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Novel Probes for the Mechanistic Study of Fatty Acid Desaturases

by
Behnaz Behrouzian, M.Sc.

A thesis submitted in
partial fulfillment of the requirements for the
degree of Doctor of Philosophy

to

The Faculty of Graduate Studies and Research
Department of Chemistry
Carleton University
Ottawa, Canada
December 1997

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the Faculty of Graduate Studies and Research
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Fatty Acid Desaturases**

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Behnaz Behrouzian, M.Sc.

in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Chair, Department of Chemistry

Thesis Supervisor

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Abstract

The intermolecular primary deuterium kinetic isotope effect (KIE) on the two C–H bond cleavage steps of fatty acid Δ^9 desaturation was measured. Competition experiments involving incubation of methyl 7-thiastearate and methyl [9,9- $^2\text{H}_2$]-7-thiastearate as well as methyl 7-thiastearate and methyl [10,10- $^2\text{H}_2$]-7-thiastearate were carried out using the Δ^9 desaturating system of *S. cerevisiae*. GC/MS analysis of the products showed that cleavage of the C-9–H bond is subject to a large deuterium isotope effect ($k_{\text{H}}/k_{\text{D}} = 7.1 \pm 0.2$) while a negligible isotope effect ($k_{\text{H}}/k_{\text{D}} = 1.03 \pm 0.09$) was observed for the C-10–H bond breaking step. This a key result since it confirms that removal of the first hydrogen is kinetically important and that oxidation is initiated at C-9 as predicted previously in our group using 9- and 10-thia fatty acid analogues.

Fluorinated fatty acids were tested as a means of intercepting possible alcohol intermediates formed during desaturation. Only (*E*)-fluoroalkenic products were obtained when racemic 9- and 10-fluoro stearates were processed by a *S. cerevisiae* Δ^9 desaturase as determined by ^1H -decoupled ^{19}F NMR and GC/MS analysis. No evidence for hydroxylated intermediates was found. α - and β -fluorine substituent effects ($k_{\text{H}}/k_{\text{F}}$) on desaturation of magnitude 6.2 and 2.4, respectively, were calculated by direct competition experiments between racemic methyl 9-fluoropalmitate and methyl 10-fluoropalmitate and between racemic methyl 10-fluoropalmitate and methyl 16-fluoropalmitate. These results did not support the involvement of a discrete carbocationic intermediate in fatty acid desaturation.

Our KIE approach was used to determine the site of initial oxidation of oleate Δ^{12} desaturation using a suitably transformed strain of *S. cerevisiae* containing a functional oleate Δ^{12} desaturase from *A. thaliana*. Mass spectral analysis of the methyl 7-thialinoleate fraction obtained from competition experiments involving methyl 7-thiastearate, methyl [12,12- $^2\text{H}_2$]-7-thiastearate and methyl [13,13- $^2\text{H}_2$]-7-thiastearate showed that cleavage of the C-12–H bond is very sensitive to isotopic substitution ($k_{\text{H}}/k_{\text{D}} = 7.3 \pm 0.4$) while a negligible isotope effect ($k_{\text{H}}/k_{\text{D}} = 1.05 \pm 0.04$) was observed for the

C-13–H bond breaking step. These results support the hypothesis that the naturally occurring ricinoleic acid is produced by a mutated Δ^{12} oleate desaturase and that the mechanistic pathways for biohydroxylation and desaturation are closely linked.

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Chapter 1

Fatty Acid Desaturation: A Perspective

1.1 Introduction

Fatty acid desaturases are a ubiquitous group of enzymes which catalyze the *syn*-dehydrogenation (desaturation) of unactivated fatty acyl hydrocarbon chains. The prototypical reaction, first discovered by Bloch in 1958¹, is the oxidation of stearoyl-CoA (1) to give oleoyl-CoA (2) as it occurs in baker's yeast (Figure 1.1). This process features the four electron reduction of O₂ to give two molecules of H₂O. Two electrons come from the two *pro-R* hydrogens at C-9 and C-10; the other two electrons are provided by NADH.

Figure 1.1. Desaturation of stearoyl-CoA.

A simplified nomenclature for desaturases specifies the position of the double bond in the product relative to the carboxyl group (i.e. Δ^9 desaturase (see above)). The unsaturated fatty acid products are named by indicating the chain length, number of the

double bonds, and the location(s) of the double bond(s) (i.e. 18:1 Δ^9 (oleic acid)). The most common desaturase by far is the Δ^9 desaturase.² However, many more desaturases with a wide variety of unique regiochemical selectivities have been identified (Figure 1.2).

Figure 1.2. Some common unsaturated fatty acids arising by action of various desaturases.

Desaturation of fatty acids occurs in a wide variety of biological species and plays an important role for the proper functioning of these organisms:

(a) Unsaturated fatty acids are of special importance as building blocks for lipids in biomembranes. Maintenance of specific fluidity of cellular membranes is essential to all organisms and is largely determined by the proportion of *cis*-olefinic fatty acids in the constituent lipid fraction. The *cis*-configuration of the double bond introduces a bend in the hydrocarbon chain of these fatty acids which increases the fluidity of the cell membrane by preventing a close-packing arrangement (Figure 1.3).²

Figure 1.3. Packing of fatty acids.

(b) Arachidonic acid ($20:4\Delta^{5,8,11,14}$) derived from linoleic acid ($18:2\Delta^{9,12}$) is an essential precursor for important signaling compounds such as prostaglandins, thromboxanes, and leukotrienes.²

(c) Unsaturated fatty acid derivatives (i.e. acetate esters, alcohols and aldehydes) are typically found in insect pheromone blends and these compounds are used to communicate between insects of the same species.³

1.2 Biochemistry of Fatty Acid Desaturation

Early work (1960's - 1970's) in the desaturase area centered on elucidating the cofactor requirements, the substrate, the nature of constituent proteins and the stereochemistry of the reaction. Two discrete classes of fatty acid desaturases have emerged: enzymes which are membrane-bound and those which are soluble. Despite differences with respect to the types of acyl derivatives that will serve as substrates and the components of electron transfer chains, these two desaturase systems share many common features.

1.2.1 Membrane-Bound Fatty Acid Desaturases

In the first study of direct desaturation by a particulate fraction in baker's yeast, Bloch *et al.* found that reducing equivalents are provided by NADH or NADPH and that oxygen is an obligatory electron acceptor.⁴ These requirements have been demonstrated in a wide variety of membrane-bound systems in different species (i.e. animal, bacteria, yeast, fungi).⁵ Further work on mammalian systems has shown that these desaturases are quite specific for acyl-CoA derivatives of fatty acid substrates.⁵⁻⁷ The only exception is the observation of Kates *et al.* who reported that the Δ^5 desaturase of rat liver can act both on the CoA or phospholipid-bound form of eicosatrienoic acid ($20:3\Delta^{8,11,14}$) to form arachidonic acid ($20:4\Delta^{5,8,11,14}$).⁸ This is the only example of a phospholipid acting as a substrate for O_2 -mediated desaturation apart from those described for polyunsaturated fatty acid biosyntheses in plants⁹ and fungi.¹⁰

The initial breakthrough in understanding the membrane-bound desaturases came from the studies of hen- and rat-liver microsomes by Holloway *et al.* and Sato *et al.* They demonstrated that the mammalian Δ^9 desaturase is composed of three proteins: NADH-cytochrome b_5 reductase, cytochrome b_5 and the desaturase protein.^{11,12} The latter protein was inhibited by ^-CN (not CO) and was thus labelled the "cyanide-sensitive" factor.

The actual arrangement of the three proteins and their orientation with respect to each other in the membrane was studied extensively by Strittmatter *et al.*¹³ who were also the first to successfully reconstitute the membrane-bound Δ^9 desaturase system from rat liver. From the detailed protein chemistry carried out by the Strittmatter's group, it is now clear that both cytochrome b_5 reductase (MW 43 kDa) and cytochrome b_5 (MW 16.7 kDa) are amphipathic proteins containing two domains: one hydrophilic and one hydrophobic.^{13,14} The hydrophilic portions are involved in electron transfer, while the hydrophobic areas serve to anchor the proteins in the phospholipid membrane.¹⁴ The terminal stearyl-CoA desaturase is a single polypeptide chain of 458 amino acids (62% non-polar, MW 33 kDa) which is more intimately associated with the lipophilic membrane (Figure 1.4).^{7,15}

Figure 1.4. Schematic representation of desaturase complex.

The reductase delivers the reducing equivalents of NADH to the heme-iron centre of cytochrome b_5 which in turn reduces the non-heme iron-containing desaturase enzyme at appropriate times in its catalytic cycle.^{16,17} This protein is capable of binding molecular oxygen and long chain saturated fatty acyl CoA derivatives and catalyzes the dehydrogenation of substrate with concomitant reduction of molecular oxygen to $2\text{H}_2\text{O}$.

However, little information about the active site was gleaned other than: (a) the enzyme was inhibited by KCN⁷ as predicted in the case of the enzyme in microsomes; (b) the protein appeared to contain one mole of iron which was essential for catalytic activity as judged by the fact that activity is destroyed by ferrous chelators;⁷ (c) desaturase was inactivated by O-acetylation of the tyrosine residues, thus raising the possibility that these amino acids may be required for catalytic activity.¹⁸

The Δ^9 desaturase was also purified from hen-liver microsomes by Parsad *et al.*⁶ and was reported to have similar properties to that described for the rat-liver enzyme. Purification of the Δ^6 desaturase from rat-liver microsomes demonstrated an electron transport protein system similar to that of Δ^9 desaturases.¹⁹

In 1990, Martin *et al.* sequenced the gene (OLE 1) responsible for Δ^9 desaturase production in yeast²⁰ and showed that the membrane-bound yeast Δ^9 desaturase is similar to the rat Δ^9 desaturase.²¹ The internal amino acid region of this protein was 36% identical and 60% similar to the rat Δ^9 desaturase enzyme. His group also compared the hydropathy of the rat and yeast desaturases and proposed a model for these proteins featuring two long hydrophobic domains (ca. 50 residues), each capable of spanning the membrane twice, such that most of the protein resides on the cytoplasmic face of the endoplasmic reticulum membrane (Figure 1.5).

Figure 1.5. Model for the orientation of the yeast (rat) desaturase in the endoplasmic reticulum membrane.

In 1994, Shanklin and Fox demonstrated, by comparative sequence analysis, that membrane-bound desaturase proteins possess eight highly-conserved histidine residues.²² Site-directed mutagenesis experiments have indicated that these amino acids are essential for catalytic activity and it has therefore been postulated that these residues act as ligands for the iron atom(s) contained in these enzymes.²² Since the requirements for desaturation (O_2 , NAD(P)H and Fe) are closely related to those involved in hydroxylation reactions ($RH + NADH + O_2 + H^+ \rightarrow ROH + H_2O + NAD^+$), it has long been suspected that desaturases may have active site features similar to those in non-heme monooxygenase enzymes.^{23,24} Indeed, it is interesting to note that the highly-conserved multi-His motif of the membrane-bound desaturases is also found in alkane ω -hydroxylase from *Pseudomonas oleovorans*. Very recently, this membrane-bound hydroxylase protein has been shown by Mössbauer spectroscopy to contain a diiron cluster.²⁵ Thus, it has been proposed that membrane-bound desaturases also contain two irons at the active site in contrast with the earlier findings of Strittmatter's group. The difficulty in purifying membrane-bound systems is a significant stumbling block to further progress in understanding these systems.

