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SULFOXIDATION OF 13-THIAOLEIC ACID BY A DESATURASE-ACTIVE ALGA

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Key Word Index—*Chlorella vulgaris*; Chlorophyceae; alga; biotransformation; 13-thiaoleic acid; methyl 13-thiaoleate *S*-oxide; oleoyl desaturase; sulfoxidation.

Abstract—The synthetic analogue of oleic acid, 13-thiaoleic acid, was readily oxidised to the corresponding *S*-oxide by the algae, *Chlorella vulgaris*. Among the thiaoleic acids tested, it was the only one that decreased the *in vivo* oleoyl desaturation rate of *C. vulgaris* substantially. It could activate the acyltransferases and cause a significant endogenous 18:1 accumulation in phospholipids. These results, as well as the time-course incorporation of 13-thiaoleic acid into the lipid classes of *C. vulgaris*, clearly showed the extreme sensitivity of this substrate for any oxidising cellular agents and was consistent with a specific but tiny intervention of the cytoplasmic oleoyl desaturase to transform it into the oxide metabolite.

INTRODUCTION

In plants, *de novo* fatty acids are first desaturated by a soluble stearoyl-acyl carrier protein desaturase located in the plastid stroma. Then, these acids are either desaturated by chloroplastic Δ12- and Δ15-desaturases or, after hydrolysis to free fatty acids and passage through the two membranes of the chloroplast envelope, thioesterified by an acyl-CoA synthetase in the outer envelope. Additional double bonds are introduced into acyl chains linked to endoplasmic lipids, mainly phosphatidylcholine (PC) and probably other phospholipids (PL), such as phosphatidylethanolamine (PE), involving membrane-bound enzymes, such as acyltransferases and desaturases [1–4]. In the last few years, several DNAs encoding membrane-bound desaturases from *Arabidopsis*, *Brassica* and cyanobacteria [5–7] have been cloned, mainly by complementation of mutants. For instance, comparisons of the deduced amino acid sequences revealed some conserved domains for the membrane-bound Δ12-desaturases from plants and cyanobacteria [8]. Nevertheless, the only membrane-bound desaturase isolated after purification is a Δ12-desaturase from spinach chloroplasts [9]. Despite these recent advances, little is known about the active site structure, as well as the specificity of the oleoyl desaturase towards its substrate. For instance, it has been thought that some ω6 and ω3-desaturases from chloroplast membranes could desaturate any fatty acids that keep a specific distance between an existing double bond and the methyl end of the acyl chain [10]. Prior to these results, other authors suggested the existence of two oleoyl desaturases in *Chlorella vulgaris* whose specificity of recognition depends on either the distance previously defined or the one between the first double bond in the chain and the carboxylic acid end [11].

In a first study [12], we were interested in a chemical approach of this enzyme-substrate interaction during *in vivo* experiments on *C. vulgaris*. Thiaoleic acids were synthesised and their influence on the desaturation of labelled oleic acid by the green algae was evaluated. In the present work, we focus on the biotransformation of these sulphur-modified fatty acids in order to demonstrate a specific interaction of the oleoyl desaturase with some of these analogues. Sulphur in the chain might influence the action of the non-heme iron containing oleoyl desaturase by sulphur-iron affinity [13]. Moreover, the Δ9-desaturase of *Saccharomyces cerevisiae* has been shown to behave as a regio- and enantioselective oxidising agent towards 9- and 10-thiastearic acids, leading to the corresponding *S*-oxide species [14–15].

