Cryptoregiochemistry of a *Brassica napus* fatty acid desaturase (*FAD3*): a kinetic isotope effect study

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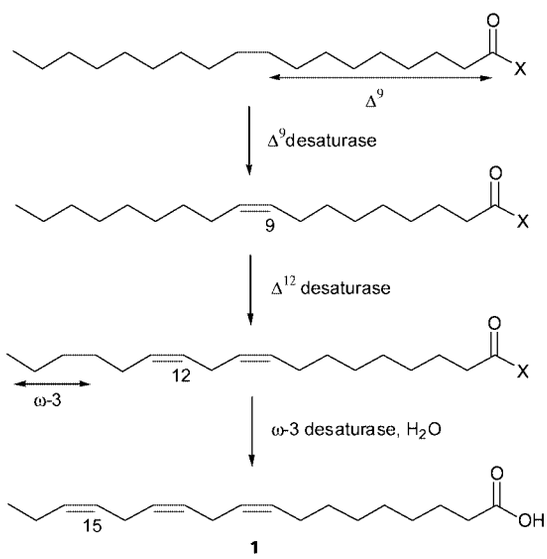
α-Linolenic acid ((*Z*,*Z*,*Z*)-octadeca-9,12,15-trienoic acid) is biosynthesized by a series of regio- and stereoselective dehydrogenation reactions which are catalyzed by a set of enzymes known as fatty acid desaturases. As part of ongoing research into the mechanism of these remarkable catalysts, we have examined the cryptoregiochemistry (site of initial oxidation) of extraplastidial ω − 3 desaturation as it occurs in the commercially important plant *Brassica napus* (oilseed rape or canola). The individual deuterium kinetic isotope effects associated with the C–H bond cleavages at C-15 and C-16 of a thiaoleoyl analogue were measured using a convenient *in vivo* yeast expression system. Competition experiments using appropriately deuterium-labelled 7-thia substrates revealed a large kinetic isotope effect (KIE) ($k_H/k_D = 7.5 \pm 0.4$) for the C–H bond-breaking step at C-15 while the C–H bond cleavage at C-16 was found to be insensitive to deuterium substitution ($k_H/k_D = 1.0 \pm 0.14$). These results point to C-15 as the site of initial oxidation in ω − 3 desaturation since the first chemical step in this type of reaction is rupture of a strong, unactivated C–H bond—an energetically difficult process which typically exhibits a large KIE.

Introduction

The search for biocatalysts which can activate C–H bonds in a selective manner remains an important scientific objective.[1] The use of soluble and membrane-bound forms of both the cytochromes P-450[2] and the emerging non-heme diiron family of enzymes[3] appears promising in this regard. A particularly impressive subset of the latter class includes the fatty acid desaturases which are capable of selectively dehydrogenating the non-activated positions of conformationally mobile substrates.[4] Some biotechnologically important examples of desaturase-mediated chemistry can be found in the biosynthetic pathway of the common plant fatty acids as shown in Scheme 1. The latter two reactions in this scheme occur in parallel on different lipid substrates within and outside the chloroplast or plastid. It is clear that the desaturation reaction is executed with exquisite chemo-, regio- and stereoselectivity. Importantly, these highly desirable properties are potentially tunable by protein engineering and recently, the first steps in this direction have been taken.[5a,b] Critical to the design and interpretation of such experiments is the identification of the methylene group which is attacked first during the introduction of the double bond. Three complementary methods for determining this parameter have been developed by the Buist group,[6a–c] based on the concept that desaturation represents a mechanistic variation[6d] of the more well-known hydroxylation reaction (Scheme 2). Our model features a highly reactive iron oxo species † which is asymmetrically located with respect to two methylene groups forced into a *gauche* ‡ conformation by the active site contours of the enzyme. Oxidation is initiated by an energetically difficult,



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Scheme 1

C–H activation step and is completed by rapid collapse of a short-lived radical "intermediate" or its organoiron equivalent (not shown) through loss of a second hydrogen to give an olefin.§ Hydroxylation would proceed by hydroxy rebound (S_H2) to give alcohol. Very recently,[9a] support for the intermediacy of a radical intermediate in the latter process as it occurs in the membrane-bound, non-heme diiron-containing ω-alkane hydroxylase[9b] has been obtained. According to our mechanistic scheme, the first step in desaturation reactions should be much more affected by isotopic substitution than the second as might

† We have chosen to use a generic iron oxo representation of the active oxidant although other structures are possible.[7]

‡ Occasionally, as in the case of insect pheromones, (*E*)-olefins are formed which presumably requires that the substrate adopt an *anti* conformation.[8] In all cases examined to date, desaturation takes place *via syn*-removal of adjacent hydrogens.[8]

§ A full discussion of other possible mechanisms for desaturation is found in ref. 6*d*. The precise nature of the switch controlling the olefin : alcohol product ratio remains a matter of speculation.

