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Mechanistic characterization of ω-3 desaturation in the green alga *Chlorella vulgaris*

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Abstract

α-Linolenic acid (ALA, 9(*Z*),12(*Z*),15(*Z*)-octadecatrienoic acid) derivatives are important plant lipids which play a critical key role in cold tolerance. The final steps of ALA biosynthesis feature a series of regio- and stereoselective dehydrogenation reactions which are catalyzed by a set of enzymes known as fatty acid desaturases. In conjunction with ongoing research into the structural biology of these remarkable catalysts, we have examined the mechanism of double bond introduction at C15,16 as it occurs in a model photosynthetic organism, *Chlorella vulgaris*. The individual deuterium kinetic isotope effects associated with the C-H bond cleavages at C-15 and C-16 of a thialinoleoyl analogue were measured via competition experiments using appropriately deuterium-labelled 7-thia substrates. A large kinetic isotope effect (KIE) ($k_H/k_D = 10.2 \pm 2.8$) was observed for the C–H bond-breaking step at C-15 while the C–H bond cleavage at C-16 was found to be relatively insensitive to deuterium substitution ($k_H/k_D = 0.8 \pm 0.2$). These results point to C-15 as the site of initial oxidation in ω-3 desaturation and imply that the *Chlorella* and corresponding plant systems share a common active site architecture.

Keywords: *Chlorella vulgaris*; *Chlorophyceae*; Microalga; Kinetic isotope effects; Desaturase; Linolenate; Fatty acid

1. Introduction

The biosynthesis of polyunsaturated fatty acids such as linoleic **1** and α-linolenic **2** acid (Fig. 1) plays an essential role in the response of plants and other organisms to chilling temperatures (Iba, 2002; Wallis and Browse, 2002; Logue et al., 2000). The introduction of one or more *cis*-olefinic linkages into the hydrocarbon chains of cellular fatty acids imparts fluidizing properties to biomembranes. A large body of work supports the role of fatty acid desaturases as part of a feedback control system in which membrane fluidity is maintained near a certain level despite changes in ambient temperature (Nishida and Murata, 1996). This homeoviscosity appears to be important in photosynthetic organisms for optimal chloroplast function at low temperatures (Routaboul et al., 2000).

Recently, *Chlorella vulgaris* has been used as a convenient eukaryotic model to study the role of lipid modification in cold acclimatization (Suga et al., 2002). A low-temperature-inducible gene *CvFad2* encoding for linoleate **1** production (Δ^{12} desaturation) has been identified and expressed in *Saccharomyces cerevisae*. It was also found that a second gene termed *CvFAD3*, responsible for α-linolenate **2** formation (ω–3 desaturation)[1] is upregulated in response to cold stress. Interestingly, the

Abbreviations: FAME, Fatty acid methyl esters; FAD-2, plant oleate Δ^{12} desaturase; FAD-3, plant linoleate ω-3 desaturase; KIE, Kinetic isotope effect; Δ^x desaturase, Δ^x designates the position of the double bond introduced relative to the acyl terminus; ω-x desaturase, ω-x designates the position of the double bond introduced relative to the methyl terminus.

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[1] ω–3 desaturations are unusual in that the double bond is inserted using the terminal (ω) methyl group rather than the acyl headgroup of the substrate as a reference point. Thus for a C18 substrate, one obtains a 15,16-olefin.

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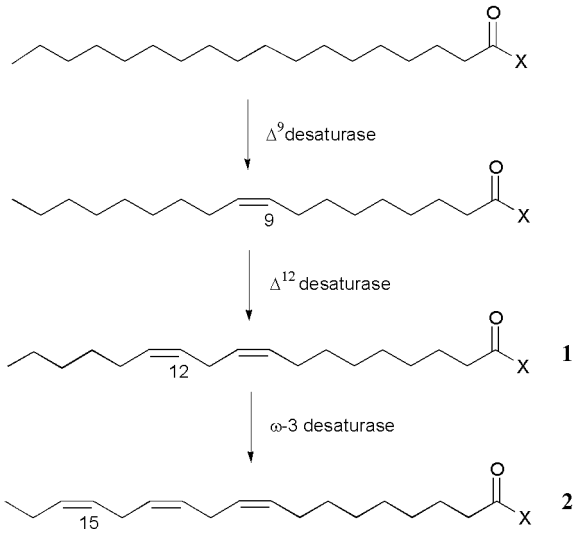