In this paper, we carefully examine the direct influence of some synthetic thiaoleic acids on the *in vivo* desaturation of labelled oleic acid. A significant inhibitory effect is observed when the sulphur occurs at the 13-position of the alkyl chain. We report different

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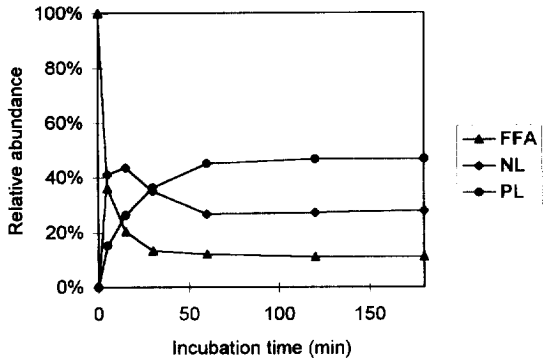


Fig. 1. Incorporation of radioactivity into the lipid classes* of *Chlorella vulgaris*. Data are mean values from three experiments. * 1 nmol of [1-^{14}C] oleic acid (55 nCi) was added to the cell suspension and incubated at 25° under 15000 lux. After appropriate treatment, lipids were separated by TLC (see Experimental).

studies concerning the *in vivo* transformation of 13-thiaoleic acid, especially in phospholipids, suggesting a specific interaction of the oleoyl desaturase with this modified substrate.

RESULTS AND DISCUSSION

Incorporation of labelled oleic acid into endoplasmic lipids

Cells in the mid-logarithmic phase were incubated with [1-^{14}C] oleic acid in a pH 7.4 phosphate buffer and the distribution of radioactivity in the lipid classes observed after a 3 hr incubation. In less than 3 min, exogenous oleic acid was readily incorporated into whole algal cells. The observed radioactivity distribution pattern in lipids (Fig. 1) showed that most of oleic acid was first incorporated into phospholipids and into the neutral lipids (NL), i.e. mainly triacylglycerols (TAG). After 1 h, the distribution of activity stabilised showing 47% in phospholipids, 28% in NL, 11% in free fatty acids pool (FFA), along with 5% in the aqueous fraction. In addition, the incorporation of activity into the chloroplastic lipids, monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and phosphatidylglycerol (PG) was poor (6% in the MGDG/DGDG fractions and 3% in the PG). As previously observed in *C. vulgaris* [16], these results underline the fact that exogenous oleic acid was incorporated by acyltransferases into phospholipids. Moreover, after 30 min, 17% of labelled oleic acid were desaturated to linoleic acid. A substantial proportion (12.5%) of labelled linoleic acid appeared in PL (i.e. PC and PE), a small amount was already transferred into TAG (3.5%), whereas a far lower proportion (1%) was found in the MGDG/DGDG/PG fractions (results not shown). Because oleoyl-PC is the substrate for endoplasmic oleoyl desaturase [2–5], the desaturase activity targeted here (48% of labelled oleic acid desaturated to linoleic acid after 3 hr) most likely concerned the extrachloroplastic oleoyl desaturase [17].

Effect of sulphur position of thiafatty acids on [1-^{14}C] *oleic acid desaturation*

[1-^{14}C] oleic acid was added to a cellular suspension and the oleoyl desaturase activity evaluated by the production of labelled linoleic acid. This rate of oleate conversion to linoleate, noted D_1, was expressed in relation to the control activity DT: $D_2 = D_1/DT$. We then compared the yields of this relative desaturation of [1-^{14}C] oleic acid, noted D2, in the presence of added unlabelled oleic acid ($D_2 = 50\%$) and, in the presence of thiaoleic acid (0.2 mM). This first experiment must be carried out for the whole of the modified fatty acids in order to reveal some particularities of one or another analogue during desaturation of labelled oleic acid. At this stage it appeared that all the thiaoleic acids tested were readily incorporated into the cells (see next section).

After 3 h incubation, the desaturation rate was particularly low (10%) in the presence of the 13-thiaoleic acid, compared with the 50% desaturation rate observed with exogenous unlabelled oleic acid (Fig. 2). These inhibition rates obviously depend on the concentration of exogenous fatty acids tested, therefore because of the strong effect of 13-thiaoleic acid, some assays were performed in order to evaluate D_2 in relation to this concentration. It appeared that when the concentration of cold oleic acid was higher than $7.6\ 10^{-3}$ mM, the desaturation rate ($D_2 = 100\%$) started to decrease. At this concentration, the 13-thiaoleic acid already had a significant effect ($D_2 = 60\%$).