1.2.2 Soluble Fatty Acid Desaturases

The situation regarding the soluble desaturases, found exclusively in plants, appears far more promising. A soluble Δ^9 stearoyl-acyl carrier protein (ACP) desaturase in photoauxotrophic *Euglena gracilis* was first discovered by Bloch *et al.* in 1966.²⁶ It was found to be highly specific for stearoyl-ACP and it required NADPH, NADPH-ferredoxin oxido-reductase, ferredoxin and molecular oxygen (Figure 1.6).²⁷ These observations have been extended to include a wide variety of plant tissues (i.e. spinach, soybean cotyledons, safflower seeds, avocado).^{27,28} Recently, several related soluble desaturases have been identified in plants that possess fatty acids with unusual double bond positions (Δ^6 , Δ^4).²⁹ It has been shown that these desaturase enzymes are also specific for the ACP derivatives and are chain length-specific.

Figure 1.6. Electron flow in the stearoyl-ACP desaturating system.

The Δ^9 stearoyl-ACP desaturase has now been purified from several sources.^{28,30} A most notable development has been the isolation and purification of a soluble plant Δ^9 desaturase from *Ricinus communis* (castor) seed by Shanklin *et al.* in 1991.³¹ The protein has been shown to be a homodimer (ca. 70 kDa) with no substantial homology with corresponding membrane-bound enzymes. Each subunit was predicted to have two atoms of catalytically essential iron. The iron atoms were demonstrated by Mössbauer³² and Resonance Raman spectroscopy³³ to reside in a μ -oxo diiron cluster with properties similar to that found in methane monooxygenase (MMO) which converts methane and small hydrocarbons to alcohols.³⁴

An important milestone in the study of fatty acid desaturases has been the crystallization of the Δ^9 stearoyl-ACP desaturase from castor seed by Lindqvist *et al.* in 1991,³⁵ followed by its X-ray structure determination in 1996.³⁶ The structure of this protein (Figure 1.7) confirms the presence of a diiron cluster as predicted by spectroscopic methods. Computer modeling of stearic acid in the presumed substrate binding pocket places C-9 in the vicinity of one of the iron atoms thought to bind molecular oxygen.

Figure 1.7. View of the postulated substrate channel in Δ^9 desaturase. The two irons are indicated by pink spheres. A model of the substrate, stearic acid is fitted into the channel.

The two iron atoms are bound in a highly symmetrical environment (Figure 1.8), with one of the irons forming interactions with the side chains of E (glutamate)196 and H (histidine)232 and the second iron with the side chains of E105 and H146. Two additional glutamic acid side chains, from E143 and E229, are also within coordination distance. A water molecule is found within the second coordination sphere of one of the iron atoms. The lack of electron density corresponding to a μ -oxo bridge suggested that the enzyme is in reduced form due to photochemical reduction by X-ray radiation.³⁶

Figure 1.8. View of the diiron center in Δ^9 desaturase (reduced form) with ligands. The two irons are indicated by black spheres.

1.3 Stereochemistry of Fatty Acid Desaturation

The fact that two hydrogens are removed in the fatty acid desaturation process raises the following questions: What is the geometry of the double bond (*cis* or *trans*)? What is the stereochemistry of the hydrogen removal (*syn*- or *anti*-elimination) and which two of the four possible hydrogen atoms are lost (enantioselectivity)? These questions were first examined by Bloch *et al.* (1965)³⁷ who investigated the Δ^9 desaturation of stearic acid as it occurs in *Corynebacterium diphtheriae*.

His group first confirmed that the chromatographic properties of the Δ^9 octadecenoic acid ($18:1\Delta^9$) methyl ester from *C. diphtheriae* were identical to those of a standard sample of methyl oleate using thin layers of AgNO_3 -impregnated silica gel.³⁸ Thus, the desaturation of the stearic acid was shown to give essentially 100% *cis*-olefin.

In order to study the stereochemistry of hydrogen removal, racemic *erythro*- and *threo*-9,10-dideuterated stearates were prepared by reduction of oleic and elaidic acids, respectively, using dideuteriodiimide ($\text{D}-\text{N}=\text{N}-\text{D}$).³⁷ Isotopic analysis of the **deuterated** olefinic products by mass spectrometry demonstrated that *erythro*-dideuterated precursors gave a preponderance of dideuterated species in the product, whereas *threo*-dideuterated precursors yielded an enrichment of monodeutero products (Figure 1.9). Thus, a *syn*-elimination process was proposed for the formation of oleic acid by the Δ^9 desaturase of *C. diphtheriae*.^{37,39}

Figure 1.9. Olefinic products from racemic *erythro*- and *threo*-dideuterated labelled precursors.

To investigate the enantioselectivity of the enzymatic conversion of stearic acid to oleic acid, (*R*)-9-, (*S*)-9-, (*R*)-10- and (*S*)-10-tritioleic acids were synthesized from the appropriate chiral stearates using stereospecific reactions (Figure 1.10).^{24,37} Methyl (*R*)-9-hydroxystearate was obtained by the reduction of naturally occurring (*R*)-9-hydroxy- Δ^{12} octadecenoic acid (*Strophanthus* oil) followed by methylation.⁴⁰ (*R*)-10-hydroxystearic acid was prepared by incubating oleic acid with a fermenting culture of a *Pseudomonas* species.⁴¹

Figure 1.10. Synthesis and desaturation of stereospecifically tritium-labelled stearic acids.

The enantioselectivity of the Δ^9 desaturase was analysed by measuring the tritium retained in the resulting oleic acid relative to a ^{14}C -labelled standard. The results of this experiment indicated that tritium from the (*S*)-9- and (*S*)-10-tritio substrates was retained in the product, whereas the (*R*)-9-, and (*R*)-10-tritium labels were lost upon desaturation.

Employing the same approach, Morris *et al.* extended these observations to the biosynthesis of oleic, linoleic ($18:2\Delta^{9,12}$) and α -linolenic ($18:3\Delta^{9,12,15}$) acids by *Chlorella vulgaris* (a green algae).⁴² The results of these studies indicated that these olefinic fatty acids were also formed by *syn*-removal of *pro-R* hydrogens from 12,13- and 15,16-positions. In the case of α -linolenic acid ($18:3\Delta^{9,12,15}$), the enantioselectivity of oxidation at C-15 and C-16 was not experimentally defined.⁴²

In 1983, Wright *et al.*⁴³ studied the stereospecificities of various Δ^9 desaturases by examining the labelled unsaturated fatty acids obtained via incubation of $^{13}\text{C}^2\text{H}_3\text{CO}_2\text{H}$ with *Saccharomyces cerevisiae*, *Chlorella pyrenoidosa*, *Anacystis nidulans* and *Phaeodactylum tricornutum* (Figure 1.11). This group had previously shown that monodeuteromethylene groups at even carbons in saturated fatty acids biosynthesized from $^{13}\text{C}^2\text{H}_3\text{CO}_2\text{H}$ have the *R*-configuration in yeast and the *S*-configuration in the three algae. Using $[^1\text{H}, ^2\text{H}]$ -decoupled ^{13}C NMR, the absence of deuterium at C-10 of palmitoleic ($16:1\Delta^9$) acid from yeast and the retention of this label at similar sites in the unsaturated acids from the three algae was demonstrated and led to the conclusion that the desaturases in all of these organisms stereospecifically remove the *pro-R* hydrogen from C-10.

Figure 1.11. Stereochemistry of Δ^9 desaturation in yeast and three algae.

In 1993, Boland *et al.*⁴⁴ reported the stereochemistry of Δ^{11} desaturation in *Mamestra brassicae*. They demonstrated that palmitic acids (16:0) undergo *syn*-elimination of the *pro-R* hydrogens at C-11 and C-12 in the pheromone gland of the moth *M. brassicae* (Figure 1.12). Interestingly, the enantioselectivity of this process was identical to that observed for desaturation reactions carried out by the Δ^9 desaturases from bacteria, plants, algae and mammals.

Figure 1.12. Stereochemistry of Δ^{11} desaturation in *M. brassicae*.

The collective evidence thus suggests that desaturases, irrespective of their origin, employ a mechanism featuring *syn*-elimination which suggests that the substrate may adopt an eclipsed conformation when bound to the active site of the enzyme. Inhibition studies with the purified rat liver desaturase showed that oleoyl-CoA and *cis*-9,10-epoxystearoyl-CoA were strong competitive inhibitors of the desaturation, while the *trans*-octadecanoyl-CoA is only a poor inhibitor.⁷ These results were consistent with an eclipsed conformation at C-9 and C-10 in the enzyme-substrate complex. In addition, the crystal structure of the soluble plant Δ^9 stearoyl-ACP reveals a crescent-shape of the presumed substrate channel which could accommodate an eclipsed conformation of the substrate at the active site (Figure 1.7).³⁶

1.4 Mechanism of Fatty Acid Desaturation

1.4.1 Mechanistic Studies by Bloch *et al.*

Despite these advances, very little work on the detailed mechanism of fatty acid desaturation has emerged over the past four decades. Based on the requirement for molecular oxygen and NAD(P)H, Bloch *et al.* first proposed that the desaturation reaction may proceed by way of a hydroxy-intermediate in a stepwise process.^{23,24} By comparing the release of tritium from the four possible tritium-labelled substrates in their studies of bacterial Δ^9 desaturation (*C. diphtheriae*), a substantial primary tritium kinetic isotope effect at C-9 position but not at the C-10 position was deduced.^{24,37} This suggested that the rate-limiting step in Δ^9 desaturation was removal of the 9*R*-hydrogen atom. They proposed that desaturation may be viewed as a monooxygenase reaction, in which the first step was hydroxylation at the 9-position followed by dehydration (Figure 1.13).

Figure 1.13. Hydroxylation/dehydration mechanism for Δ^9 desaturation.

In an attempt to support this hypothesis, they prepared and incubated the four possible 9- and 10-hydroxy fatty acids and their CoA/ACP derivatives with Δ^9 desaturase system from *S. cerevisiae* and *E. gracilis*, respectively.^{23,45} However, no olefinic products were formed. In addition, attempts to implicate a hydroxy olefinic acid as a precursor for linoleate ($18:2\Delta^{9,12}$) gave negative results. Thus, ricinoleic ((*R*)-12-hydroxyoleic) acid, a naturally occurring compound, did not appear to be an intermediate in the conversion of oleate ($18:1\Delta^9$) to linoleate ($18:2\Delta^{9,12}$).

1.4.2 Mechanistic Studies by Morris *et al.*

The results obtained by this group in their studies of Δ^9 desaturation of *C. vulgaris*⁴² differed from those obtained by Bloch *et al.*^{23,24} The isotopic analysis of deuterated products by mass spectrometry appeared to demonstrate that the amount of dideuterated-labelled oleoyl product (5.45 mol/100 mol) from the *erythro*-9,10-dideuterated stearate is considerably greater than the amount of monodeuterated-oleate (1.55 mol/100 mol) obtained from *threo*-9,10-dideuterated stearate. Non-deuterated products were not analyzed due to mass spectral interference by endogenous oleate. They reasoned that if a large kinetic isotope effect operated at the C-9 position only, as observed by Bloch, then *erythro*-(9*R*,10*R*)- and the *threo*-(9*R*,10*S*)-9,10-dideuterated substrates would both be processed more slowly than the corresponding enantiomer (Figure 1.14). Thus, comparable amounts of dideuterated- and monodeuterated-labelled products would have been produced from *erythro*- and *threo*-precursors, respectively.⁴²

Figure 1.14. Olefinic products from racemic *erythro*- and *threo*-dideuterated labelled precursors assuming isotope effect at C-9 position only.