Fig. 1. Aerobic biosynthesis of the major C18 unsaturated fatty acids: oleate, 9(*Z*)-octadecenoate; linoleate **1**, 9(*Z*),12(*Z*)-octadecadienoate; linolenate **2** (9(*Z*)12(*Z*),15(*Z*)-octadecatrienoate) in plants. X denotes a thioester or phospholipid head group.

deduced amino acid sequence of each clone was highly similar to the corresponding microsomal plant protein: Δ^{12} *FAD2* and *ω-3 FAD 3* (66 and 60% respectively). These data strongly suggest that each pair of gene products is closely related at the structural level. A more detailed comparison in terms of 3-D motifs is currently not possible due to the difficulty in obtaining X-ray or NMR data for membrane-bound proteins. Clearly, other approaches are required.

As part of a comprehensive investigation into the bioorganic mechanism of fatty acid desaturation, we have introduced the use of various probes which allows detailed comparison of enzymes from different sources (Buist and Marecak, 1992; Buist and Behrouzian, 1996; Buist et al., 1996). The most versatile of these mechanistic tools is a measurement of the individual primary deuterium kinetic isotope effects associated with the dehydrogenation process (Buist and Behrouzian, 1996). To date, we have found that in the case of membrane-bound desaturases, only one of the C–H cleavage steps is subject to a large primary deuterium kinetic isotope effect. These observations are consistent with the currently accepted mechanistic model for desaturation (Behrouzian and Buist, 2002) (Fig. 2). That is, an initial, energetically difficult, and therefore kinetically important, hydrogen abstraction step generates a very short-lived, carbon-centered radical intermediate which collapses rapidly to give unsaturated product via a second hydrogen abstraction or a one electron oxidation/deprotonation sequence (not shown). Using this model, KIE measurements have been used to pinpoint the site of initial oxidation (crypto-regiochemistry) for over a dozen fatty acid desaturation reactions (Behrouzian and Buist, 2002). Corroborating evidence for the correctness of these crypto-regiochemical assignments is available in most cases: for example, varying amounts of hydroxylated byproducts (Broadwater et al., 2002) are produced at the site of initial oxidation presumably by an apparent competing hydroxyl transfer (S_H2) reaction (Fig. 2). The factor(s) controlling the ratio of desaturation/hydroxylation is currently an active area of research.

We have recently compared the mechanism of Δ^{12} desaturation as it occurs in *Chlorella* (Behrouzian et al., 2001) with that found for the corresponding enzyme found in the model plant *Arabidopsis thaliana* (Buist and Behrouzian, 1998). We have shown that for both the plant and algal systems, cleavage of the C–H bond at C-12 is subject to a large, intermolecular deuterium kinetic isotope effect ($k_H/k_D \sim 7$) whereas bond rupture at C-13 is insensitive to isotopic substitution ($k_H/k_D \sim 1$). These observations correlate well with those of Suga et al., *vide supra*, who have found a significant degree of amino acid sequence similarity between the plant and algal enzymes (Suga et al., 2002).

Given the paucity of mechanistic information currently available for the ω-3 desaturation process and its

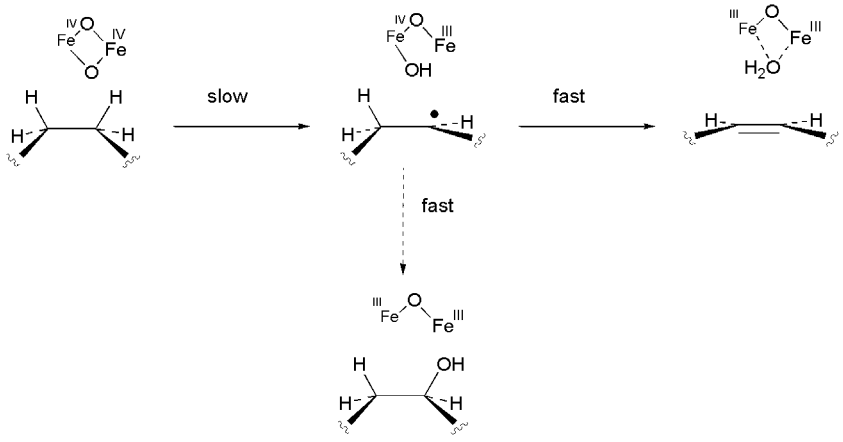

Fig. 2. Generalized mechanistic scheme for fatty acid desaturation showing stepwise removal of hydrogens and the relationship to corresponding hydroxylation reaction. The exact structure of the putative diiron oxidant remains to be determined.