The very different influence of 13-thiaoleic acid

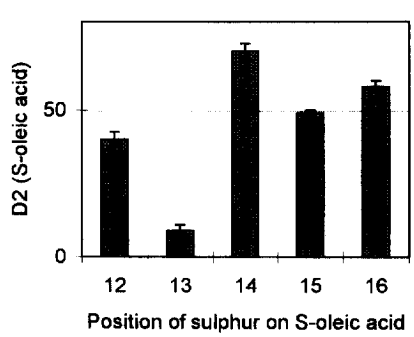


Fig. 2. Effect of sulphur position of thiafatty acids on [1-^{14}C] oleic acid desaturation by *Chlorella vulgaris**. * Desaturation rates were determined on 1 ml of cell suspension incubated with 1 nmol of [1-^{14}C] oleic acid (55 nCi nmol^{-1}) and 0.2 μmol of an exogenous fatty acid for 3 h at 25° under 15000 lux. Desatuation rate (D_2) is expressed relative to control activity of oleoyl desaturase ($DT = 47\%$):

$$D_2 = D_1/DT \times 100$$

and $$D_1 = \frac{[1\text{-}^{14}\text{C}]\ \text{linoleic acid}}{[1\text{-}^{14}\text{C}]\ \text{linoleic acid} + [1\text{-}^{14}\text{C}]\ \text{oleic acid}}$$

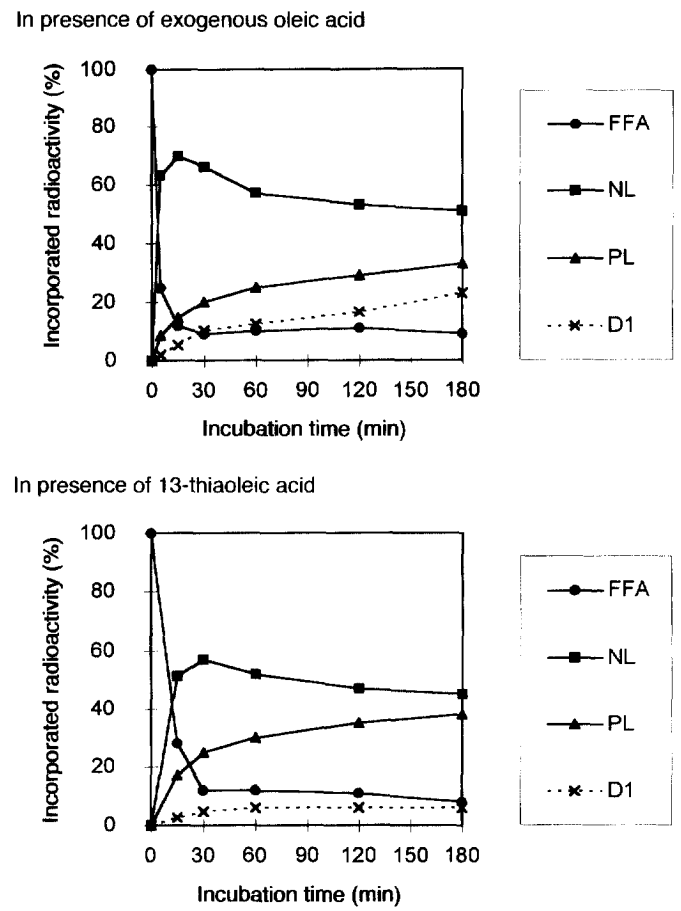


Fig. 3. Time-courses of [1-^{14}C] oleic acid incorporation* into lipid classes of *Chlorella vulgaris*. Data are mean values from three experiments. *Incubations were performed with 1 ml of cell suspension, 1 nmol of [1-^{14}C] oleic acid and 0.2 μmol of exogenous fatty acid (oleic and thiaoleic) for 3 h at 25° under 15000 lux.

from the rest of the analogues was striking and we then explored this regioselective effect on the radioactivity distribution in lipids, as well as the biotransformation of this modified fatty acid.