They argued that the observed results were consistent with the presence of a substantial kinetic isotope effect at both *pro-R* positions. In this scenario, neither of the *threo*-enantiomers would be as efficient a substrate as the *erythro*-(9*S*,10*S*)-9,10-dideuterated enantiomer, leading to a considerable amount of the dideuterated-labelled product from *erythro*-precursors relative to the amount of monodeuterated products from *threo*-substrates (Figure 1.15). Thus, a “simultaneous concerted” removal of hydrogens was proposed by this group.^{39,42} (If there was no kinetic isotope effect at C-9 and C-10, there should be twice as much enrichment of monodeuterated-labelled product from the *threo*-substrates as there is dideuterated-labelled product from the *erythro*-substrates.)

Figure 1.15. Olefinic products from racemic *erythro*- and *threo*-dideuterated labelled precursors assuming isotope effects at both C-9 and C-10 positions.

Morris' results were apparently confirmed by Johnson *et al.*⁴⁶ They compared the rate of desaturation of [1-¹⁴C]-stearoyl-CoA with that of racemic *erythro*- and *threo*-9,10-ditritiated-stearoyl-CoA acids using a cell-free Δ^9 desaturase preparation from hen liver. The tritiated substrates were prepared by reduction of an appropriate unsaturated precursor using carrier-free tritium gas and thus each tritiated substrate bore two tritium atoms. An apparent $V_{\max}$ (nmol/30 min/0.6 mg protein) for the desaturation of each labelled substrate was calculated and the following ratio was obtained: ¹⁴C (3.3) > *threo*-9,10-ditritium (2.0) > *erythro*-9,10-ditritium (1.0). Thus it appeared that the isotope effect involved in the removal of the two tritium atoms at the C-9 and C-10 was twice that observed for the removal of one tritium at either the C-9 or C-10 positions. This in turn suggested that an isotope effect at both carbons was operative.

Based on these data, in 1971, a highly speculative disulfide bridged model was postulated for the desaturases by James *et al.*⁴⁷ which involved simultaneous removal of two *pro-R* hydrogens to form an olefin and two thiols (Figure 1.16). In light of what we now know about the non-heme iron active site of desaturases, this model can be discarded. However, the issue of whether C-9-H and C-10-H bond cleavage is concerted or stepwise has never been resolved.

Figure 1.16. Hypothetical scheme for desaturation as proposed by James *et al.*

1.4.3 Mechanistic Studies by Strittmatter *et al.*

Strittmatter *et al.*¹³ demonstrated isotopic discrimination in their studies with the reconstituted rat liver stearoyl-CoA desaturase system by comparing $V_{\max}$ of completely deuterated stearoyl-CoA, 9,10-tetradedeuterated stearoyl-CoA and stearoyl-CoA. The magnitude of k_H/k_D values ($k_H/k_D = 6$) was within the range expected for a primary isotope effect. Thus, they proposed that hydrogen removal is the rate-limiting step in enzymatic desaturation as suggested by Bloch *et al.* for the whole cell bacterial system. However, they did not distinguish between the presence of an isotope effect at one or both carbons (C-9 and C-10).

1.4.4 Mechanistic Studies on Iron-Based hydroxylases

As alluded to in 1.2.2 (page 7), desaturation and hydroxylation are closely linked at the structural level (Figure 1.17). It is therefore not surprising that the modern view of desaturation mechanisms is usually discussed in terms of the mechanism of the more well-known hydroxylation process. A detailed discussion of the various mechanistic models for the latter reaction is therefore in order.

Figure 1.17. Overall stoichiometry of hydroxylation and desaturation reactions.

Two major classes of biological iron-based hydroxylases have been found. The first class consisting of the heme-containing cytochrome P-450 hydroxylase has been extensively characterized.⁴⁸ The second class of hydroxylases is exemplified by two non-

heme diiron systems: MMO and alkane ω -hydroxylase.^{25,49} Historically, mechanistic thinking in this area has been guided by early work on cytochrome P-450 and this is discussed first.

1.4.4.1 Heme Iron-Containing Cytochrome P-450 Hydroxylase

The cytochrome P-450 monooxygenase system consists of three protein components: a cytochrome P-450, a electron transfer protein and a reductase (flavoprotein).⁵⁰ The classification of a protohemoprotein as a P-450 is defined by its absorption spectrum; the Fe(II)-CO complex has a characteristic absorption maximum near 450 nm due to coordination of a sulfhydryl sulfur atom (cysteine thiolate) to the heme iron of the enzyme.⁵¹ A large family of related proteins has been discovered, not only in hepatic microsomes and mitochondria but also in bacteria.⁵² The P-450_{camphor} protein from *Pseudomonas putida* was the first P-450 to be crystallized and has been subjected to detailed studies.⁵³ The protein has a triangular shape and consists of 414 amino acid residues. The heme prosthetic group is deeply embedded in the hydrophobic interior with no significant exposure of the heme to the protein surface. The heme iron is hexacoordinate, with cysteine-357 as the proximal axial ligand and water molecule or hydroxide ion as the distal axial ligand.⁵⁴ Very recently, the three dimensional structure of a P-450 fatty acid monooxygenase from *Bacillus megaterium*⁵⁵ and P-450 _{α -terpineol} from a *Pseudomonas*⁵⁶ species has also been determined; the overall structures are similar to that of the P-450_{camphor}.

The catalytic mechanism of cytochrome P-450-mediated monooxygenation can be considered in two parts: oxygen activation and substrate oxidation. The current proposal for the formation of activated oxygen in cytochrome P-450 is illustrated in Figure 1.18. It is thought that an oxoiron(IV) porphyrin radical cation species is the likely oxidant in these reactions.⁴⁸

Figure 1.18. Formation of iron-oxo intermediate in heme hydroxylase enzymes (P-450).

The substrate oxidation mechanism has remained unclear due to the instability of the intermediates in the catalytic cycle and the lack of simple model systems with similar reactivity. Early mechanistic studies (1950's-1960's), showed that the substrate stereochemistry appeared to be conserved and the C-H bond cleavage was subject to low kinetic isotope effects.⁵⁷ This suggested that oxygen atom delivery to the substrate occurred via a concerted "insertion" pathway (Figure 1.19).

Figure 1.19. Concerted "insertion" pathway for hydrocarbon hydroxylation.

In 1978, Groves *et al.* showed that hydrogen removal for hydroxylation of *exo*-tetra-deuterionorbornane by cytochrome P-450 is accompanied by a very large isotope effect ($k_H/k_D = 10-12$).⁵⁸ His group also reported that the oxidation event occurs with significant loss of stereochemistry at the oxidized carbon. Thus, they proposed, a stepwise, homolytic path for hydroxylation which involves an initial hydrogen abstraction by an iron-oxo species followed by hydroxyl rebound (Figure 1.20).

Figure 1.20. Hydroxyl rebound mechanism for hydrocarbon hydroxylation.

Further evidence for the radical intermediate, came from studies with probe substrates such as deuterium-substituted cyclohexene (**3**) (Figure 1.21).⁵⁹ It was demonstrated that this compound is oxidized by cytochrome P-450 to give allylic rearranged products. This observation was extended by Ortiz de Montellano *et al.* They showed that a probe substrate such as bicyclo[2.1.0]pentane (**4**) (Figure 1.22) is hydroxylated by cytochrome P-450 to give both unrearranged and rearranged products.⁶⁰ A requisite cationic intermediate was excluded because substrates such as methylcyclopropane (**5**) (Figure 1.22) were oxidized at the cyclopropylcarbinyl positions without rearrangement as would be expected for a cationic intermediate.

Figure 1.21. Allylic scrambling observed for cytochrome P-450-catalyzed hydroxylation reactions.

In 1991, Ingold *et al.* estimated the life time of the radical intermediate using “radical clock” substrates that have highly strained carbocyclic structures such as (**4**) (Figure 1.22).⁶¹ A carbon radical localized adjacent to the cyclopropane ring of such substrate (i.e. **U**[•]) rearranges to another ring-opened carbon radical at a known rate ($k_r = 2.1 \times 10^9 \text{ s}^{-1}$). Hence, the rate of oxygen rebound ($k_{OH} = 1.4 \times 10^{10} \text{ s}^{-1}$) can be estimated by examining the ratio of unrearranged to rearranged products ($\text{UOH}:\text{ROH} = 6.5\text{--}7$).⁶⁰⁻⁶² This group employed a series of fast-clock substrates (i.e. **6**, Figure 1.22) and calculated k_{OH} values which were varied from 10^{11} to 10^{12} s^{-1} (1-2 orders of magnitude higher than the value determined for (**4**)).^{61,63} These high values were interpreted as being most consistent with the possibility that the steric effects at the enzyme active site might slow the radical clocks and yield overestimated k_{OH} values. Ingold’s group also proposed that the hydroxyl rebound step might better be viewed as a bimolecular homolytic substitution (S_H2) (Figure 1.20).

Figure 1.22. Some typical radical clock substrates employed in the mechanistic study of cytochrome P-450-catalyzed reactions.

This consensus mechanism has recently been re-evaluated by Newcomb *et al.* They showed that the constrained substrate (7) (Figure 1.22),⁶⁴ which would be less susceptible to steric effects, gives an incredibly high apparent k_{OH} value ($1.3 \times 10^{13} \text{ s}^{-1}$). This value is essentially equal to the rate constant for the decomposition of a transition state. In the same study, this group showed that there is no correlation between the ring opening rate of a range of probes and the amount of ring-opened products observed after oxidation of those probes by cytochrome P-450. Thus, they proposed that the origins of rearranged products are uncertain and the variable k_{OH} values might be a reflection of ionic rearrangements that cannot be discerned from radical rearrangements.

Later work by Newcomb's group, employing a probe (8) (Figure 1.23)⁶⁵ designed to discriminate between radical and cation intermediates, demonstrated that rearranged products are seen to arise from both radical and cationic pathways.⁶⁶ The calculated oxygen rebound rate constant (k_{OH}) for this probe was very similar to that determined for (7). From this value, they determined the life time of the radical to be ca. 70 fs. They proposed that a life time of less than 100 fs is too short for formation of a true intermediate but it can be a component in a reacting ensemble produced in a "nonsynchronous, concerted" mechanism (Figure 1.24).

In addition, this group recently reported that reaction of methyl cubane with *tert*-butoxyl radical ($^t\text{BuO}^\bullet$)⁶⁷ gives mainly cube-substituted products while oxidation of this compound by P-450 gives hydroxylated methyl cubane at all positions. They argued that this difference in regioselectivity is consistent with their mechanistic proposal. However, Jones *et al.*,⁶⁸ very recently, demonstrated that there is a good correlation between P-450 and $^t\text{BuO}^\bullet$ isotope effects for a variety of substrates which is consistent with the formation of a carbon centered radical by Groves/Ingold hydrogen abstraction mechanism. Thus, Newcomb's "insertion" mechanism remains controversial.