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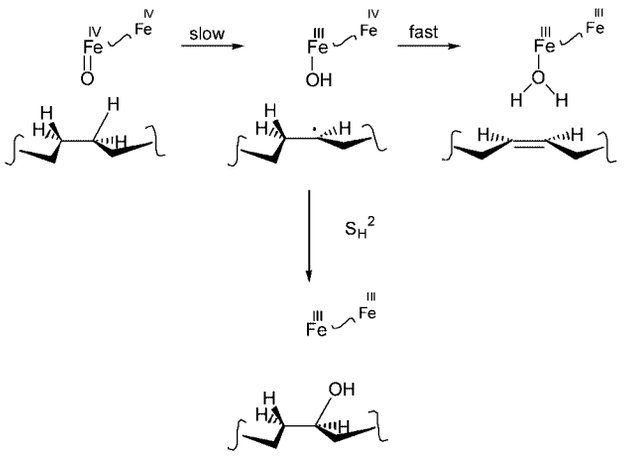


Scheme 2

be expected, *a priori*, for a disproportionation reaction.[10] Indeed, precisely such a pattern of kinetic isotope effects (one large, one negligible) has been found by ourselves and others for all membrane-bound desaturases examined to date[6b,6d,11a–g] and this information has been used to pinpoint the site of initial oxidation. In a number of cases, corroborating evidence is available to support the cryptoregiochemical assignment: that is, when a desaturase behaves as an oxygenase or *vice versa*, the regioselectivity of oxygenation matches that of the initial hydrogen abstraction step involved in desaturation.[5b,6a,6d,11e,11h] In addition, a strong primary deuterium KIE ($k_H/k_D = 7.8$) on the initial hydrogen abstraction step has been found in the case of the alkane ω-hydroxylase (*vide supra*).[9b] Finally, our observation of a strong α-fluoro effect[6c] at the predicted site of initial oxidation for a Δ^9 desaturase is indicative of hydrogen abstraction by a strongly electrophilic reagent such as the putative iron oxo species.

An interesting trend has emerged from these studies: in all cases examined to date, it appears that it is the C–H bond closest to the acyl terminus which is attacked first by the iron oxidant. Most of the desaturases which have been analyzed are of the Δ^x type; *i.e.* the double bond is introduced at a fixed distance from the carbonyl recognition point over a range of substrate chain lengths (*e.g.* Δ^9, Scheme 1). Recently, we have looked at the cryptoregiochemistry of a fatty acid desaturase from the nematode *Caenorhabditis C. elegans* (*FAT-1*) which inserts a double bond using the terminal methyl group as a reference point (ω − *x* type). The initial site of oxidation in this case was again found to be the carbon closest to C-1 as determined by our KIE methodology.[11f] This is an intriguing result since it suggests that two disparate subclasses of desaturases might share common cryptoregiochemistry. To test the generality of this phenomenon, we thought it would be useful to study the extraplastidial ω − 3 desaturase (*FAD3*) from plants—an enzyme which is involved in the biosynthesis of α-linolenic acid (ALA, (*Z*,*Z*,*Z*)-octadeca-9,12,15-trienoic acid) **1** (Scheme 1) in the endoplasmic reticulum. ALA plays an important role in plant[12] and animal[13] physiology and has found many applications in the coatings industry. Comparison of predicted amino acid sequences[14] suggests that the plant and nematode ω − 3 desaturases are two structurally divergent proteins, which were independently derived from Δ^{12} desaturases. Furthermore, the enzymes are thought to act on different lipid substrates—the plant extraplastidial enzyme utilizing phosphatidylcholine derivatives and the animal enzyme recognizing acyl-coenzyme A thioesters. Consequently, it is of interest to probe the cryptoregiochemistry of the plant desaturase and compare it to a corresponding enzyme from the animal kingdom. Covello *et al.*[15] have recently provided a convenient expression system for the *FAD3* desaturase from *Brassica napus*, thus offering a unique opportunity to determine the cryptoregiochemistry of this enzyme using the KIE approach. In this paper, we document the design, synthesis and desaturation of our cryptoregiochemical probes.¶

Results and discussion

Previous studies[6b,7,11f] have shown that baker's yeast, *Saccharomyces cerevisiae*, is an extremely convenient *in vivo* microbial system for studying the kinetic isotope effects associated with fatty acid desaturation. There are two principal reasons for this: firstly, cultures of this organism readily incorporate labelled exogenous fatty acid methyl esters and convert them to the appropriate substrate required by the desaturase under study. Secondly, *S. cerevisiae* has proven to be the eukaryotic host of choice for the heterologous expression of desaturases from a diverse array of sources.[11f,14,15] Our KIE method involves incubating an equimolar mixture of the appropriate regiospecifically dideuterated (–CD_2–) fatty acid analogue and the non-deuterated parent compound with cultures of a yeast strain bearing the appropriate desaturase gene. Evaluation of the d_1/d_0 ratio in the olefinic products by mass spectral examination allows one to compare the competitive deuterium kinetic isotope effect for each individual C–H cleavage involved in desaturation. We typically use deuterated substrates in which a remote methylene group is replaced by a sulfur atom in order to eliminate interference by endogenous olefinic fatty acids in the mass spectral analyses. (It is known that substitution of a C-7 methylene group with a sulfur atom in the substrate does not change the cryptoregiochemistry of double bond introduction[11d,e]). In general, our estimates of the KIE correlate well with those originally measured by Strittmatter using 9,9,10,10-d_4-stearoyl CoA and a highly purified, reconstituted rat liver Δ^9 desaturase system.[11i] *In vitro* measurements using a related sterol desaturase has revealed that the primary deuterium kinetic isotope effects on *V*/*K* and *V* are similar.[11g]

A preliminary experiment was carried out involving the administration of methyl 7-thiastearate **2**[16] (100 mg L^{-1}) to cultures (50 cm^3) of the pRS131/MKP-o strain of *S. cerevisiae*[15] incubated at 20 °C for 3 days for relatively rapid growth and then at 15 °C for a further 3 days to reach saturation at a temperature which has been found to give better substrate conversion rates. The cell cultures were harvested by centrifugation and the cellular fatty acids were isolated *via* a standard hydrolysis–methylation sequence.[15] Analysis of the fatty acids as methyl esters by GC-MS revealed a typical fatty acid profile for this organism (Fig. 1A) when it is grown in the presence of thia fatty acid analogues. We were pleased to observe that the 7-thiastearoyl starting material **2** had been transformed into the corresponding 7-thiaoleate **3** by the endogenous yeast Δ^9 desaturase, as expected,[16] and then desaturated further to a 9,15-dienoic material **4** by *FAD3* (Scheme 3) as determined by mass spectral analysis.[11f]¶ The amount of **4** could be significantly enriched by HPLC fractionation of the sample (Fig. 1B). Control experiments with the yeast strain MKP-o containing the pSE936 plasmid without the *fad3* gene indicated that the production of **4** was dependent on the presence of the *FAD3* enzyme.