[1-^{14}C] *oleic acid desaturation and radioactivity distribution into lipid classes in the presence of oleic acid or 13-thiaoleic acid*

Incubation assays were performed under the conditions described previously for the determination of desaturation rate (Fig. 2) in the presence of either exogenous oleic acid or 13-thiaoleic acid. Time-course of radioactivity incorporation in the lipid classes for both fatty acids were followed for 3 h (Fig. 3). When incubation was performed with cold oleic acid or 13-thiaoleic acid, [1-^{14}C] oleic acid was rapidly and mainly incorporated into diacylglyceride (DAG) and TAG (66% in presence of 18:1 and 57% in presence of the 13-thiaoleic acid after 30 min) and in a lower proportion into PL (respectively, 20% and 25%). This favoured storage of [1-^{14}C] oleic acid in TAG could be explained by activation of transfer to NL rather than PL, resulting from the large amount of exogenous substrate [18]. These results suggested that 13-thiaoleic acid could be recognised as a 18:1-like substrate by acyltransferases. During a 3 h incubation, the radioactivity increased in the PL (33% in presence of 18:1 and 38% in presence of 13-thiaoleic acid) and decreased in the NL (respectively, 51% and 45%). The other lipid classes (MGDG, DGDG and PG) were not affected by the presence of exogenous substrates and the radioactivity in every chloroplastic lipid remained low and unchanged during incubation. In both cases, incorporation of labelled substrate into PL was slowly regulated by the labelled oleic acid transfer from TAG to PC. Despite this good incorporation of labelled oleic acid into PL, the time-course of oleic acid desaturation in the presence of 13-thiaoleic acid demonstrated a specific inhibition of oleoyl desaturase activity (Fig. 4). By comparison with the control experiment, it appeared to be difficult to restore such desaturation activity in the case of 13-thiaoleic acid, which has a lasting influence. On the contrary, the decreased desaturation rate observed for the experiment conducted with exogenous oleic acid

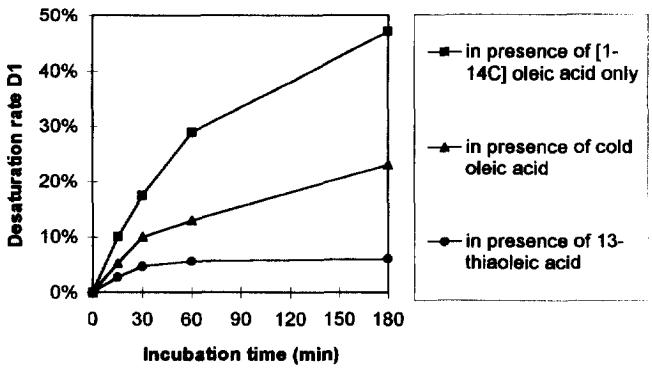


Fig. 4. Time-course* of [1-^{14}C] oleic acid desaturation in *Chlorella vulgaris* in the presence of exogenous fatty acids. * Time-course experiments were performed under the same conditions as described in Fig. 2.

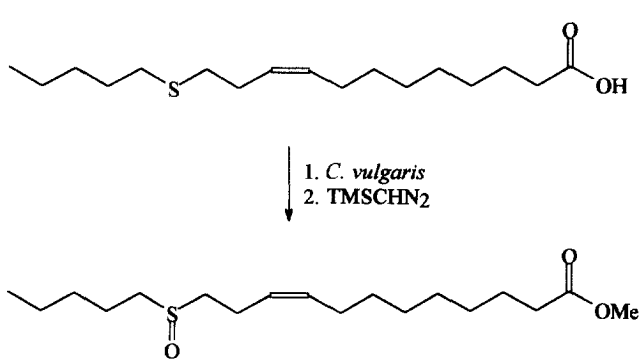
regularly increased after 1 h incubation, tending to control desaturation rate. Such disturbance of the oleoyl desaturase activity might have an effect on the fatty acid profile of phospholipids.