The relevance of this work to the desaturase mechanism is heightened by recent confirmation that the Δ^{22} desaturation of sterols is catalyzed by a cytochrome P-450 catalyst.⁶⁹ In addition, 4,5-desaturation of valproic (2-*n*-propylpentanoic) acid (9) (Figure 1.28, page 31) is catalyzed by hepatic cytochrome P-450, an enzyme which also catalyses 4- and 5-hydroxylation of valproic acid.⁷⁰

Figure 1.23. Mechanistic probe employed for distinguishing between radical and carbocation intermediates.

Figure 1.24. Nonsynchronous, concerted model for cytochrome P-450 hydroxylation.

1.4.4.2 Non-Heme Diiron-Containing MMO Hydroxylase

Despite the obvious structural differences between cytochrome P-450 and MMO, the reaction chemistries they catalyze are strikingly similar. Thus, the studies of MMO have made it possible to compare the proposed mechanism of P-450 with that of structurally dissimilar non-heme diiron systems.^{49,71}

The soluble MMO system consists of three protein components: a hydroxylase (MMOH), a reductase, and a coupling protein, all three of which are required for enzymatic activity.⁷² The crystal structure of the oxidized form of MMOH has been determined for both *Methylococcus capsulatus* (Bath)⁷³ and *Methylosinus trichosporium*⁷⁴ enzymes (Figure 1.25). Further crystallographic studies led to the structures of both the oxidized and reduced forms of MMOH from *M. capsulatus* (Bath)⁷⁵ at -160 °C. The overall structures of enzymes from these two different sources are remarkably similar. Each iron in the MMOH cluster is six coordinate, having one nitrogen ligand (His) and five oxygen ligands comprised of glutamic acid residues, bridging hydroxides, and a terminal water. Upon reduction, one of the terminal carboxylates shifts to a bridging ligand between the two iron ions; such carboxylate shifts⁷⁶ may play an important but as yet undefined role in oxygen activation.

Figure 1.25. Schematic representation of the diiron center of MMOH in reduced and oxidized forms.

Recently, there has been significant progress in understanding the oxygen activation mechanism of non-heme diiron containing MMOH. It has been shown, by Lipscomb⁷⁷ and Lippard *et al.*,⁷⁸ that the catalytic cycle of MMOH proceeds through several discrete intermediates (Figure 1.26). They postulated that the cycle is first initiated with the reaction of oxygen with MMOH_{red} to form intermediate P, which is spontaneously converted to the intermediate Q. “Q” then reacts with substrate to afford hydroxyl product and MMOH_{ox}, the diiron(III) form. On the basis of their spectroscopic

properties, “P” and “Q” are thought to be a (μ -peroxo)diiron(III) and bis(μ -oxo)diiron(IV) species, respectively.⁷⁹ The latter intermediate is also depicted as a bis(oxo)iron(IV) species which resembles the iron(IV)oxo (porphyrin⁺) species postulated for cytochrome P-450. The second iron can be thought of as fulfilling the role of the heme of P-450 as an electron storage site to reduce the oxidation level of its partner from V to IV.

Figure 1.26. A mechanistic framework for oxygen activation in MMOH.

Studies of model complexes that mimic some spectroscopic features of intermediates P and Q have also contributed to understanding the structure of the diiron center in these complexes.⁸⁰ Very recently, Que and Münck *et al.*⁸¹ reported that Fe(III)Fe(IV) synthetic complexes with bis(μ -oxo)diiron diamond core (i.e. $[\text{Fe}_2(\mu\text{-O})_2(\text{TPA})_2]^{3+}$, TPA = tris(2-pyridylmethyl)amine) have spectroscopic features very similar to those of intermediate Q. They proposed that a species with highvalent bis(μ -oxo)diiron diamond core serves as the oxidizing species in MMO hydroxylation.

The substrate oxidation mechanism by MMO was first discussed in terms of the Groves “hydroxyl rebound” mechanism proposed for cytochrome P-450 (Figure 1.20). The catalytic cycle involving hydrogen atom abstraction by a high-valent iron-oxo intermediate followed by hydroxylation was proposed by several authors.⁸² However, the results of radical clock reactions for MMO were less consistent than those reported for cytochrome P-450. Newcomb *et al.*⁸³ demonstrated that the enzyme from *M. trichosporium* catalyzed the oxidation of a range of radical clock substrates and gave varying extents of rearranged products, whereas the MMOH from *M. capsulatus* (Bath) gave little or no rearranged product.

In 1992, Floss *et al.*⁸⁴ reported that 35% inversion of configuration and an intramolecular deuterium kinetic isotope effect (KIE) of 4.2 are observed in hydroxylation of chiral ethane (CH_3CHDT) with *M. trichosporium*. These results were interpreted as being most consistent with a substrate radical which is not entirely free but constrained in some way by the enzyme active site. Lippard and Floss *et al.*,⁸⁵ very recently, reported that hydroxylation of chiral ethane (CH_3CHDT) and primary chiral butane ($\text{CH}_3\text{CH}_2\text{CH}_2\text{CHDT}$) with *M. capsulatus* (Bath) give 72 and 77% retention of stereochemistry, respectively. They also demonstrated an intramolecular $k_{\text{H}}/k_{\text{D}}$ ratios of 3.4 and 2.2 for these two enzymatic reactions, respectively, and ca. 90% retention of stereochemistry at labelled carbon for the hydroxylation of (2- ^3H)-butane. These results were explained by a process in which O–H bond formation, C–H bond breaking and C–O bond formation occur in a concerted but not totally synchronous manner as proposed by Newcomb (Figure 1.27). They argued that this mechanistic model is consistent with the observed kinetic isotope effect and explains the lack of ring-opened products in the radical clock studies, since a free substrate radical is never formed. It also explains the greater retention of stereochemistry for the C-2 chiral butane hydroxylation; steric bulk limits back side attack and inversion of stereochemistry.

Very recently, Siegbahn *et al.*⁸⁶ reported the quantum mechanical studies of the mechanism of C–H activation by MMO. They proposed that the reaction mechanism is most consistent with hydrogen atom abstraction followed by rapid collapse of the transient radical with the metal center (Figure 1.30, page 32). It is clear that mechanisms for this class of reactions remains highly speculative.

Since the crystal structure of the soluble plant Δ^9 stearoyl-CoA desaturase in reduced form is very similar to that characterized for MMOH (Figures 1.8 and 1.25), it is therefore thought that the MMO hydroxylase system can be considered as a guide in the mechanistic study of fatty acid desaturases.

Figure 1.27. Nonsynchronous concerted mechanistic model for oxidation of an alkane by MMO.

1.4.5 Mechanistic Studies by Buist *et al.*

The mechanistic link between desaturases and the hydroxylases was first brought into sharper focus by Ortiz de Montellano in 1989.⁸⁷ He outlined the formation of radical and cationic intermediates in the unusual 4,5-dehydrogenation of valproic acid (**9**) by cytochrome P-450 (Figure 1.28). In 1991, Collins *et al.*⁸⁸ calculated the ionization potential of the various radical species obtained by H-abstraction and demonstrated that the resultant carbocation formed at C-4 can be stabilized by a five-membered, protonated lactone formation. Thus, terminal olefin formation at either n-propyl chain was rationalized by postulating a C-4, C-4' carbocation intermediate.

Figure 1.28. Alternative mechanisms for the 4,5-dehydrogenation of valproic acid (9) by cytochrome P-450.

By analogy to this work, Buist's group adopted a mechanistic model relating desaturation to hydroxylation (Figure 1.29):⁸⁹ one might envisage a hydrogen atom abstraction step by a highvalent iron-oxo species which generates a carbon-centered radical "intermediate". This species could collapse to olefin via a rapid Lewis acid (Fe³⁺)-catalyzed dehydration of an alcohol intermediate (pathway a), simple disproportionation (pathway b), or by a one electron oxidation/deprotonation⁹⁰ sequence (pathway c). This group proposed that desaturases might lower the oxidation potential of the carbon radical by a negatively charged carboxylate or by cation- π interactions⁹¹ using an aromatic amino acid residue (pathway c). There is also another possible pathway (Figure 1.30) which involves formation of an organo-iron bond followed by reductive elimination as proposed by Siegbahn *et al.* in a theoretical study of the MMO system.⁸⁶

Figure 1.29. Different pathways for the mechanism of desaturation.

Figure 1.30. Proposed pathway involving the formation of organo-iron intermediate.

Work in the Buist group has primarily focused on studying the site of initial attack by oxidant using the whole cells of baker's yeast (*S. cerevisiae*) as a convenient source of the Δ^9 desaturase. It was shown that sulfoxidation of a number of thia fatty acid analogues by Δ^9 desaturase of *S. cerevisiae* is most efficient when sulfur replaces C-9 of stearic acid (Figure 1.31).^{89,92} Substantially less sulfoxide was obtained when sulfur was at C-10 position, and sulfoxidation at the C-8 and C-11 positions was negligible.⁹³ In addition,

this group showed that sulfoxidation of a 9-thia analogue of stearic acid does not occur in a Δ^9 desaturase-deficient mutant of *S. cerevisiae*.⁹⁴ The stereochemistry of sulfoxidation at the C-9 and C-10 positions was consistent with that reported for hydrogen removal during desaturation (*R*-configuration).⁸⁹ Thus, it was postulated that the non-heme iron oxidant initiates Δ^9 desaturation of *S. cerevisiae* by hydrogen atom abstraction at C-9 as originally proposed by Bloch *et al.* ... **a testable hypothesis.**

Figure 1.31. Thia fatty acid probes employed in the mechanistic study of yeast Δ^9 desaturase.

1.5 Research Goals

The purpose of this thesis was to develop and apply new probes for the mechanistic study of fatty acid desaturation as it occurs in *S. cerevisiae*:

(a) Use of primary deuterium kinetic isotope effects (KIEs) to firmly establish the cryptoregiochemistry (site of initial oxidation) of yeast Δ^9 desaturation. This study was required in order to determine the fidelity with which thia fatty acid analogues report the location of the oxidant since: (a) introducing a sulfur atom with the change in bond lengths and the bond angles along the chain might alter the proper conformation of the substrate in the active site of enzyme, (b) the possibility of autoxidation of the sulfur atom decreases the accuracy of the thia test when studying desaturase-mediated reactions at the “trace” level. Thus, we decided to investigate the site of initial oxidation employing a primary kinetic isotope effect approach. This approach involved the synthesis of 9,9- and 10,10-dideuterated 7-thiasubstrates followed by determination of the intermolecular kinetic isotope effect (KIE) on C–H bond cleavage at C-9 and C-10. The kinetic isotope effects (KIEs) can be evaluated via direct competition between labelled and non-labelled materials.