unique mode of substrate recognition', it is of interest to conduct a detailed comparison of this reaction as it occurs in *Chlorella* and in higher plants. The KIE signature for the latter enzymes as represented by the extraplastidial ω-3 desaturase found in *Brassica napus* has recently been determined using a heterologous yeast expression system (Savile et al., 2001). The results clearly indicate that ω-3 desaturation of a C18 substrate proceeds in a stepwise fashion since only C–H bond cleavage at C-15 (ω-3 position where ω = 18) is subject to a significant deuterium KIE ($k_H/k_D = 7.5 \pm 0.4$ at C-15, $k_H/k_D = 1.0 \pm 0.14$ at C-16). In addition, the presence of trace 15-hydroxylinoleate in a linolenate-rich seed oil (flax) has been demonstrated (Broadwater et al., 2002), an observation which is consistent with our cryptoregiochemical analysis. The results of very early experiments suggested that the production of α-linolenate **2** in *Chlorella* also occurred in a stepwise manner but the methodology used was indirect and a definitive conclusion regarding the site of initial oxidation could not be reached (Morris et al., 1968). Herein, we report the results of experiments which clearly demonstrate that ω-3 desaturation as it occurs in *C. vulgaris* proceeds with a large KIE at C15 but not at C16.

2. Results and discussion

Our method of determining the intermolecular primary deuterium KIE on each C–H cleavage step in fatty acid desaturation has been described previously (Buist and Behrouzian, 1996, 1998). Briefly, our experimental approach involves incubating a mixture of the appropriate, regiospecifically dideuterated (–CD_2–) fatty acid analogue and the non-deuterated parent compound with a convenient source of the desaturase. The condition of low % conversion as required for these types of KIE measurements (Melander and Saunders, 1980) is typically fulfilled. Evaluation of the d_1/d_0 ratio in the olefinic products by mass spectral examination allows one to estimate the competitive primary deuterium KIE. [It should be noted that secondary deuterium KIE's may also be operating due to the presence of the spectator deuterium atom but this effect is expected to be small (< 10% of observed KIE) (Jones and Trager, 1987]. The competitive design of this approach is ideal for the study of intact membrane-bound desaturases—systems which have proven to be extremely difficult to purify to homogeneity. This technique and variations thereof has been used in several laboratories to extract valuable mechanistic information on microsomal desaturases (e.g. Abad et al., 2000; Meesapyodsuk et al., 2001; Beckmann et al., 2002).

In order to examine the *C. vulgaris* ω-3 desaturase, we needed to establish conditions which maximized the incorporation and desaturation of exogenous substrates. Thus a trial incubation involving the administration of methyl 7-thiastearate **3** (70 mg/l) to mid-logarithmic cells of *C. vulgaris* (*strain 211/8K*) was carried out. [We label substrates with sulfur as a methylene isostere to eliminate mass spectral interference by endogenous olefinic fatty acids. Experiments have shown that the introduction of a remote sulfur atom into the substrates does not bias the results of the KIE test (cf. Baenziger et al., 1990; Fauconnot and Buist, 2001).] Analysis of the cellular fatty acids as methyl esters by GC–MS (Fig. 3) revealed that reasonable incorporation of substrate was achieved under these conditions and the 7-thiastearoyl starting material **3** had been transformed into the desired 7-thialinolenoyl product **5** by Δ^9, Δ^{12} and ω-3 desaturations (Fig. 4). We found that it was important to carry out the incubations at 25 °C in order to maximize the amount of ω-3 desaturated product **5** formed. Trial experiments using a lower (17 °C) culture temperature resulted in poor uptake of thiastearate and at higher incubation temperature (30 °C) the ω-3 desaturation of the 7-thialinoleate intermediate **4** was found to be negligible.