13-*Thiaoleic acid transformation*

In time-course experiments, 0.4 mg of 13-thiaoleic acid were incubated with 2 ml of cellular suspension of *C. vulgaris*. Crude homogenate (cellular fraction) was then separated from soluble fraction (supernatant) by centrifugation. The exogenous and endogenous fatty acids were extracted, methylated and separated by GC analysis for both cellular and supernatant fractions. Biotransformation of thiafatty acid proceeded quickly to give the corresponding *S*-oxide. After 10 min, 95% of 13-thiaoleic acid was incorporated into cells and 80% was oxidised to the corresponding sulfoxide, whereas 20% was unoxidised and located in the cells as free fatty acids and CoA-esters.

After 1 h incubation, only traces of thiafatty acid could be detected in cells, whereas a control experiment (0.4 mg of 13-thiaoleic acid incubated in 2 ml of 0.5 M phosphate buffer pH 7.4) showed that only 13% of the thiafatty acid was oxidised by atmospheric and chemical oxidation. This non-cellular oxidation increased to 30% of the total thiafatty acid after a 3 hr incubation period. It should be emphasised that these procedures were carried out with excellent percentage recoveries (97–98%). Moreover, we also checked that when we supplied the *S*-oxide as free fatty acid in the extracellular medium to be incubated, only 15% could be incorporated into the cells, even after a 3 h incubation time. This experiment clearly showed that at least 85% of the sulfoxide detected into the cells after 1 h comes from cellular biotransformation.

After 3 h incubation, no 13-thiaoleic acid was detected either in the cell fraction or in the supernatant. Also no sulfone was detected. The sulfoxide isolated from the bulk (93% of total sulfoxide was trapped in the cell, whereas 7% was free in the supernatant) 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). Moreover, the lipids separation by TLC (results not shown) indicated that 13-thiaoleic acid incorporated into the cells, was readily transformed to its CoA ester (50% after 3 min), confirming that this thiafatty acid competes with oleic acid for long-chain acyl-CoA synthetase. These results could be related to those of Bremer *et al.* [19], these authors demonstrating that long-chain 3-thiafatty acids were substrates for long-chain acyl-CoA synthetase in rat



Scheme 1.

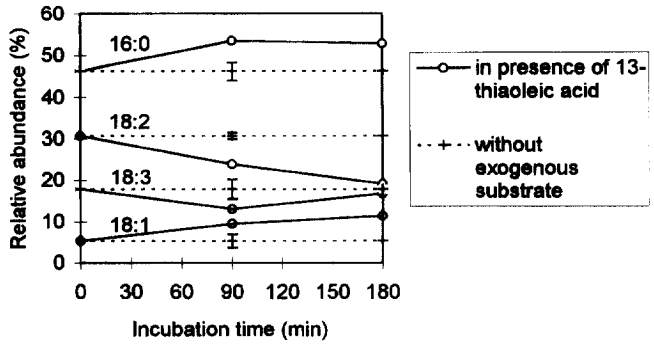


Fig. 5. Fatty acid composition of phospholipids* of *Chlorella vulgaris* in the presence of 13-thiaoleic acid. * Incubations were performed with 2 ml of cell suspension and 0.4 mg of 13-thiaoleic acid for 3 h at 25° under 25 000 lux. Lipids were separated by TLC. PL were transesterified and fatty acids analysed by GC.

liver microsomes. Moreover, a small part of 13-thiaoleic acid was detected after less than 1 h in PL, whereas 25% accumulated as free *S*-oxide fatty acid.