(b) Use of monofluorinated fatty acids as a new mechanistic probe for possible hydroxyl or carbocationic intermediates. If Newcomb’s “non-synchronous, concerted” mechanism for biological hydroxylation (1.4.4, Figures 1.24, 1.27)^{85,66} is correct, then the closely-related fatty acid desaturation process may indeed involve an initial hydroxylation event (pathway a, Figure 1.29). On the other hand, the observation that carbocations are formed in those cytochrome P-450 reactions involving neighboring group participation (e.g. the role of carboxyl group in 4,5-dehydrogenation of valproic acid (**9**) (Figure 1.28)) would suggest that desaturases may operate by pathway c (Figure 1.29). We decided to investigate the possibility of a fluorine substituent as a means of examining the formation of an alcohol or carbocation intermediate during desaturation. The approach involved the synthesis of a racemic mixture of methyl 9- and 10-fluorostearates followed by incubation using the whole cell yeast Δ^9 desaturase system. Analysis of the substrates and products was carried out by ¹H-decoupled ¹⁹F NMR and/or GC/MS.

(c) Application of primary deuterium kinetic isotope effects (KIEs) to establish the cryptoregiochemistry of oleate Δ^{12} desaturation. The site of initial oxidation in Δ^{12} desaturation was to be determined via the measurement of primary isotope effects on the individual C–H bond cleavage steps involved in linoleic acid ($18:2\Delta^{9,12}$) biosynthesis using 12,12- and 13,13-dideuterated 7-thiasubstrates. In this manner, evidence for or against the hypothesis that the production of ricinoleic ((*R*)-12-hydroxyoleic) acid in the castor plant is carried out by a modified Δ^{12} desaturase could be obtained.

Chapter 2

Deciphering the Cryptoregiochemistry of Δ^9 Desaturase: A Kinetic Isotope Effect (KIE) Study

2.1 Experimental Approach

A major reason for determining kinetic isotope effects (KIEs) in enzymology has been to determine the “rate determining step” of the enzymatic reactions.⁹⁵ It is now well understood that if cleavage of the C–H bond is the slow, rate-determining step in a enzyme-catalyzed reaction, then substituting hydrogen with the heavier deuterium atom should in principle decrease the reaction rate. This decrease in the rate is called a “deuterium isotope effect” and it is expressed as the ratio between the rate constants for the reactions involving hydrogen and deuterium isotopes (k_H/k_D), (Appendix A).⁹⁶ One problem with the determination of isotope effects for enzymatic reactions is that the isotopically-sensitive step (i.e. C–H bond cleavage) is not always the only rate-determining step in the reaction sequence. This can lead to masking of the so-called “intrinsic isotope effect”.⁹⁷ Masking can be minimized by the use of intramolecular isotope effects, where both isotopes in equivalent positions within the same substrate compete in a reaction.⁹⁸

We decided to probe the cryptoregiochemistry of the Δ^9 desaturase enzyme by measuring the deuterium kinetic isotope effect (KIE) on the two C–H cleavage steps involved in fatty acid Δ^9 desaturation. In this study, we took advantage of this fact that, according to our mechanistic scheme (Chapter 1, Figure 1.29), initial hydrogen abstraction should be slow relative to the second C–H bond cleavage and therefore more sensitive to the isotope effect. The enzymatic isotope effects in our study were determined via intermolecular experiments where both labelled and unlabelled substrates were present and compete in the same incubation.⁹⁹ This competitive method has several

advantages: (a) exactly the same conditions are provided for the reactions of both labelled and unlabelled substrates; (b) time as well as the absolute concentration of labelled and unlabelled substrates do not have to be measured accurately; and (c) the same purity of labelled and unlabelled substrates is not required.⁹⁹ Thus, it was hoped that if complete masking does not occur, the results obtained from this study will provide the closest estimate of the observed isotope effect. An intramolecular method could not be used for our kinetic isotope effect (KIE) studies since the *pro-S* hydrogens at the C-9 and C-10 positions of the stearyl substrates do not undergo reaction.

Our approach involved comparing the d_0/d_1 ratio of 7-thiaoleate products derived from a ca. 1:1 mixture of methyl 7-thiastearate (**10**) and methyl [9,9-²H₂]-7-thiastearate ([9,9-²H₂]-**10**) with that obtained from a ca. 1:1 mixture of methyl 7-thiastearate (**10**) and methyl [10,10-²H₂]-7-thiastearate ([10,10-²H₂]-**10**). It should be pointed out that the use of thia-substituted analogues eliminates the problem of mass spectral interference by endogenous (nondeuterated) oleate when the d_0/d_1 ratio of the olefinic products is evaluated. The sulfur was placed at position "7" in order to facilitate the synthesis of the deuterated substrates. In addition, it was previously shown in our group that the replacement of C-7 with sulfur does not affect the yeast Δ^9 desaturation process.

The selected source of the Δ^9 desaturase for this isotope effect study was *S. cerevisiae* since previous mechanistic studies of desaturation using sulfur probes by our group were carried out using this system. Furthermore, it is a readily available yeast, relatively easy to grow, and known to contain a fairly active desaturase which can accept a wide variety of substrates to produce milligram amounts of products. Use of the whole cell system is thus ideal for the accurate measurement of product isotopic ratios since only the desaturation step would be sensitive to isotope substitution at the *pro-R* positions at C-9 and C-10.

2.2 Results and Discussion

Part 1: Synthesis of Substrates

2.2.1 Methyl 7-thiastearate (**10**)

The route that was used to prepare methyl 7-thiastearate (**10**) is outlined in Figure 2.1. The reaction of 6-bromohexanoic acid with thiourea yielded 6-mercaptohexanoic acid.¹⁰⁰ S-alkylation of the dilithio salt of this material using 1-bromoundecane followed by methylation¹⁰¹ and purification by flash chromatography (silica gel, 4% EtOAc/hexanes) gave methyl 7-thiastearate (**10**) in 30% est. overall yield. GC/MS (Figures 2.2 and 2.3), ¹H NMR (Figure 2.4), and ¹³C NMR (Figure 2.5 and Appendix B) data were in accord with our structural assignment.

The compound was essentially pure by GC (Figure 2.2). The elemental analysis results confirmed the molecular formula of C₁₈H₃₆O₂S for this material. The mass spectrum (Figure 2.3) showed the following characteristic fragment ions: m/z 316 (M⁺), 285 [(M - OCH₃)⁺], 187 [(CH₃(CH₂)₁₀S)⁺], 129 [(M - CH₃(CH₂)₁₀S)⁺] which were consistent with the assigned structure. In the ¹H NMR spectrum (Figure 2.4), the signals of hydrogen atoms α to the sulfinyl group appeared as two overlapping triplets at 2.47 ppm, a typical value for the chemical shift of aliphatic thioethers.¹⁰² The complete ¹³C NMR spectrum assignment of methyl 7-thiastearate (**10**) was carried out using literature¹⁰² values for methyl stearate as well as ¹³C NMR obtained for deuterated methyl 7-thiastearates ([9,9-²H₂]-, [10,10-²H₂]-, [12,12-²H₂]-, [13,13-²H₂]-**10**) (Appendix B).

Figure 2.1. Synthesis of methyl 7-thiastearate (10).

Runname : 8t1926 Acquired Nominal data (Zero baseline). 22 Aug 94 5:24
Unsmoothed data only:

Figure 2.2. Gas chromatogram of methyl 7-thiastearate (10).

9t3116 Scan 753 RT=16:43 100%=159489 mv 6 Jan 97 8:29
LRP +EI d0

Figure 2.3. Mass spectrum of methyl 7-thiastearate (10).

Figure 2.4. ^1H NMR spectrum of methyl 7-thiastearate (10).

Figure 2.5. Partial ^{13}C NMR spectrum of methyl 7-thiastearate (10).

2.2.2 Methyl [9,9-²H₂]-7-thiastearate ([9,9-²H₂]-10)

[9,9-²H₂]-10 (8% est. overall yield) was synthesized as shown in Figure 2.6. The sequence of steps was as follows: [1,1-²H₂]-1-*p*-toluenesulfonyloxydecane, obtained from the reduction¹⁰³ of the corresponding acid followed by tosylation,¹⁰⁴ was converted to [3,3-²H₂]-dodec-1-yne using lithium acetylide-ethylenediamine complex.^{103,105} This material was cleaved¹⁰⁶ to yield crude [2,2-²H₂]-undecanoic acid which was contaminated with ca. 10% of the overoxidation product (decanoic acid). The crude [2,2-²H₂]-undecanoic acid was reduced¹⁰³ to provide [2,2-²H₂]-undecan-1-ol. The corresponding tosylate,¹⁰⁴ [2,2-²H₂]-1-*p*-toluenesulfonyloxyundecane, was used to S-alkylate the dilithio salt of 6-mercaptohexanoic acid.¹⁰⁰ Methylation of the carboxyl function via BF₃/MeOH¹⁰¹ followed by purification (silica gel, 4% EtOAc/hexanes) yielded various fractions containing methyl [9,9-²H₂]-7-thiastearate, contaminated to various degrees with methyl 7-thiaheptadecanoate derived from decanoic acid (see above). An essentially pure fraction of the title compound, as determined by GC (Figure 2.7), could be obtained in this manner.

The deuterated substrate consisted entirely of dideuterated species (within experimental error) as determined by MS. The mass spectrum (Figure 2.8) was in accord with our assigned structure: *m/z* 318 (M⁺), 287 [(M - OCH₃)⁺], 189 [(CH₃(CH₂)₈-CD₂CH₂S)⁺], 129 [(M - CH₃(CH₂)₈CD₂CH₂S)⁺]. The ¹H and ¹³C NMR spectra were consistent with the location of the deuterium label. In the ¹H NMR spectrum (Figure 2.9), the signal at 2.47 ppm present as two overlapping triplets in the unlabelled compound, was collapsed to an overlapping singlet (C-8-H₂) and triplet (C-6-H₂). The signal assigned to C-9 at 29.77 ppm had disappeared in the ¹³C NMR spectrum of methyl [9,9-²H₂]-7-thiastearate ([9,9-²H₂]-10) (Figure 2.10 and Appendix B). An upfield β-isotope shift (0.19 ppm) was observed for the ¹³C resonances at C-8 and C-11 and the signal assigned to C-12 experienced a γ-isotope shift (0.06 ppm).

Figure 2.6. Synthesis of methyl [9,9-²H₂]-7-thiastearate ([9,9-²H₂]-10).

Runname : 81927 Acquired Nominal data (Zero baseline). 22 Aug 94 5:52
Unsmoothed data only:

Figure 2.7. Gas chromatogram of methyl [9,9-²H₂]-7-thiastearate ([9,9-²H₂]-10).

81927 Scan 774 RT=17:15 100%=10377 mv 22 Aug 94 5:52
LRP +EI s-942

Figure 2.8. Mass spectrum of methyl [9,9-²H₂]-7-thiastearate ([9,9-²H₂]-10).

Figure 2.9. ^1H NMR spectrum of methyl $[9,9\text{-}^2\text{H}_2]$ -7-thiastearate ($[9,9\text{-}^2\text{H}_2]$ -10).

Figure 2.10. Partial ^{13}C NMR spectrum of methyl $[9,9\text{-}^2\text{H}_2]$ -7-thiastearate ($[9,9\text{-}^2\text{H}_2]$ -10).