The two regiospecifically dideuterated methyl 7-thiastearates, [15,15-$^2H_2$]-**3** and [16,16-$^2H_2$]-**3**, required for this study were available from previous work (Savile et al., 2001). Mixtures of nondeuterated substrate with each isotopomer were administered to growing cultures of *C. vulgaris* under conditions worked out in the trial experiments. After a further incubation period of ca. 48–60 h, the cells were harvested by centrifugation and the fatty acid fraction was isolated from the cell pellet via a hydrolysis/methylation sequence (see Section 3). In order to maximize the sensitivity of the mass spectrometric measurements, aliquots of the crude isolate were further fractionated by HPLC and the thia fatty acid fractions pooled and analyzed for isotopic content by GC–MS as described in the Section 3. The isotope ratios

Table 1
Isotopic ratios of mixtures of methyl 7-thiastearate **3**/[15,15-d_2]-methyl 7-thiastearate and methyl 7-thiastearate **3**/[16,16-d_2]-methyl 7-thiastearate and their Δ^9, $\Delta^{9,12}$ and $\Delta^{9,12,15}$ desaturated products

Isotopic ratios[a]				
Analyte	$d_0/15d_2$	$d_0/15d_1$	$d_0/16d_2$	$d_0/16d_1$
7*S*-18:0[b]	2.0±0.3		2.1±0.3	
7*S*-18:0[c]	1.7±0.3		1.8±0.3	
7*S*-(9*Z*)-18:1	1.6±0.3		1.7±0.3	
7*S*-(9*Z*,12*Z*)-18:2	1.5±0.3		1.7±0.3	
7*S*-(9*Z*,12*Z*,15*Z*)-18:3		20.4±5.0		1.6±0.3
KIE[d]		10.2±2.8		0.8±0.2

[a] Each value represents the mean of 2–4 mass spectrometric measurements. Each incubation was repeated twice.

[b] Isotopic ratio for the starting mixture of deuterated thiastearate with its nondeuterated analogue.

[c] Isotopic ratio of thiastearate found in the cellular extract.

[d] KIE (k_H/k_D) = [%d_0 (7*S* 18:3) /%d_1 (7*S* 18:3)]/ [%d_0 (7S-18:0)/ %d_2 (7*S*-18:0)].