The two regiospecifically dideuterated methyl 7-thiastearates ([15,15-$^2H_2$]-**2**, [16,16-$^2H_2$]-**2**) required for the KIE study were prepared *via* synthetic routes similar to that previously described[6d] in overall yields of 4% and 3% respectively (Scheme 4).

¶ This work was presented at the recent 51st Harden Conference on Fatty Acid Desaturases held in Wye, Kent, UK, 30 July to 2 August, 2000.

‖ *S. cerevisiae* does not possess a native Δ^{12} desaturase. It has been shown[11f,15] that the ω − 3 desaturase will operate on substrates which lack a (*Z*)-double bond at C-12,13.

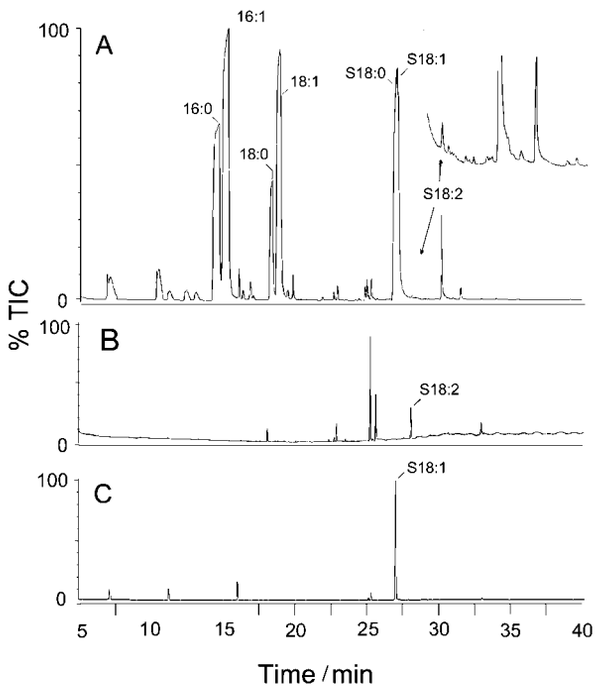
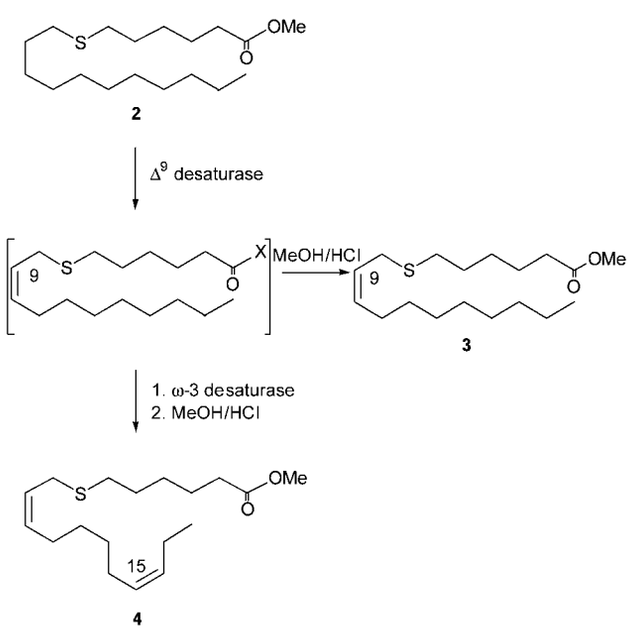
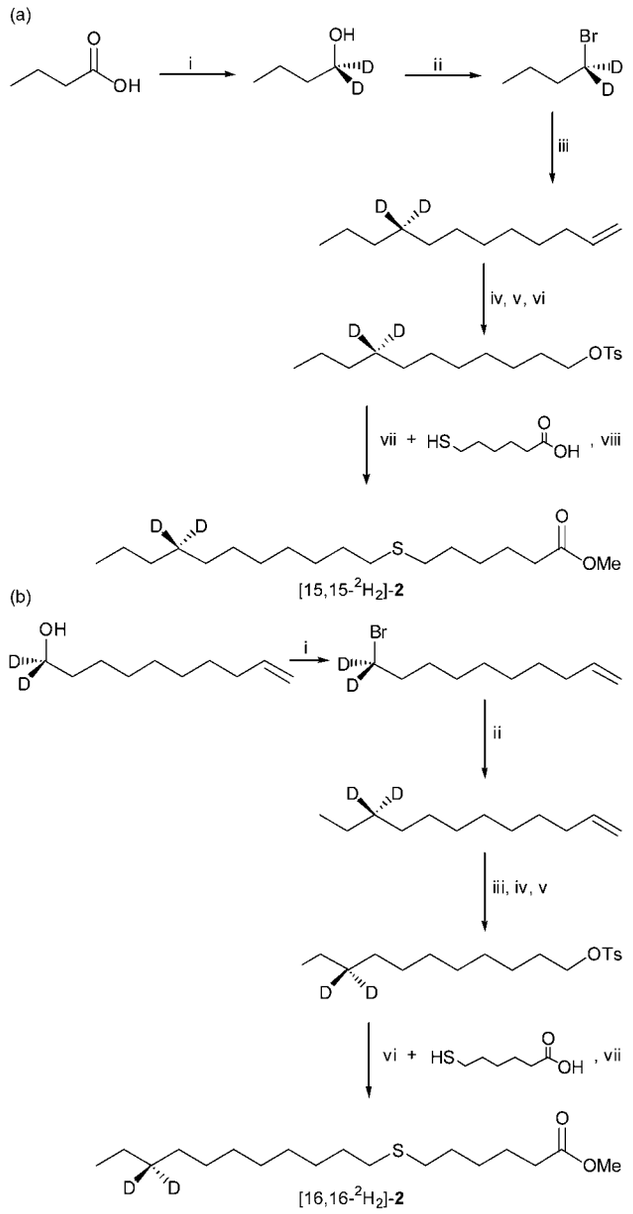