2.2.3 Methyl [10,10-²H₂]-7-thiastearate ([10,10-²H₂]-10)

The synthesis of [10,10-²H₂]-10 (7.4% overall est. yield) was carried out as outlined in Figure 2.11. Malonate extension¹⁰⁷ of the [1,1-²H₂]-1-bromononane, prepared by reduction¹⁰³ of the corresponding acid followed by bromination,¹⁰⁸ gave [3,3-²H₂]-undecanoic acid. This compound was reduced¹⁰³ to provide [3,3-²H₂]-undecan-1-ol which in turn was tosylated¹⁰⁴ to yield [3,3-²H₂]-1-*p*-toluenesulfonyloxyundecane. S-alkylation¹⁰⁰ of 6-mercaptohexanoic acid using [3,3-²H₂]-1-*p*-toluenesulfonyloxyundecane, followed by methylation¹⁰¹ and purification (silica gel, 4% EtOAc/hexanes) gave methyl [10,10-²H₂]-7-thiastearate ([10,10-²H₂]-10) (Figure 2.12).

The deuterium content of the substrate was shown to be essentially 100% d₂ (within experimental error) by GC/MS. The MS, ¹H and ¹³C NMR data were in accord with our assigned structure. The ¹H NMR and MS data were similar to those obtained for methyl 7-thiastearate (10) (Figure 2.4) and methyl [9,9-²H₂]-7-thiastearate ([9,9-²H₂]-10) (Figure 2.8), respectively. The ¹³C NMR spectrum (Figure 2.13 and Appendix B) was consistent with the location of the deuterium label: the signal assigned to the C-10 at 28.99 ppm had disappeared. ¹³C resonances assigned to C-8, C-9, C-11 and C-12 were isotope shifted upfield: 0.09 ppm (γ-effect), 0.26 ppm (β-effect), 0.22 ppm (β-effect) and 0.06 ppm (γ-effect), respectively.

Figure 2.11. Synthesis of methyl [10,10-²H₂]-7-thiastearate ([10,10-²H₂]-10).

Runname : 8t1972 Acquired Nominal data (Zero baseline). 24 Aug 94 7:19
Unsmoothed data only:

Figure 2.12. Gas chromatogram of methyl $[10,10\text{-}^2\text{H}_2]$ -7-thiastearate ($[10,10\text{-}^2\text{H}_2]$ -10).

Figure 2.13. Partial ^{13}C NMR spectrum of methyl $[10,10\text{-}^2\text{H}_2]$ -7-thiastearate ($[10,10\text{-}^2\text{H}_2]$ -10).

Part 2: Trial Incubation of Substrates

This experiment was carried out in order to examine the formation of the unlabelled and labelled 7-thiaolefinic products (**11**, [9-²H₁]-**11** and [10-²H₁]-**11**) (Figure 2.14) from the corresponding substrates. Thus, ca. 25 mg of each substrate (**10**, [9,9-²H₂]-**10** and [10,10-²H₂]-**10**) was incubated for ca. 24 h at 30 °C with growing cultures (150 mL) of *S. cerevisiae* ATCC 12341 (baker's yeast).⁸⁹ The yeast cells grew out to a normal extent in each experiment and were collected by centrifugation. Examination of the methylated dichloromethane extracts of the culture medium (Chapter 5, 5.14.2) obtained in each experiment revealed only the presence of starting material. The cellular fatty acid fraction from each incubation experiment was isolated by a standard hydrolysis (1 M 50% ethanolic KOH solution, (Chapter 5, 5.14.1))/methylation (CH₂N₂, (Chapter 5, 5.15)) procedure and analyzed by GC/MS (Figures 2.15-2.18 and Table 2.1). Interestingly, a dramatic reduction in the percent desaturation of [9,9-²H₂]-**10** relative to **10** (9-fold) and [10,10-²H₂]-**10** (12-fold) was noted which pointed to a possible large isotope effect at C-9 (Table 2.1). The slight increase (1.4 fold) in percent desaturation of [10,10-²H₂]-**10** relative to **10** (Table 2.1) cannot be interpreted with any degree of confidence and demonstrates the importance of using a competitive approach in our intermolecular kinetic isotope effect (KIE) studies. No evidence for ω-oxidized (chain-shortened) products was found. This excluded the possibility of extraneous isotopic enrichment in the substrate or product.

The deuterated methyl 7-thiaoleate products ([9-²H₁]-**11**, [10-²H₁]-**11**) consisted entirely of monodeuterated species (within experimental error) as determined by GC/MS. The mass spectra (Figures 2.19 and 2.20) were in accord with proposed structures: for unlabelled methyl thiaoleate (**11**): m/z 314 (M⁺); m/z 152 [(CH₃(CH₂)₆CH=CH-CH=CH₂)⁺], and for labelled methyl thiaoleates ([9-²H₁]-**11**, [10-²H₁]-**11**): m/z 315 (M⁺); m/z 153 [(CH₃(CH₂)₆CH=CHCD=CH₂)⁺]. The (Z)-stereochemistry of the product derived from methyl 7-thiastearate (**10**) had previously been confirmed using ¹H NMR (300 MHz, AB quartet of triplets, J=10-11 Hz at ca. 5 ppm).⁹³ In addition, a comparison between ¹³C NMR spectra of the cell extracts obtained from incubation of methyl 7-

thiastearate (**10**) (Figure 2.21) and methyl $[10,10\text{-}^2\text{H}_2]\text{-7-thiastearate}$ ($[10,10\text{-}^2\text{H}_2]\text{-10}$) (Figure 2.22) was used to confirm the location of the deuterium label in methyl $[10\text{-}^2\text{H}_1]\text{-7-thiaoleate}$ ($[10\text{-}^2\text{H}_1]\text{-11}$). The signal assigned to C-10 at 132.78 ppm had disappeared. A β -isotope shift (0.13 ppm) for the signal assigned to C-9 (125.63 ppm) was consistent with the presence of deuterium label at C-10 in methyl $[10\text{-}^2\text{H}_1]\text{-7-thiaoleate}$ ($[10\text{-}^2\text{H}_1]\text{-11}$) (Figure 2.22). Because of the very low conversion of the C-9-dideuterated substrate ($[9,9\text{-}^2\text{H}_2]\text{-10}$), the spectrum of the corresponding methyl $[9\text{-}^2\text{H}_1]\text{-7-thiaoleate}$ ($[9\text{-}^2\text{H}_1]\text{-11}$) could not be observed in the ^{13}C NMR spectrum of the cell extracts obtained in this experiment. However, there is no reason to suspect that the label had been scrambled in the desaturation-catalyzed reaction.

Figure 2.14. Biosynthesis of methyl 7-thiaoleate (**11**), methyl $[9\text{-}^2\text{H}_1]\text{-7-thiaoleate}$ ($[9\text{-}^2\text{H}_1]\text{-11}$), and methyl $[10\text{-}^2\text{H}_1]\text{-7-thiaoleate}$ ($[10\text{-}^2\text{H}_1]\text{-11}$).

Runname : 81908 Acquired Nominal data (Zero baseline). 11 Aug 94 8:53
Unsmoothed data only:

Figure 2.15. Cellular fatty acid profile of control culture of *S. cerevisiae*.

Runname : 81907 Acquired Nominal data (Zero baseline). 11 Aug 94 8:29
Unsmoothed data only:

Figure 2.16. Cellular fatty acid profile of *S. cerevisiae*, grown in a medium supplemented with methyl 7-thiastearate (10).

Runname : 81929 Acquired Nominal data (Zero baseline). 22 Aug 94 6:51
Unsmoothed data only:

Figure 2.17. Cellular fatty acid profile of *S. cerevisiae*, grown in a medium supplemented with methyl [9,9-²H₂]-7-thiastearate ([9,9-²H₂]-10).

Runname : 81971 Acquired Nominal data (Zero baseline). 24 Aug 94 6:44
Unsmoothed data only:

Figure 2.18. Cellular fatty acid profile of *S. cerevisiae*, grown in a medium supplemented with methyl [10,10-²H₂]-7-thiastearate ([10,10-²H₂]-10).

Table 2.1. Incubation of methyl 7-thiastearate (**10**), methyl [9,9-²H₂]-7-thiastearate ([9,9-²H₂]-**10**) and methyl [10,10-²H₂]-7-thiastearate ([10,10-²H₂]-**10**) using *S. cerevisiae*: Fatty acid profiles.

Exp.	Substrate Incubated	% Composition of Fatty Acids (FA) ^a						% Incorp. Thia FA ^b	% Desat. Thia FA ^c
		16:1	16:0	18:1	18:0	Thia-18:1	Thia-18:0		
Control	-	45	13	39	3	-	-	-	-
1	10	40	12	27	3	8	10	18	44
2	[9,9- ² H ₂]- 10	42	12	26	1	1	18	19	5
3	[10,10- ² H ₂]- 10	41	13	33	3	6	4	10	60

^a The relative amounts of each fatty acid component are expressed as a % of the total fatty acids; they were determined using GC/MS Total Ion Current (TIC) chromatograms. Each entry represents an average value based on two to three GC/MS runs (standard deviation = 0.5%).

^b % Incorp. Thia FA refers to the ratio: $[(\text{Thia-18:0} + \text{Thia-18:1})_{\text{cell}} \times 100] / (\text{Total FA})_{\text{cell}}$.

^c % Desat. Thia FA refers to the ratio: $[(\text{Thia-18:1})_{\text{cell}} \times 100] / (\text{Thia-18:0} + \text{Thia-18:1})_{\text{cell}}$.

8:1907 Scan 756 RT=16.55 100%=40801 mv 11 Aug 94 8:29
LRP +EI p-do

Figure 2.19. Mass spectrum of methyl 7-thioleate (**11**).

81929 Scan 755 RT=16.54 100%=4891 mv 22 Aug 94 6:51
LRP +EI p-9d2

Figure 2.20. Mass spectrum of methyl [9-²H₁]-7-thiaoleate ([9-²H₁]-11).

Figure 2.21. Partial ¹³C NMR spectrum of the cell extracts obtained from incubation of methyl 7-thiastearate (10).

Figure 2.22. Partial ^{13}C NMR spectrum of the cell extracts obtained from incubation of methyl $[10,10\text{-}^2\text{H}_2]\text{-7-thiastearate}$ ($[10,10\text{-}^2\text{H}_2]\text{-10}$).

Part 3: Intermolecular Kinetic Isotope Effect (KIE) Studies

For the determination of the competitive intermolecular isotope effect,⁹⁹ a mixture consisting of approximately equal amounts of each deuterated and nondeuterated material was prepared (total ca. 25 mg). Each incubation was carried out three times and the six starting mixtures were individually prepared. A small portion of this mixture was examined by GC/MS to determine the exact d_0/d_2 ratio. The remaining portion of the starting sample was administered as a 5% w/v ethanolic solution to growing cultures (150 mL) of *S. cerevisiae*.⁸⁹ After 24 h of growth at 30 °C, the cellular fatty acids (ca. 30 mg) were isolated by a standard hydrolysis (1 M 50% ethanolic KOH, (Chapter 5, 5.14.1))/methylation (CH_2CN_2 , (Chapter 5, 5.15)) sequence, and the deuterium content of thiaoleate products (d_0/d_1) was assessed by GC/MS. The fatty acid profile from incubation of each mixture is shown in Figures 2.23, 2.24 and Table 2.2.

Runname : 812665 Acquired Nominal data (Zero baseline). 5 Dec 94 8:13
Unsmoothed data only:

Figure 2.23. Cellular fatty acid profile of *S. cerevisiae*, grown in a medium supplemented with a mixture of methyl 7-thiastearate (10)/methyl [9,9- $^2\text{H}_2$]-7-thiastearate ([9,9- $^2\text{H}_2$]-10).