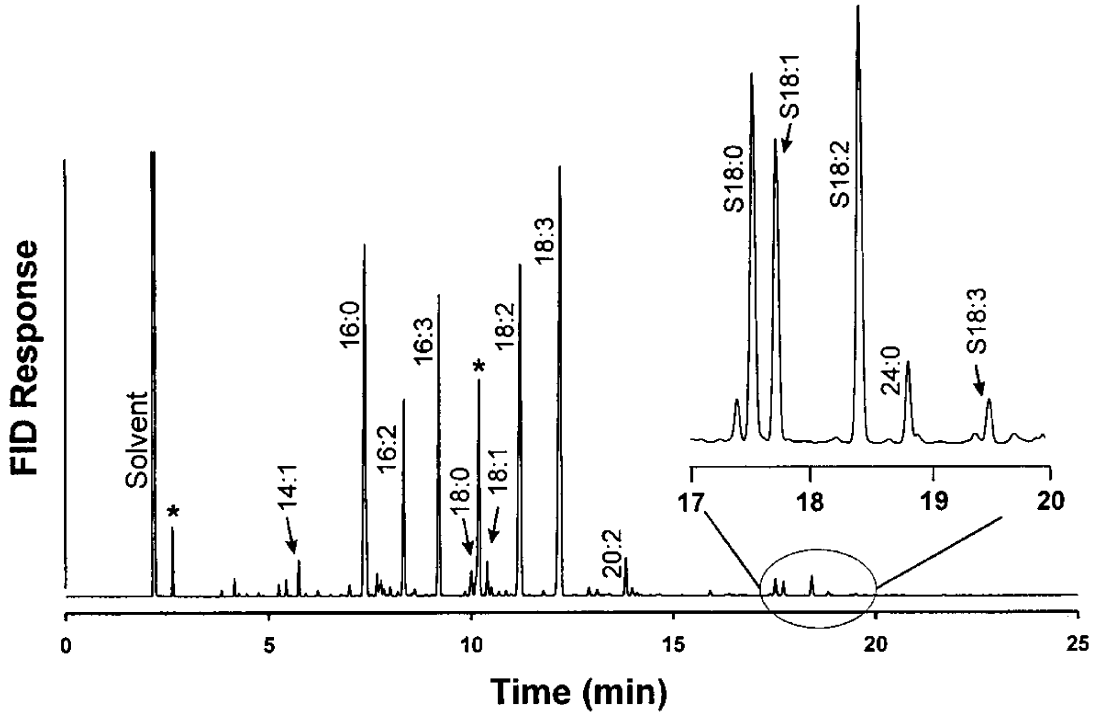

Fig. 3. Cellular fatty acid profile of *C. vulgaris* (*strain 211/8K*), grown in a nutrient medium (see Experimental) supplemented with methyl 7-thiastearate **3** (0.24–0.3 mM), as determined by GC–MS (DB-23 capillary column). 14:1 = methyl 9(*Z*)-tetradecenoate; 16:0 = methyl palmitate (hexadecanoate); 16:2 = methyl 7(*Z*),10(*Z*)-hexadecadienoate; 16:3 = methyl 7(*Z*),10(*Z*),13(*Z*)-hexadecatrienoate; 18:0 = methyl stearate (octadecanoate); 18:1 = methyl oleate (9(*Z*)-octadecenoate); 18:2 = methyl linoleate (9(*Z*),12(*Z*)-octadecadienoate); 18:3 = methyl linolenate (9(*Z*),12(*Z*), 15(*Z*)-octadecatrienoate); *S*18:0 = methyl 7-thiastearate **3**; S18:1 = methyl 7-thiaoleate; S18:2 = methyl 7-thialinoleate **6**; S18:3 = methyl 7-thialinolenate **7** (*denotes peaks of unknown identity).

of the thia analytes is given in Table 1. Product kinetic isotope effects (k_H/k_D) for the ω-3 desaturation reaction were calculated using the ratio: [% d_0 (product)/%d_1 (product)]/[%d_0 (substrate)/%d_2 (substrate)]. A large primary deuterium isotope effect (10.2±2.8) was found for the C–H bond cleavage at C-15 while the C16–H bond breaking step was shown to be comparatively insensitive to deuterium substitution (K.I.E. = 0.8±0.2).[2] The latter calculations were based on the assumption that the d_2/d_0 ratio of the intermediate 7-thiaoleate and 7-thialinoleates in this experiment was similar to that of the starting 7-thiastearoyl **3** mixture. Mass spectrometric examination of the appropriate fractions (S18:1, and S18:2, Fig. 3, Table 1) confirmed that this was the case within experimental error as expected—the position of the deuterium label being remote from the site of reaction.

This study represents the first mechanistic examination of a ω-3 desaturase operating in its native cellular environment. The markedly different KIEs we observe for C–H cleavage at C15 and at C16 (one large, one small) clearly support a stepwise mechanism for α-linolenate **2** formation as it occurs in *C. vulgaris*. Furthermore, according to our mechanistic paradigm (Fig. 2), the data indicate that ω-3 desaturation is initiated at C-15 rather than at C-16. This result is essentially identical to that obtained for the corresponding plant (*B. napus*) ω-3 desaturase (see Section 1) and suggests that the similarity between the *Chlorella* and plant enzymes extends beyond that of high amino acid sequence identity (Suga et al., 2002) to include a common active site architecture. This information can now be used to guide ongoing efforts aimed at constructing a more detailed topological model for the substrate binding site of ω-3 and related desaturases.

3. Experimental

3.1. Labelled substrates

Methyl 7-thiastearate **3** and the deuterated analogues were available from a previous study (Savile et al., 2001).

[2] Experimental errors on these KIE's are larger than those computed for Δ^9 and Δ^{12} desaturation in our previous experiments using *Chlorella* (Behrouzian et al., 2001) since the amount of 7-thialinolenate analyzed was substantially lower (0.08% of total FAME) than that of the precursor 7-thialinoleate and 7-thiaoleate (0.8 and 0.6% of total FAME respectively).