Fig. 1 (A) GC-MS analysis of FAME (fatty acid methyl esters), total extract derived from pRS131/MKP-o yeast cultures supplied with undeuterated methyl 7-thiastearate **2**. The thia starting material, monoenoic intermediate and the dienoic product are indicated as S18:0, S18:1 and S18:2, respectively. 16:0 = methyl palmitate (hexadecanoate); 16:1 = methyl palmitoleate ((*Z*)-hexadec-9-enoate); 18:0 = methyl stearate (octadecanoate); 18:1 = methyl oleate ((*Z*)-octadec-9-enoate). S18:1 appeared as an unresolved shoulder on the peak due to S18:0. (B) GC-MS analysis of HPLC fraction (reaction time = 4–5 min) containing S18:2. (C) GC-MS analysis of HPLC fraction (reaction time = 5–6 min) containing S18:1; See Experimental for culture conditions and instrumental parameters for GC-MS and HPLC.



Scheme 3

The key step in these syntheses is the Grignard coupling reaction between two alkyl bromides which depends on the presence of a Cu(I) catalyst for success.[17] The final products were purified by careful flash chromatography to remove small amounts of C-17 material produced by overoxidation in the $KMnO_4$-mediated alkene cleavage step.[18] GC-MS analysis of the final deuterated products revealed that each isotopomer consisted essentially entirely of dideuterated species.



Scheme 4 *Reagents and conditions*: (a) (i) $LiAlD_4$; (ii) HBr, H_2SO_4; (iii) 8-bromooct-1-ene, Mg, THF; (iv) $KMnO_4$, H^+; (v) $LiAlH_4$; (vi) TsCl, pyridine; (vii) *n*-BuLi (2 equiv.), THF; (viii) BF_3–MeOH. (b) (i) PBr_3; (ii) ethylmagnesium bromide, THF; (iii) $KMnO_4$, H^+; (iv) $LiAlH_4$; (v) TsCl, pyridine; (vi) *n*-BuLi (2 equiv.), THF; (vii) BF_3–MeOH.

A ~1 : 1 mixture of each deuterated material with its non-deuterated parent (5 mg) was administered to growing cultures (50 cm^3) of the *S. cerevisiae* transformant (pRS131/MKP-o) using conditions identical to that of the trial experiment. All incubations were carried out three times. The deuterium content of the olefinic fatty acid methyl esters in the cellular lipid extract was assessed by GC-MS as described in the Experimental section. As expected, the $d_2 : d_0$ ratio of the methyl 7-thiaoleate isotopomers (**3**, S18:1) was essentially identical to that of the starting material in both incubations, indicating that the condition for low conversion (<10%) which is required for these types of competitive KIE measurements has been met.[19] Indeed, as can be seen in Fig. 1, the amount of ω − 3-desaturated product (**4**, S18:2) is less than 1% of the thiaoleate (**3**, S18:1) fraction and thus no significant isotopic enrichment is observed for the latter compound (*vide infra*). Mass spectral analysis of the product of the second desaturation reaction (**4**, S18:2) revealed that in both incubations this material consisted entirely of a d_0–d_1 mixture as expected,

Table 1 Intermolecular kinetic isotope effects on the ω − 3 desaturation of 7-thiaoctadec-9-enoate catalyzed by *B. napus FAD3*[a]

FAME mixture supplied	Isotope effect
15,15-d_2S18:0Me–S18:0Me	7.5 ± 0.4
16,16-d_2S18:0Me–S18:0Me	1.0 ± 0.14

[a] Assays and intermolecular isotope effect calculations were performed as described under Experimental. Values are the means and standard deviations of separate calculations for 3 cultures.

indicating a loss of one deuterium from the d_2-substrate. Product kinetic isotope effects (k_H/k_D) were calculated using the ratio: [% d_0 (product)/% d_1 (product)] : [% d_0 (substrate)/% d_2 (substrate)] and this analysis (Table 1) indicated the presence of a large deuterium isotope effect (7.5 ± 0.4, average of 3 experiments) ** for the carbon–hydrogen bond cleavage at C-15 while the C16–H bond breaking step was shown to be essentially insensitive to deuterium substitution (KIE = 1.0 ± 0.14, average of 3 experiments). According to our mechanism (Scheme 2), these results demonstrate that the site of initial oxidation for ω − 3 desaturation is C-15 rather than C-16. This result echoes a similar finding obtained recently for an ω − 3 desaturase from the animal kingdom where the site of initial oxidation for the *C. elegans* desaturase was also determined to be at C-15 for a C18 substrate (k_H/k_D = 7.8 ± 0.4 (C-15), k_H/k_D = 0.99 ± 0.04 (C-16)). The common cryptoregiochemistry shared between these two enzymes points to similar active site motifs despite the differences in substrate acyl head group recognition (see Introduction). The precise details of these architectural elements will hopefully be unveiled when the 3-D structures of membrane-bound desaturases eventually become available.