Runname : 812664 Acquired Nominal data (Zero baseline). 5 Dec 94 7:43
Unsmoothed data only:

Figure 2.24. Cellular fatty acid profile of *S. cerevisiae*, grown in a medium supplemented with a mixture of methyl 7-thiastearate (10)/methyl [10,10- $^2\text{H}_2$]-7-thiastearate ([10,10- $^2\text{H}_2$]-10).

Table 2.2. Incubation of a mixture of methyl 7-thiastearate (**1**)/methyl [9,9-²H₂]-7-thiastearate ([9,9-²H₂]-**10**) and methyl 7-thiastearate (**10**)/methyl [10,10-²H₂]-7-thiastearate ([10,10-²H₂]-**10**) using *S. cerevisiae*: Fatty acid profiles.

Exp.	Mixture Incubated	% Composition of Fatty Acids (FA) ^a						% Incorp. Thia FA ^b	% Desat. Thia FA ^{c,d}
						Thia-18:1	Thia-18:0		
		16:1	16:0	18:1	18:0	18:1	18:0		
1	10 , [9,9- ² H ₂]- 10	46	19	24	1	2	8	10	20
2	10 , [9,9- ² H ₂]- 10	45	19	25	1	2	8	10	20
3	10 , [9,9- ² H ₂]- 10	43	21	24	1	2	9	11	18
1	10 , [10,10- ² H ₂]- 10	42	22	25	2	6	3	9	67
2	10 , [10,10- ² H ₂]- 10	41	22	27	2	5	3	8	63
3	10 , [10,10- ² H ₂]- 10	43	21	26	1	6	3	9	67

^a The relative amounts of each fatty acid component are expressed as a % of the total fatty acids; they were determined using GC/MS Total Ion Current (TIC) chromatograms. Each entry represents an average value based on two to three GC/MS runs (standard deviation = 0.5%).

^b % Incorp. Thia FA refers to the ratio: [(Thia-18:0 + Thia-18:1)_{cell} × 100] / (Total FA)_{cell}.

^c % Desat.^d Thia FA refers to the ratio: [(Thia-18:1)_{cell} × 100] / (Thia-18:0 + Thia-18:1)_{cell}.

^d % Desat. Thia FA is different from overall % conv. of this compound (see below) which refers to the ratio: [Thia-18:1 (mg) × 100] / [Mixture Incubated (mg)].

The total fatty acid content in each cell extract was estimated to be ca. 20 mg using methyl heptadecanoate as an internal standard and the amount of thia olefinic products was calculated for each incubation: **11**, [9-²H₁]-**11** mixture (ca. 0.4 mg) and **11**, [10-²H₁]-**11** mixture (ca. 1 mg). Thus, the overall conversion of substrate (total ca. 25 mg) to product was estimated as follows: **10**, [9,9-²H₂]-**10** (ca. 2%) and **10**, [10,10-²H₂]-**10** (ca. 4%). Since the overall percent conversion of incubated mixture to products was less than 10% for each incubation, the intermolecular kinetic isotope effects (KIEs) (k_H/k_D) was measured via the following equation (Appendix C):⁹⁹

$$\frac{k_H}{k_D} = \frac{P_H / P_D}{S_H^0 / S_D^0}$$

where, P_H / P_D is the ratio of the nondeuterated to monodeuterated methyl 7-thiaoleate (11) and S_H^0 / S_D^0 is the ratio of the nondeuterated to dideuterated methyl 7-thiasubstrates (10). Thus,

$$\frac{k_H}{k_D} = \frac{(\%d_0)_P}{(\%d_1)_P} / \frac{(\%d_0)_S}{(\%d_2)_S}$$

It should be noted that k_H/k_D can be calculated as the ratio of isotopic species in the products or in the unchanged substrates relative to the ratio in the original substrate mixture. However, it is self-evident that the measurement of isotopic species in the unreacted substrates for small amounts of conversion does not constitute an accurate method of determining rate ratios.⁹⁹

The analytical data is displayed in Table 2.3. A large primary deuterium isotope (7.1 ± 0.2 , average of 3 experiments $\pm$ standard deviation) was determined for the carbon-hydrogen bond cleavage at C-9, while the C-10-H bond-breaking step was shown to be essentially insensitive to deuterium substitution ($KIE = 1.03 \pm 0.09$, average of 3 experiments $\pm$ standard deviation). The observed isotope effect at C-9 is close to the theoretical maximum value for a primary deuterium isotope effect at 30 °C namely $k_H/k_D = 6.8$.⁹⁶ However, it should be noted that a small proportion (<10%) of the observed isotope effect may be due to an α -secondary isotope effect.¹⁰⁹ On the other hand, the magnitude of the intrinsic primary deuterium isotope effect at C-9 may well be higher since our estimated value is derived via an intermolecular competition experiment and is therefore subject to partial masking by other enzymatic steps. Large intrinsic primary deuterium isotope effects are not uncommon for biological hydrocarbon activation thought to involve iron-oxo species.¹⁰⁹

Table 2.3. Intermolecular isotopic discrimination in Δ^9 desaturation of methyl [9,9- $^2\text{H}_2$]-7-thiastearate ([9,9- $^2\text{H}_2$]-10) and methyl [10,10- $^2\text{H}_2$]-7-thiastearate ([10,10- $^2\text{H}_2$]-10).

Exp.	Isotopic Composition (% mol) ^a						KIE
	Substrates ^b			Products			
	d ₀	9-d ₂	10-d ₂	d ₀	9-d ₁	10-d ₁	
1	47.2 ± 0.7	52.8 ± 0.7		86.8 ± 0.4	13.2 ± 0.4		7.4 ± 0.3
2	47.0 ± 0.7	53.0 ± 0.7		86.1 ± 0.8	13.9 ± 0.8		7.0 ± 0.4
3	46.8 ± 0.3	53.2 ± 0.3		86.8 ± 0.4	14.0 ± 0.4		7.0 ± 0.2
							(7.1 ± 0.2) ^c
1	49.2 ± 0.4		50.8 ± 0.4	52.3 ± 0.8		47.7 ± 0.8	1.13 ± 0.03
2	50.9 ± 0.3		49.1 ± 0.3	50.3 ± 0.1		49.7 ± 0.1	0.98 ± 0.01
3	49.7 ± 0.4		50.3 ± 0.4	49.2 ± 0.6		50.8 ± 0.6	0.98 ± 0.02
							(1.03 ± 0.09) ^d

^a The % mol of each isotopic species is given as an average value based on two to three GC/MS runs. ^b The six starting mixtures were individually prepared. ^c The average KIE at C-9 ± standard deviation. ^d The average KIE at C-10 ± standard deviation.

These results confirmed that desaturation is initiated at C-9 by a relatively slow, isotopically sensitive step while the second C–H cleavage at C-10 appears to be fast as might be expected for the collapse of an unstable intermediate such as radical (pathway b) or a carbocation (pathway c) as shown in Figure 1.29 (Chapter 1). Pathway a, in this mechanistic scheme, can be an option but only if the dehydration reaction is a very fast process. Thus, Δ^9 desaturation involves stepwise, not synchronous removal of the two hydrogens and the position of the initial oxidation is clearly at C-9 as predicted by previous studies in our group using 9,10 sulfur-substitution as a probe of cryptoregiochemistry (Chapter 1, 1.4.5).^{89,92}

Interestingly, other laboratories have obtained a similar pattern of kinetic isotope effects (KIEs) (one high, one low) for other desaturations. Bloch *et al.*^{24,37} inferred the existence of a kinetic isotope effect at C-9 but not C-10 for the whole cell bacterial system by measuring the ratio of $^3\text{H}/^{14}\text{C}$ (a competitive method) in unchanged (*R*)-9 and (*R*)-10-tritiated stearic acid (Chapter 1, 1.4.1). The $^3\text{H}/^{14}\text{C}$ ratio of unchanged (*R*)-9-

tritiated stearic acid was considerably higher than the original value, indicating a substantial kinetic tritium isotope effect at the (*R*)-9-position. There was no such effect at the (*R*)-10-position. Thus, they suggested that the removal of the two hydrogens is not synchronous. In addition, a large isotope effect at C-6 but not at C-7 was deduced for a Δ^6 desaturase in *Tetrahymena* by measuring the incorporation of various deuterium-labelled substrates.¹¹⁰ Finally, Baillie *et al.*¹¹¹ reported a substantial intramolecular primary deuterium isotope effect at C-4 (5.58) and a significantly smaller value at C-5 (1.62) for the cytochrome P-450-mediated 4,5-dehydrogenation of valproic acid (9) (Chapter 1, Figure 1.28)-a reaction thought to be initiated by hydrogen abstraction at C-4. These data suggest a common mechanistic model for these systems of the type proposed in Figure 1.29 (Chapter 1).

Our results and those cited above differ from those of Morris⁴² and Johnson *et al.*⁴⁶ who inferred that a primary isotope effect was operating on the C-H cleavage at both C-9 and C-10 for Δ^9 desaturases of *C. vulgaris* and hen liver (Chapter 1, 1.4.2). These groups carried out a number of non-competitive isotope effect experiments using *erythro*- and *threo*-9,10-labelled stearates which led to the suggestion that the removal of hydrogen from C-9 and C-10 is a "simultaneous, concerted" process for these systems. However, it should be pointed out that:

(a) The stereochemical purity of the synthetic dideuterated and ditritiated *erythro*- and *threo*-substrates was not determined by these groups.^{42,46} Results could only be interpreted if the purity of the *erythro*- and *threo*-substrates was the same.

(b) The results of non-competitive experiments are always less secure than competitive methods since identical conditions and very high purity of substrates are required for a correct evaluation of the kinetic isotope effect.⁹⁹

(c) No errors were reported for the proportion of methyl dideuterated (d_2) and monodeuterated (d_1) oleate, derived from 1-2% incorporation of the dideuterated *erythro*- and *threo*-stearates, respectively.⁴² The mass spectral interference of these isotopic species with large amounts of endogenous methyl oleate (d_0) clearly increases the uncertainty in the results. Further investigation is obviously required to determine the kinetic mechanisms of Δ^9 desaturases from these sources.

Summary: The work described here,¹¹² represents the first direct determination of the intermolecular primary deuterium isotope effects involved in yeast Δ^9 desaturation. The success of our methodology is in large measure due to the fact that the first C–H bond breaking step in fatty acid desaturation is a kinetically significant step in the catalytic cycle. Our findings validate the results of previous studies of the desaturation process which were obtained using thia-substrates (Chapter 1, 1.4.5).^{89,92}

Buist, P. H.; Behrouzian, B. *J. Am. Chem. Soc.* **1996**, *118*, 6295.

Future Work: In the light of our results, the *C. vulgaris* Δ^9 desaturating system should be re-examined using our approach. In addition, it would be interesting to apply our methodology to the study of other desaturases such as the recently-discovered soluble, diiron plant Δ^9 desaturase for which no mechanistic information is currently available.