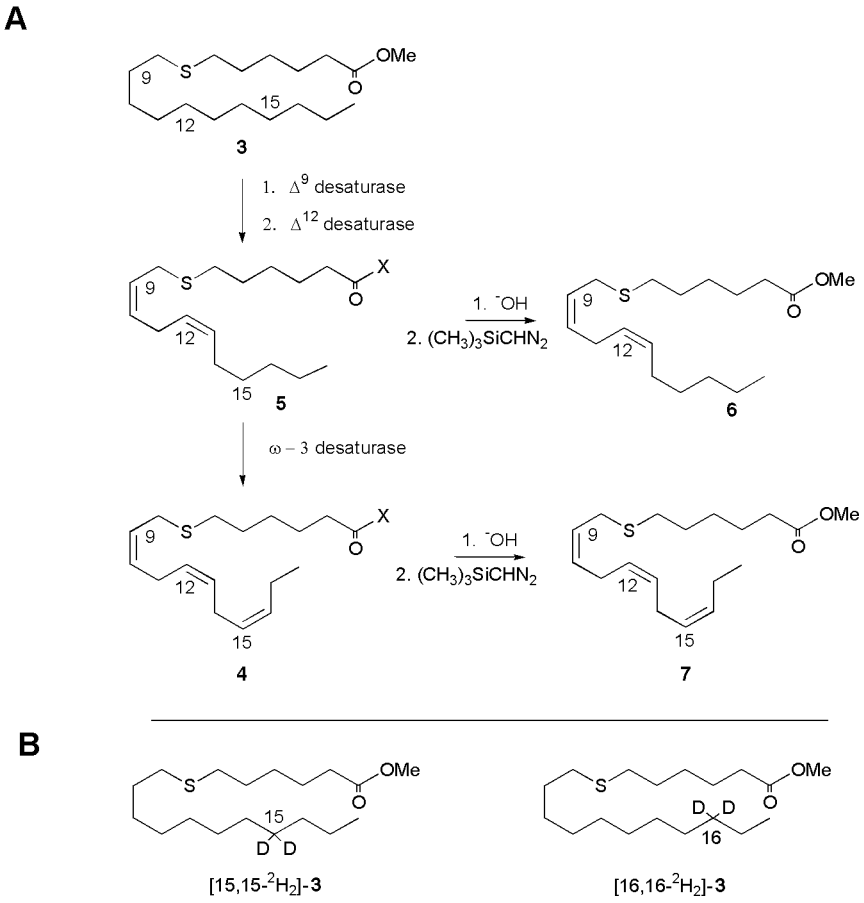

Fig. 4. (A) Consecutive Δ^9, $\Delta^{12,}$ Δ^{15} (ω-3) desaturation of methyl 7-thiastearate **3** in *C. vulgaris*. We assume that the thia-analogues are converted intracellularly to the appropriate acyl derivative (X) prior to desaturation. (B) Isotopomers of **3** used to probe the KIE effects for Δ^{15} (ω-3) desaturation.

3.2. Incubation experiments

C. vulgaris (*strain 211/8K*) was purchased from the Culture Collection of Algae and Protozoa (Cambridge, UK). The incubation of substrates with *Chlorella* and the subsequent isolation of product mixtures was carried out essentially as previously described (Behrouzian et al., 2001). Two adjustments to the experimental protocol were made: (1) Cultures were grown at 25 °C; and (2) the crude fatty acid extract containing products was fractionated by HPLC prior to GC–MS analysis. The latter step required removal of the non-saponifiable lipids which was accomplished by saponifying the crude FAME fraction (sealed tube with 5 ml 10% KOH, 5% H_2O in methanol at 80 °C for 2 h) followed by extraction with 3×3 ml hexane. The aqueous layer was then acidified to pH 2 with 50% HCl and extracted three times with hexane. The extract was taken to dryness under nitrogen and the free fatty acids were methylated in a sealed tube with 3 ml 10% H_2SO_4 in methanol at 60 °C for 30 min. The FAME were extracted into hexane, dried, redissolved in acetonitrile and fractionated by HPLC, the details of which have been previously described (Savile et al., 2001). The appropriate HPLC fractions containing thia fatty acids were pooled and evaporated prior to GC–MS analysis.

3.3. Mass spectrometric determination of substrate and product deuterium content

The GC–MS conditions which were used for the isotopic analysis of fatty acid methyl esters (FAME) from incubation experiments have been published previously (Savile et al., 2001). Deuterium content was determined using ion clusters at *m/z* 316, M^+ (methyl-7-thiastearate **3**); *m/z* 152, $(CH_3(CH_2)_6–CH=CHCH=CH_2)^+$ (methyl-7-thiaoleate); *m/z* 150, $(CH_3–(CH_2)_4–CH=CH–CH=CH–CH=CH_2)^+$ (methyl-7-thialinoleate **6**); *m/z* 148 $(CH_3–CH_2–CH=CH–CH_2–CH=CH–CH=CH–CH=CH_2)^+$ (methyl-7-thialinolenate **7**).

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