After 1 h incubation, the same experiment showed that the completely oxidised thiafatty acid was recovered mainly as free fatty acid in the cells (52.5%) and, to a lesser extent, esterified as 6.5% as CoA ester in the aqueous fraction, 4.3% in PL and 3.4% in NL. Whether the sulfoxide present in these different lipid classes was transferred from the 13-thiaoleoyl-CoA or from the *S*-oxide thiaoleoyl-CoA, was still not clear. Surprisingly, after a 3 h incubation time, we observed an increased amount of sulfoxide in PL, especially in PC (from 3 to 8%), whereas it appeared less abundant in NL (1–2%) and in the acyl-CoA fraction, suggesting that the *S*-oxide thiaoleate was transferred from the CoA fraction to the PL classes. However, Aarland and Berge [20] reported that such initial sulfoxidation of their thiafatty acids prevented activation to acyl CoA esters, as well as incorporation into complex lipids.

In conclusion, the above observations might suggest that oleoyl desaturase is partly and, to a limited extent, involved in the transformation of 13-thiaoleic acid into the corresponding sulfoxide in the PL classes.

Influence of 13-thiaoleic acid on endogenous fatty acid distribution in phospholipids

Fatty acid composition of PL of *C. vulgaris* was affected by incubation with 13-thiaoleic acid (Fig. 5). After a 3 h incubation, 18:1 was accumulated (from 5.3 to 11.4%), while 18:2 decreased significantly (from 30.7 to 19.1%). For the same experiment carried out without exogenous fatty acid, endogenous fatty acids abundances were constant. This result was consistent with the highly decreased desaturation rate observed when incubation was performed in the presence of 13-thiaoleic acid (Fig. 2), demonstrating an inhibitory effect of this compound on the desaturase complex.

In the present study, it has been shown that the 13-thiaoleic acid, supplied in the extracellular medium, is oxidised after a 3 h incubation by *C. vulgaris* whole cells, into the corresponding *S*-oxide, in a good yield (>85%). Moreover, in this type of experiment, desaturation of oleic acid decreased by 90% (compared with 50% in presence of exogenous oleic acid) and the distribution of fatty acids in PL showed an accumulation of endogenous oleic acid and a small proportion of *S*-oxide 13-thiaoleic acid in this lipid class. These results might suggest that the oleoyl desaturase could behave as an oxygenase in this oxidative pathway, as previously demonstrated for the stearoyl desaturase from *S. cerevisiae*, towards 9- and 10-thiastearic acids [14, 15]. However, it was clear that this partial oxidation could not be largely extended and that other oxidative enzymes must be responsible for biotransformation of 13-thiaoleic acid, such as FAD monoxygenase, which was supposed to be responsible for the oxidation of thiafatty acids in rat liver microsomes [21, 22]. In order to further explore the influence of sulphur atom position and the regiospecificity of the likely oleoyl desaturase substrate interaction, study of metabolism of 14-thiaoleic acid (which had a very weak effect on desaturation) is in progress.

EXPERIMENTAL

Culture conditions

Chlorella vulgaris (strain 211/8K) was purchased from CCAP (Cambridge, U.K.). Microalgae were maintained on nutrient agar at 20°. Cells were then used to inoculate 40 ml autoclaved culture medium [23] added to (gl^{-1}): glucose 5, $MgSO_4$, 7 H_2O 1, peptone 0.1 and yeast extract 0.1.

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 soln (gl^{-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 15000 lux. 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 fr. was washed ($\times 3$) 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}.

Incubation with labelled substrate

To 1 ml of the algal suspension previously described, 1 nmol of an EtOH soln of [1-^{14}C] oleic acid (sp. act. 55 mCi $mmol^{-1}$) was added and EtOH soln of modified fatty acids added to a final conc. of 0.20 mM. The reaction mixt. was stirred for 3 h at 25° and illuminated from above at 15000 lux.