Our data also have implications for protein engineering experiments aimed at converting desaturases into highly regioselective and enantioselective hydroxylators. Broun *et al.* [5b] have shown recently that a bifunctional Δ^{12} desaturase/12-hydroxylase can be tuned in the direction of dehydrogenation or hydroxylation by the exchange of only 6 protein residues. We had previously shown [6d] using our KIE methodology that a related plant Δ^{12} desaturase initiates oxidation at C-12 which implies that the protein switch controlling chemoselectivity probably operates after a common initial first step (Scheme 2). On the basis of the results reported in this paper, one would then predict that our ω − 3 desaturase could potentially be engineered to give a chiral 15-hydroxylated product from a C_{18} substrate.

In summary, we have successfully uncovered the cryptic site of initial oxidation for an ω − 3 desaturase which introduces the third double bond of a central plant lipid—α-linolenic acid **1**. The success rate of our KIE approach in elucidating the cryptoregiochemistry of membrane-bound desaturases remains 100%.

Experimental

General

All ^{1}H and ^{13}C NMR spectra were obtained at 200 and 50 MHz respectively on a Varian XL 200 spectrometer using dilute $CDCl_3$ solutions. Chemical shifts are expressed in parts per million (ppm) and are referenced to TMS. *J*-Values are in Hz. Deuterium isotope effects on ^{13}C NMR shifts were estimated by running spectra of mixtures (1 : 2) of labelled and unlabelled material. Mass spectra of substrates were obtained by GC-MS using a Kratos Concept 1H mass spectrometer equipped with a 70 eV EI ionization chamber. Samples were introduced using an HP 5980 Series 2 gas chromatograph equipped with a J. & W. 30 m × 0.21 mm, DB-5 capillary column. GC-MS analyses of fatty acid methyl esters (FAME) from incubation experiments were performed using a Fisons VG TRIO 2000 mass spectrometer (VG Analytical UK) controlled by Masslynx version 2.0 software, coupled to a GC 8000 Series gas chromatograph equipped with a 30 m × 0.253 mm DB-23 (0.25 μm film thickness) fused silica column (temperature program isothermal 200 °C for 1 min, gradient 10 °C min^{-1} to 240 °C and then isothermal at 240 °C for 25 min). Deuterium content was measured using selected ion monitoring in EI mode (70 eV) of pertinent ion clusters with a cycle time of 0.1 s per channel (4 channels for methyl 7-thiaoleate and 5 channels for methyl 7-thiaisolinoleate) corresponding to *ca*. 20–25 scans per peak. The integrated intensities of the individual ions were corrected for natural isotopic abundance and the isotopic ratios were determined using these corrected intensities for the ions: *m/z* 316, M^+ (methyl 7-thiastearate); *m/z* 152, $(CH_3(CH_2)_6CH{=}CHCH{=}CH_2)^+$ (methyl 7-thiaoleate); *m/z* 150, $(CH_3{-}CH_2{-}CH{=}CH{-}(CH_2)_3{-}CH{=}CH{-}CH{=}CH_2)^+$ (methyl 7-thiaisolinoleate). Flash chromatography using silica gel (230–400 mesh) was used to purify substrates. Analytical TLC was performed using Merck glass plates pre-coated with silica G/UV 254. Visualization of UV-inactive materials was accomplished by using a combination of I_2 vapour followed by a water spray.

Materials

Unless otherwise stated, all reagents and starting materials were purchased from Aldrich Chemical Co. and used without purification. Tetrahydrofuran (THF) and diethyl ether (Et_2O) were freshly distilled from Na and LAH, respectively. All air- and moisture-sensitive reactions were performed under N_2. Organic extracts were typically shaken with saturated NaCl and dried over Na_2SO_4, and solvents were evaporated on a rotary evaporator. Methyl 7-thiastearate **2** was prepared as previously described.[16]

Synthesis of deuterated intermediates

[1,1-$^2H_2$]Butan-1-ol. Butanoic acid (4.85 g, 55 mmol) was added under N_2 to a slurry of $LiAlD_4$ (2.51 g, 60 mmol) in Et_2O (25 cm^3) at 0 °C over 30 minutes. After stirring at RT for 4 h, the reaction was quenched with the dropwise addition of H_2O (10 cm^3) followed by HCl (3 M, 20 cm^3) and extracted with Et_2O (2 × 25 cm^3). The combined organic layers were washed with HCl (3 M, 30 cm^3), dried and evaporated to yield the title compound as a colourless liquid (3.14 g, 75%). For the unlabelled material: δ_H 0.91 (3H, t, *J* 6.5, C*H*$_3$), 1.36 (2H, sextet, *J* 7.6, $CH_3CH_2CH_2$), 1.51 (2H, quintet, *J* 7.5, CH_2CH_2OH), 3.63 (2H, t, *J* 6.5, CH_2OH); δ_C[22] 13.75 (4-C), 18.83 (3-C), 34.66 (2-C), 62.23 (1-C). The title compound showed similar spectral characteristics except for δ_H 1.47 (2H, br t, *J* 7.4, CH_2CD_2OH), 3.63 (absent); δ_C 18.78 (3-C, upfield γ-isotope shift (0.05 ppm)), 34.45 (2-C, upfield β-isotope shift (0.21 ppm)), 62.23 (1-C, absent).

