.7. Stereochemical analysis of the methyl-S-oxide 13-thiaoleate

The ee were determined by HPLC on a ChiralCel 03 column (Daicel Chemical Industries Ltd, 250 × 4.6 mm). The separation of the enantiomers was optimised with the racemic synthetic sulfoxide. The elution was realised under isocratic conditions using a mixture of hexane–iso-PrOH (91:9) with a flow rate of 0.7 ml min^{-1}. Sulfoxides were detected by UV at 208 nm.

3.8. Methyl-S-oxide 13-thiaoleate

CIMS (NH_3) 70 eV, *m/z* (rel. int.) 331 $[M+H]^+$ (100), 348 $[M–H_2O]^+$ (85), 365 $[M–NH_4–NH_3]^+$ (50). ^1H NMR (400 MHz, $CDCl_3$): δ 5.51 (1H, m, H-9), 5.38 (1H, m, H-10), 3.64 (3H, s, H-19), 2.68 (4H, m, H-12 and H-14), 2.53 (2H, dt, ^3J = 7.6 Hz, H-11), 2.31 (2H, t, ^3J = 7.6 Hz, H-2), 2.06 (2H, m, H-8), 1.77 (2H, m, H-3), 1.5–1.6 (14H, m, H-4, H-5, H-6, H-7, H-15, H-16, H-17), 0.92 (3H, t, ^3J = 7.1 Hz, H-18).

3.9. Methyl-S-oxide 14-thiaoleate

^1H NMR (400 MHz, $CDCl_3$): δ 5.43 (1H, m, H-9), 5.23 (1H, m, H-10), 3.59 (3H, s, H-19), 2.72 (4H, m, H-13 and H-15), 2.24 (2H, t, ^3J = 7.5 Hz, H-2), 2.17 (2H, dt, ^3J = 7.6 Hz, H-11), 1.90 (2H, dt, ^{3}J = 7.6 Hz, H-8), 1.78–1.55 (6H, m, H-3, H-12 and H-16), 1.42–1.30 (10H, m, H-4, H-5, H-6, H-7, H-17), 0.86 (3H, t, ^3J = 7.5 Hz, H-18).

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