Δ12-desaturase activity assays

The desaturation reaction was stopped by addition of 1 ml of 12% KOH in EtOH. After saponification at 70° for 30 min, 2 ml of 10% NaCl soln and 10 drops of conc. H_2SO_4 were added. Free fatty acids were extracted ($\times 3$) with 3 ml of Et_2O. Efficient decantation was achieved by centrifugation at 1500 g for 3 min. Solvent was evapd under N_2 and fatty acids methylated by addition of 1 ml of MeOH-H_2SO_4 (10:1). After reaction at 70° for 30 min, fatty acid Me esters were extracted with petrol and analysed by HPLC. Oleate and linoleate, resuspended in hexane, were separated by HPLC using; a 15×0.15 cm column of Nucleosyl C18, (Chrompack). A mixt. MeCN-H_2O was used as eluent with a gradient from 80:20 to 100:0 in 10 min, with a flow rate of 1 ml min^{-1}. Esters were detected by UV at 192 nm. Oleate and linoleate frs were collected and radioactivity determined by liquid scintillation counting.

Biotransformation assays

To 2 ml of cellular suspension previously described, an EtOH soln of 13-thiaoleic acid was added to a final conc. of 0.2 mg ml^{-1}. The reaction mixt. was stirred at 25° under 15000 lux illumination. For kinetic studies, lipids were fixed in boiling EtOH. Then, tubes were centrifuged at 1500 g for 10 min and supernatants and cellular frs were separated. Cellular frs containing total lipids were transesterified by addition of the MeOH-H_2SO_4 (2.5%). The reaction mixt. was stirred during 2 h at 70°. Fatty acid Me esters were extracted ($\times 3$) by 2.5 ml of petrol. Supernatants were acidified to pH 1–2 by addition of conc. H_2SO_4. Then, free fatty acids were extracted ($\times 3$) by 2.5 ml of Et_2O. Solvent was evapd under N_2 and endogenous and exogenous fatty acids methylated under mild conditions: 10 μl of MeOH, 75 μl of benzene and 25 μl of 0.2 M Me_3SiCHN_2 in hexane. Reaction mixts. were stirred under N_2 for 30 min at 20°. After addition of 3 ml of NaCl soln, fatty acid Me esters were extracted ($\times 3$) by 2.5 ml of petrol.

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 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. Inj. and det. heater temps were 250°. The split ratio used in the injection system was 7 ml min^{-1} and the injection vol. for all samples was 0.5 μl. For measuring amounts of fatty acid Me esters, 17:0 was added as int. standard before transmethylation.

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 mixt. was heated at 70° for 30 min. Then, 1 ml of H_2O and 1 ml of a 2M soln of NaCl in KPi buffer (pH 7.4) were added. After briskly stirring and decanting, the organic phase was collected. Total lipids were extracted ($\times 3$) by addition of 3 ml of $CHCl_3$. The solvent was then evapd under N_2.

Lipid separation

Lipids were separated by TLC. When cells were incubated with labelled substrate, the residue was resuspended in 100 μl of $CHCl_3$-MeOH (1:1). Exactly 10 μl of this lipid soln were 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-CH_3COOH-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. Spots were removed and dissolved into the liquid scintillation fluid for radioactivity measurements. When cells were incubated without labelled substrate, the residue was resuspended in 300 μl of $CHCl_3$-MeOH (1:1). The entire lipid soln was deposited on activated TLC plates. After detection with I_2 vapour, lipids were removed and transesterified by addition of 3 ml of MeOH-H_2SO_4 (2.5%). The reaction mixt. was stirred for 2 h at 70°. Fatty acid Me esters were extracted and analysed by capillary GC as described above.

Purification of metabolic products

At the end of the incubation period, supernatant and cellular frs were separated by centrifugation. Lipids were saponified and free fatty acids were then methylated as described above. Fatty acid Me esters were resuspended in hexane and analysed by HPLC.

The sulfoxide was separated from endogenous fatty acid Me esters by HPLC on a Hypersil BDS C18 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.