[1,1-$^2H_2$]-1-Bromobutane. [1,1-$^2H_2$]Butan-1-ol (3.08 g, 34 mmol) was added to a solution of HBr (48%, 7.5 g) and conc. H_2SO_4 (1.2 cm^3) followed by additional conc. H_2SO_4 (1.0 cm^3). The solution was refluxed under N_2 for 3 h. Distillation directly from the reaction vessel gave the title compound as a colourless liquid (3.35 g, 71%). For the unlabelled material: δ_H 0.94 (3H, t, *J* 7.5, C*H*$_3$), 1.50 (2H, sextet, *J* 7.0, $CH_3CH_2CH_2$), 1.83 (2H, quintet, *J* 7.3, CH_2CH_2Br), 3.40 (2H, t, *J* 6.8, CH_2Br); δ_C[22,23] 13.17 (4-C), 21.30 (3-C), 33.66 (1-C), 34.76 (2-C). The title compound showed similar spectral characteristics except for

** As has been pointed out on a previous occasion,[6b] the magnitude of the large primary deuterium KIE such as that observed for C-15–H cleavage must be regarded as an estimate since the observed value may incorporate a small (<10%) α-secondary isotope effect.[20] In addition, partial masking of the "intrinsic" KIE by other enzymic steps in the catalytic cycle such as substrate binding may also be occurring.[21] None of these considerations affect the conclusions reached in this paper.

δ_H 1.81 (2H, br t, *J* 7.8, CH_2CD$_2$Br), 3.40 (absent); δ_C 21.25 (3-C, upfield γ-isotope shift (0.05 ppm)), 33.66 (1-C, absent), 34.52 (2-C, upfield β-isotope shift (0.24 ppm)).

[1,1-$^2H_2$]Dec-9-en-1-ol. Dec-9-enoic acid (prepared by Jones oxidation[24] of the commercially available dec-9-en-1-ol) (11.78 g, 69 mmol) was reduced with $LiAlD_4$, as described above, to give [1,1-$^2H_2$]dec-9-en-1-ol as a colourless liquid (9.62 g, 88%). For the known[25] unlabelled material: δ_H 1.28 (10H, br s, CH$_2$(CH_2)$_5$CH$_2$), 1.54 (2H, quintet, *J* 6.8, CH_2CH$_2$OH), 2.02 (2H, m, CH$_2$=CHCH_2), 3.62 (2H, t, *J* 6.4, CH_2OH), 4.95 (2H, m, CH_2=CH), 5.80 (1H, m, CH$_2$=C*H*); δ_C 25.71 (3-C), 28.85, 29.03, 29.36, 29.42, 32.66 (2-C), 33.75 (8-C), 62.71 (1-C), 114.08 (10-C), 139.09 (9-C). The title compound showed similar spectral characteristics except for δ_H 1.53 (2H, br t, CH_2CD$_2$OH), 3.62 (absent); δ_C 25.66 (3-C, upfield γ-isotope shift (0.05 ppm)), 32.45 (2-C, upfield β-isotope shift (0.21 ppm)), 62.71 (1-C, absent).

[1,1-$^2H_2$]-1-Bromodec-9-ene. PBr_3 (9.98 g, 36 mmol) was added to a solution of [1,1-$^2H_2$]dec-9-en-1-ol (8.24 g, 52 mmol) in anhydrous Et_2O (35 cm^3) at 0 °C under N_2. After reflux (2.5 h), the product was cooled and quenched with H_2O (10 cm^3). The organic layer was separated and the aqueous layer was extracted with Et_2O (2 × 20 cm^3). The combined organic extracts were washed, dried (Na_2SO_4), evaporated and purified by chromatography on silica, with hexanes as eluent, to give [10,10-$^2H_2$]-1-bromodec-9-ene as a colourless liquid (7.47 g, 65%). For the known[26] unlabelled material: δ_H 1.30 (10H, br s, CH$_2$(CH_2)$_5$CH$_2$), 1.82 (2H, quintet, *J* 7.0, CH_2CH$_2$Br), 2.02 (2H, m, CH$_2$=CHCH_2), 3.41 (2H, t, *J* 7.0, CH_2Br), 4.95 (2H, m, CH_2=CH), 5.80 (1H, m, CH$_2$=C*H*); δ_C 28.16 (3-C), 28.72, 28.86, 29.01, 29.28, 32.82 (2-C), 33.78 (8-C), 34.00 (1-C), 114.18 (10-C), 139.08 (9-C). The title compound showed similar spectral characteristics except for δ_H 1.81 (2H, br t, *J* 7.1, CH_2-CD$_2$Br), 3.41 (absent); δ_C 28.11 (3-C, upfield γ-isotope shift (0.05 ppm)), 32.58 (2-C, upfield β-isotope shift (0.24 ppm)), 34.00 (1-C, absent).

Synthesis of substrates

The title compounds were prepared using a sequence of well-established synthetic procedures.