Methyl S-*oxide* 13-*thiaoleate*

CIMS (NH_3) 70 eV, *m/z* (rel. int.) 331 $[M+H]^+$ (100), 348 $[M\text{-}H_2O]^+$ (85), 365 $[M\text{-}NH_4\text{-}NH_3]^+$ (50). ^1H NMR (400 MHz, $CDCl_3$): δ 5.51 (1H, *m*, H-9), 5.38 (1H, *m*, H-10), 3.64 (*s*, 3H, H-19), 2.68 (4H, *m*, H-12 and H-14), 2.53 (2H, *dt*, $^3J = 7.6$, $^3J = 7.6$ 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).

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REFERENCES

1. Kader, J. C., Baillet, B., Grosbois, M., Galle, A. M., Hirsch, A. M., Jolliot, A. and Oursel, A., *Acta Botanica Gallica*, 1993, **140**, 735.
2. Mazliak, P., *Journal of Plant Physiology*, 1994, **143**, 399.
3. Roughan, P. G. and Slack, C. R., *Annual Review of Plant Physiology*, 1982, **33**, 97.
4. Browse, J. and Somerville, C., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1991, **42**, 467.
5. Falcone, D. L., Gibson, S., Lemieux, B. and Somerville, C., *Plant Physiology*, 1994, **106**, 1453.
6. Arondel, V., Lemieux, B., Hwang, I., Gibson, S., Goodman, H. M. and Somerville, C. R., *Science*, 1992, **258**, 1353.
7. Gombos, Z., Wada, H., Varkonyi, Z., Los, D. A. and Murata, N., *Biochimica et Biophysica Acta*, 1996, **1299**, 117.
8. Sakamoto, T., Wada, H., Nishida, I., Ohmori, M. and Murata, N., *Plant Molecular Biology*, 1994, **24**, 643.
9. Schmidt, H., Dresselhaus, T., Buck, F. and Heinz, E., *Plant Molecular Biology*, 1994, **26**, 631.
10. Norman, H. A. and St. John, J. B., *Plant Physiology*, 1986, **81**, 731.
11. Howling, D., Morris, L. J. Gurr, M. I. and James, A. T., *Biochimica et Biophysica Acta*, 1972, **260**, 10.
12. Poulain, S., Noiret, N., Nugier-Chauvin, C. and Patin, H., *Liebigs Annalen/Recueil*, 1997, 35.
13. Ferrante, G. and Kates, M., *Biochimica et Biophysica Acta*, 1986, **876**, 429.
14. Buist, P. H. and Marecak, D. M., *Journal of American Chemical Society*, 1992, **114**, 5073.
15. Buist, P. H. and Marecak, D. M., *Canadian Journal of Chemistry*, 1994, **72**, 176.
16. Appleby, R. S., Safford, R. and Nichols, B. W., *Biochimica et Biophysica Acta*, 1971, **248**, 205.
17. Harris, P. and James, A. T., *Biochimica et Biophysica Acta*, 1965, **106**, 456.
18. Grenier, G., Guyon, D., Roche, O., Dubertret, G. and Trémolières, A., *Plant Physiology and Biochemistry*, 1991, **29**, 429.
19. Wu, P. and Bremer, J., *Biochimica et Biophysica Acta*, 1994, **1215**, 87.
20. Aarsland, A. and Berge, R. K., *Biochemical Pharmacology*, 1991, **41**, 53.
21. Hvattum, E., Bergseth, S., Pedersen, C. N., Bremer, J. Aarsland, A. and Berge, R. K., *Biochemical. Pharmacology*, 1991, **41**, 945.
22. Skrede, S., Sorensen, H. N., Larsen, L. N., Steineger, H. H., Hovik, K., Spydevold, O. S., Horn, R. and Bremer, J., *Biochimica et Biophysica Acta*, 1997, **1344**, 115.
23. Sorokin, C. and Krauss, R. W., *Plant Physiology*, 1960, **33**, 109.