Methyl [15,15-$^2H_2$]-7-thiastearate (7-thiaoctadecanoate) [15,15-$^2H_2$]-2. Oct-1-enylmagnesium bromide was prepared by addition of 8-bromooct-1-ene (5.92 g, 31 mmol) to a mixture of magnesium (0.76 g, 31 mmol) and I_2 (100 mg) in THF (30 cm^3) under N_2. After an exothermic reaction had subsided, the Grignard reagent was added, *via* syringe, to a solution of [1,1-$^2H_2$]-1-bromobutane (4.24 g, 31 mmol) in THF (25 cm^3) at –10 °C followed by 1.7 cm^3 of a freshly prepared dilithium tetrachlorocuprate solution (LiCl (0.21 g, 0.5 mmol) and $CuCl_2$ (0.34 g, 0.25 mmol) in THF (25 cm^3)). The reaction was stirred for 3 h at 0 °C and then quenched with ice (25 g) and extracted with Et_2O (3 × 25 cm^3). The combined organic layers were washed with NH_4OH (2 × 25 cm^3), $Na_2S_2O_3$ (1 × 25 cm^3), H_2O (2 × 25 cm^3), dried over Na_2SO_4 and evaporated to give [9,9-$^2H_2$]dodec-1-ene as a colourless liquid (4.01 g, 77% est.). For the unlabelled compound: δ_H 0.87 (3H, t, *J* 6.7, CH_3), 1.25 (16H, br s, CH$_3$(CH_2)$_8$-CH$_2$), 2.02 (2H, m, CH_2CH=CH$_2$), 4.95 (2H, m, CH=CH_2), 5.80 (1H, m, C*H*=CH$_2$); δ_C[27] 14.17 (12-C), 22.78 (11-C), 29.06, 29.27, 29.46, 29.63, 29.73, 29.74 (8-C), 32.03 (10-C), 33.93 (3-C), 114.12 (1-C), 139.25 (2-C); *m*/*z* 168 (M$^+$). The labelled material showed similar spectral characteristics except for δ_C 22.74 (11-C, upfield γ-isotope shift (0.04 ppm)), 29.53 (8-C, upfield β-isotope shift (0.21 ppm)), 31.82 (10-C, upfield β-isotope shift (0.21 ppm)); *m*/*z* 170 (M$^+$). This material was contaminated with a small amount (*ca.* 10%) of the homo-coupled product, hexadeca-1,15-diene, as judged by GC-MS. This intermediate was used without further purification.

Crude [9,9-$^2H_2$]dodec-1-ene (3.53 g, 21 mmol) was oxidized with acidic $KMnO_4$, as previously described,[18] to give the chain-shortened carboxylic acid [8,8-$^2H_2$]undecanoic acid (3.57 g, 90% est.) as a white solid. This material was reduced with LAH (see above) to give the corresponding alcohol, [8,8-$^2H_2$]-undecan-1-ol (2.24 g, 68% est. yield) and subsequently treated with tosyl chloride to give a low melting white solid, [8,8-$^2H_2$]-1-(tolyl-*p*-sulfonyloxy)undecane (2.91 g, 70% est. yield). For the known[28] unlabelled compound: δ_H 0.87 (3H, t, *J* 6.0, CH_3), 1.20 (16H, br s, CH$_3$(CH_2)$_8$CH$_2$), 1.80 (2H, quintet, *J* 6.6, CH_2-CH$_2$OTs), 2.43 (3H, s, Ar-CH_3), 4.00 (2H, t, *J* 6.6, CH_2OTs), 7.32 (2H, d, Ar*H*), 7.77 (2H, d, Ar*H*); δ_C 14.06 (11-C), 21.55 (Ar-CH$_3$), 22.62 (10-C), 25.25 (2-C), 28.73, 28.85, 29.25 (8-C), 29.33, 29.43, 29.50, 31.83 (9-C), 70.66 (1-C), 127.79, 129.75, 133.10, 144.58. The labelled tosylate showed similar spectral characteristics except for δ_C 22.57 (10-C, upfield γ-isotope shift (0.05 ppm)), 29.25 (8-C, absent), 31.62 (9-C, upfield β-isotope shift (0.21 ppm)).

A 1.6 M *n*-butyllithium (12.5 cm^3) reagent was added, *via* syringe, to a solution of 6-mercaptohexanoic acid (1.36 g, 9.2 mmol) in 3 : 1 THF–HMPA (28 cm^3) under N_2 at 0 °C. After stirring at 0 °C for 30 min, a solution of [8,8-$^2H_2$]-1-(tolyl-*p*-sulfonyloxy)undecane (2.79 g, 8.5 mmol) in THF (3 cm^3) was added *via* syringe and stirred at RT for 24 h. The reaction was quenched with the addition of H_2O (30 cm^3), acidified to a pH of 2 with 3 M HCl (15 cm^3) and extracted with hexanes (4 × 30 cm^3). The combined organics were washed, dried (Na_2SO_4) and evaporated to give crude [15,15-$^2H_2$]-7-thiastearic acid (2.14 g, 82% est.) as a white solid. This compound was methylated with BF_3–MeOH (pre-prepared reagent) and purified by flash chromatography using 4% EtOAc–hexanes to give methyl [15,15-$^2H_2$]-7-thiastearate, [15,15-$^2H_2$]-**2**[6d] (580 mg, 26%) as a colourless oil which solidified at 20 °C. The physical and spectral data of the title compound were similar to those of known unlabelled **2**[6d] except for δ_C 22.66 (17-C, upfield γ-isotope shift (0.05 ppm)), 29.32 (15-C, absent), 29.43 (14-C, upfield β-isotope shift (0.21 ppm)), 31.77 (16-C, upfield β-isotope shift (0.21 ppm)); *m*/*z* 318 (M$^+$), 287 ((M − OCH$_3$)$^+$), 189 ((CH$_3$(CH$_2$)$_2$-CD$_2$(CH$_2$)$_7$S)$^+$), 129 ((M − CH$_3$(CH$_2$)$_2$CD$_2$(CH$_2$)$_7$S)$^+$).

Methyl [16,16-$^2H_2$]-7-thiastearate (7-thiaoctadecanoate) [16,16-$^2H_2$]-2. A solution of [1,1-$^2H_2$]-1-bromodec-9-ene (6.98 g, 32 mmol) in THF (5 cm^3) was added to 1.0 M ethylmagnesium bromide (50 cm^3) followed by 2.0 cm^3 of a freshly prepared dilithium tetrachlorocuprate solution (LiCl (0.21 g, 0.5 mmol) and $CuCl_2$ (0.34 g, 0.25 mmol) in THF (25 cm^3)). The reaction was stirred for 3 h at 0 °C and then quenched with ice (25 g) and extracted with Et_2O (3 × 25 cm^3). The combined organic layers were washed with NH_4OH (2 × 25 cm^3), $Na_2S_2O_3$ (1 × 25 cm^3), H_2O (2 × 25 cm^3), dried over Na_2SO_4 and evaporated to give [10,10-$^2H_2$]dodec-1-ene as a colourless liquid (4.25 g, 78% est.). The labelled material showed similar spectral characteristics to those described above for [9,9-$^2H_2$]dodec-1-ene except for δ_C 14.12 (12-C, upfield γ-isotope shift (0.05 ppm)), 22.57 (11-C, upfield β-isotope shift (0.21 ppm)), 32.01 (10-C, absent). This material was contaminated with a small amount (*ca.* 20%) of the starting compound, [1,1-$^2H_2$]-1-bromodec-9-ene, as judged by GC-MS. This intermediate was used without further purification.

Crude [10,10-$^2H_2$]dodec-1-ene (4.08 g, 24 mmol) was oxidized with acidic $KMnO_4$, as previously described,[18] to give the chain-shortened carboxylic acid [9,9-$^2H_2$]undecanoic acid (4.25 g, 94% est.) as a white solid. This material was reduced with $LiAlH_4$ (see above) to give the corresponding alcohol [9,9-$^2H_2$]undecan-1-ol (2.43 g, 61% est.) and subsequently treated with tosyl chloride to give a low melting white solid, [9,9-$^2H_2$]-1-(tolyl-*p*-sulfonyloxy)undecane (2.49 g, 57% est.). The labelled material showed similar spectral characteristics to

those described above for [8,8-$^2H_2$]-1-(tolyl-*p*-sulfonyloxy)-undecane except for δ_C 14.01 (11-C, upfield γ-isotope shift (0.05 ppm)), 22.41 (10-C, upfield β-isotope shift (0.21 ppm)), 29.04 (8-C, upfield β-isotope shift (0.21 ppm)), 31.83 (9-C, absent).

[9,9-$^2H_2$]-1-(Tolyl-*p*-sulfonyloxy)undecane (2.32 g, 7 mmol) was reacted with 6-mercaptohexanoic acid (1.18 g, 8 mmol), as described above, to give crude [16,16-$^2H_2$]-7-thiastearic acid (1.78 g, 85% est.) as a white solid. This compound was methylated with BF_3–MeOH (pre-prepared reagent) and purified by flash chromatography using 4% EtOAc–hexanes to give methyl [16,16-$^2H_2$]-7-thiastearate, [16,16-$^2H_2$]-**2** (466 mg, 1.5 mmol, 25%) as a colourless oil which solidified at 20 °C. The physical and spectral data of the title compound were similar to those of known unlabelled **2**[6d] except for δ_C 14.07 (18-C, upfield γ-isotope shift (0.05 ppm)), 22.50 (17-C, upfield β-isotope shift (0.21 ppm)), 29.16 (15-C, upfield β-isotope shift (0.21 ppm)), 31.98 (16-C, absent); *m*/*z* 318 (M^+), 287 ($(M - OCH_3)^+$), 189 ($(CH_3CH_2CD_2(CH_2)_8S)^+$), 129 ($(M - CH_3CH_2CD_2(CH_2)_8S)^+$).

Incubation experiments using *S. cerevisiae*

To determine the cryptoregiochemistry of the *FAD3* desaturase, the pRS131/MKP-o strain of *S. cerevisiae* containing a plasmid with the *B. napus fad3* gene under the control of the yeast *GAL 1* promoter[15] was used. Cultures were grown in minimal medium lacking uracil and containing 2% galactose (CM gal-ura) supplemented with 1 : 1 mixtures of **2** : [15,15-$^2H_2$]-**2** or **2** : [16,16-$^2H_2$]-**2** (100 mg L^{-1}) and Tergitol (Type NP-40, 0.1% [v/v]) at 20 °C for 3 days followed by 15 °C for 3 days. The plasmid vector pSE936 in the yeast strain MKP-o was used as a control.

Fatty acid methyl esters (FAME) were prepared from yeast cultures using methanolic HCl as described previously.[15] To facilitate GC-MS analysis in the isotope effect experiments, the FAME were fractionated on a Hewlett Packard 1100 Series HPLC system (Hewlett Packard, Palo Alto, USA) equipped with an auto-sampler, solvent degasser, quaternary pump, column heater and diode array detector (monitoring at 205 and 254 nm), which were all controlled by HP Chemstation software. The samples were separated on a series of two 4.6 × 125 mm Whatman Partisphere C-18 reverse phase columns (Whatman Inc., Clifton, NJ), eluting at a rate of 1 mL min^{-1} with 5 mL acetonitrile followed by a 0% to 30% acetone gradient (15 mL). Fractions eluting from 2 to 7 min (0.5 mL) were concentrated and analyzed by GC to confirm fractions containing thia fatty acids. These were pooled for MS analysis.

The position of newly introduced double bonds in the desaturated products was determined by preparing the GC-MS analysis of fatty acyl diethylamides as described previously.[29a,b]

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