Chapter 3

Fluorinated Fatty Acids: New Mechanistic Probes for Desaturases

3.1 Experimental Approach

After establishing the cryptoregiochemistry of yeast Δ^9 desaturation,¹¹² the next step was to determine how the putative radical intermediate collapses to olefin (Figure 3.1). As mentioned in Chapter 1 (Figures 1.24 and 1.27),^{66,85} Newcomb *et al.* recently postulated that hydroxylation of unactivated hydrocarbons mediated by cytochrome P-450 and MMO proceeds by a “concerted, non-synchronous” mechanism. If this mechanism is operative in the closely-related fatty acid Δ^9 desaturation reaction, then the intermediacy of a C-9 hydroxylated species (Figure 3.1, pathway a) merits serious consideration.

Figure 3.1. Different pathways for the mechanism of desaturation.

We decided to examine the possibility of using the fluorine substituent as a new mechanistic probe for the hydroxylated intermediate in fatty acid desaturation reaction (Figure 3.1, pathway a). It has been known for some time that fluorine can have profound effects on biological activity.¹¹³ Fluorine is typically regarded as a sterically unobtrusive substituent, since its small van der Waals radius (1.35 Å) closely mimics that of hydrogen (1.20 Å). This allows fluorinated analogues to be incorporated into the organism and to follow the metabolic pathway of the parent hydrogen compounds. In addition, fluorine is a good leaving group, and a large number of analogues have been discovered that inhibit enzymes irreversibly by covalent modification of nucleophilic moieties in the catalytic site.¹¹³

Our experimental approach involved the synthesis of racemic methyl 9-fluorostearate (**12**) and methyl 10-fluorostearate (**13**) followed by incubation using Δ^9 desaturase enzyme. Since it is known that the yeast Δ^9 desaturase abstracts the *pro-R* hydrogens at C-9 and C-10 (Chapter 1, 1.3), and that oxidation is initiated at C-9 (Chapter 2),¹¹² the (*R*)-9-fluoro enantiomer cannot be oxidized (Figure 3.2). If a discrete 9-hydroxy intermediate is involved in Δ^9 desaturation, then the corresponding (*S*)-9-fluoro enantiomer would give a 9,9-fluorohydrin which could collapse spontaneously to give ketone. (The α -hydroxylation of a fluorinated steroid has been shown to give a keto product).¹¹⁴ (*R*)-10-fluoro enantiomer would give an *erythro*-1,2-fluorohydrin.

Figure 3.2. 9- and 10-Fluoro fatty acids as probes for a putative 9-hydroxy intermediate.

3.2 Results and Discussion

Part 1: Synthesis of Substrates

3.2.1 Methyl (*R,S*)-9-fluorostearate (12)

The methyl (*R,S*)-9-fluorostearate (12) was synthesized in 21.4% est. overall yield (from aldehyde) as shown in Figure 3.3. The coupling reaction¹¹⁵ of nonylmagnesium bromide with 9-decenal, prepared by oxidation of 9-decen-1-ol using dimethyl sulfoxide “activated” by oxalyl chloride,¹¹⁶ yielded (*R,S*)-1-nonadecen-10-ol. Fluorination of this compound using diethylaminosulfur trifluoride (DAST)¹¹⁷ gave (*R,S*)-10-fluoro-1-nonadecene. Oxidation¹⁰⁶ of the terminal double bond of this material followed by methylation¹⁰¹ yielded the title compound.

The reaction of DAST with alcohols to replace the hydroxyl group with fluorine appears to be a broadly general reaction under mild conditions.¹¹⁷ Different runs of this reaction showed that a better yield of fluorinated compound was obtained for reaction time between three and four hours at low temperatures (ca. - 45 °C). The only byproducts for all fluorinations were various olefinic regioisomers via an elimination side reaction. These byproducts were cleaved to give mono- and diacidic compounds in the subsequent oxidation step. After methylation, these chain-shortened products were removed by flash chromatography (silica gel, 2% EtOAc/hexanes) and essentially pure methyl (*R,S*)-9-fluorostearate (12) was obtained as determined by GC (Figure 3.4).

The elemental analysis confirmed the molecular formula of C₁₉H₃₇FO₂ for this compound. The mass spectral (Figure 3.5), ¹H NMR (Figure 3.6) and ¹⁹F NMR (Figure 3.7) data were in accord with our structural assignments. The mass spectrum of this material (Figure 3.5) featured the following characteristic fragment ions: *m/z* 296 [(M - HF)⁺], *m/z* 264 [(M - HF) - MeOH)⁺]. In the ¹H NMR spectrum (Figure 3.6), the signal of the hydrogen atom at C-9 (CHF) appeared at 4.45 ppm as doublet of multiplets with

coupling constant of ca. 48 Hz ($J_{\text{HF}} = 48$ Hz). The fluorine resonance appeared at -181.50 ppm in the ^1H -decoupled ^{19}F NMR spectrum (Figure 3.7).

Figure 3.3. Synthesis of methyl (R,S)-9-fluorostearate (12).

Runname : 9t0515 Acquired Nominal data (Zero baseline). 1 Jun 95 3:25
Unsmoothed data only:

Figure 3.4. Gas chromatogram of methyl (*R,S*)-9-fluorostearate (12).

9t2448 Scan 706 RT=15:50 100%=64958 mv 12 Aug 96 7:57
LRP +EI

Figure 3.5. Mass spectrum of methyl (*R,S*)-9-fluorostearate (12).

Figure 3.6. ^1H NMR spectrum of methyl (*R,S*)-9-fluorostearate (12).

Figure 3.7. ^1H -decoupled ^{19}F NMR spectrum of methyl (*R,S*)-9-fluorostearate (12).

3.2.2 Methyl (*R,S*)-10-fluorostearate (**13**)

Methyl (*R,S*)-10-fluorostearate (**13**) was prepared employing a synthetic route similar to that described for methyl (*R,S*)-9-fluorostearate (**12**) (Figure 3.8). Fluorination¹¹⁷ of (*R,S*)-1-nonadecen-11-ol, synthesized from the Grignard reaction of octylmagnesium chloride with 10-undecenal,¹¹⁵ gave (*R,S*)-11-fluoro-1-nonadecene which in turn was oxidized¹⁰⁶ to (*R,S*)-10-fluorostearic acid. Methylation¹⁰¹ of this compound followed by purification by flash chromatography (silica gel, 2% EtOAc/hexanes) yielded the essentially pure title compound in 19.5% est. overall yield (Figure 3.9).

The molecular formula of $C_{19}H_{37}FO_2$ for methyl (*R,S*)-10-fluorostearate (**13**) was confirmed by elemental analysis. The spectroscopic data was in accord with our assigned structure. Mass and 1H NMR spectra were similar to those obtained for methyl (*R,S*)-9-fluorostearate (**12**) (Figures 3.5 and 3.6, respectively). The fluorine resonance of this compound in 1H -decoupled ^{19}F NMR appeared at -180.46 ppm (Figure 3.10).

Figure 3.8. Synthesis of methyl (*R,S*)-10-fluorostearate (**13**).

Runname : 9t0483 Acquired Nominal data (Zero baseline). 18 May 95 12:52
Unsmoothed data only:

Figure 3.9. Gas chromatogram of methyl (*R,S*)-10-fluorostearate (13).

Figure 3.10. ^1H -decoupled ^{19}F NMR spectrum of methyl (*R,S*)-10-fluorostearate (13).

Part 2: Synthesis of Standards

3.2.3 Racemic methyl *erythro*-9(10)-fluoro,10(9)-hydroxystearate (**14**)

In general, preparation of 1,2-fluorohydrins is difficult. It has been reported that pyridinium polyhydrofluoride as the hydrofluorinating agent for epoxides, is a convenient way to carry out the reaction under mild conditions with minimal complications from polymerization.¹¹⁸ Thus, we decided to synthesize the title compound (**14**) following the pathway shown in Figure 3.11. Epoxidation of methyl elaidate via *m*-chloroperoxybenzoic acid¹¹⁹ gave racemic methyl *trans*-9,10-epoxystearate. The reaction of this compound with pyridinium polyhydrofluoride¹¹⁸ followed by flash chromatography (silica gel, 15% EtOAc/hexanes) yielded racemic methyl *erythro*-9(10)-fluoro,10(9)-hydroxystearate (**14**) as a colourless oil. Only one peak was observed on the gas chromatogram of this compound (Figure 3.12). The mass spectral (Figure 3.13), ¹H NMR (Figure 3.14) and ¹H-decoupled ¹⁹F NMR (Figure 3.15) data were in accord with the proposed structure (see 3.2.4). The following ions were detected in the mass spectrum (Figure 3.13) of this compound: m/z 312 [(M - HF)⁺], m/z 294 [((M - HF) - H₂O)⁺]. The ¹H-decoupled ¹⁹F NMR (Figure 3.15) of this compound demonstrated two fluorine resonances due to two regioisomers at -191.48 and -191.56 ppm. It should be pointed out that this material contained a minor fluorinated contaminant of unknown structure which was detected by ¹⁹F NMR but not by GC/MS and ¹H NMR.

Figure 3.11. Synthesis of racemic methyl *erythro*-9(10)-fluoro,10(9)-hydroxystearate (14).

Runname : 9t0630 Acquired Nominal data (Zero baseline). 26 Jun 95 6:15
Unsmoothed data only:

Figure 3.12. Gas chromatogram of racemic methyl *erythro*-9(10)-fluoro,10(9)-hydroxystearate (14).

9t1156 Scan 764 RT=17:15 100%=131726 mv 20 Nov 95 5:23
LRP +EI

Figure 3.13. Mass spectrum of racemic methyl *erythro*-9(10)-fluoro,10(9)-hydroxystearate (14).

Figure 3.14. ¹H NMR spectrum of racemic methyl *erythro*-9(10)-fluoro,10(9)-hydroxystearate (14).

Figure 3.15. ¹H-decoupled ¹⁹F NMR spectrum of racemic methyl *erythro*-9(10)-fluoro,10(9)-hydroxystearate (14). × denotes impurities.

3.2.4 Racemic methyl *threo*-9(10)-fluoro,10(9)-hydroxystearate (15)

Racemic methyl *threo*-9(10)-fluoro,10(9)-hydroxystearate (**15**) was prepared following the route which was used to synthesize the corresponding *erythro* stereoisomer (Figure 3.16). Racemic methyl *cis*-9,10-epoxystearate, obtained by epoxidation¹¹⁸ of methyl oleate, was treated with pyridinium polyhydrofluoride¹¹⁹ to yield the crude product. This material was purified by flash chromatography (silica gel, 15% EtOAc/hexanes) to give the title compound as a low melting waxy solid which was essentially pure by GC (Figure 3.17). The mass spectrum of this compound was similar to that obtained for racemic methyl *erythro*-9(10)-fluoro,10(9)-hydroxystearate (**14**) (Figure 3.13). The ¹H NMR (Figure 3.18) and ¹H-decoupled ¹⁹F NMR (Figure 3.19) data were in accord with the *threo* structure for this compound. In the ¹H NMR spectrum of **15** (Figure 3.18), the signals assigned to hydrogens α to hydroxyl and fluorine in this material were shifted upfield (0.2 and 0.07 ppm, respectively) relative to those observed for the *erythro* analogue. Typically, an upfield shift has been reported for the ¹H resonance assigned to adjacent *trans*-hydrogens in three-, four-, five-, and six-membered rings relative to those assigned to the corresponding *cis* analogues.¹²⁰ In the ¹H-decoupled ¹⁹F NMR spectrum of **15** (Figure 3.19), an upfield-shift of ca. 4 ppm was observed for the fluorine resonances (-195.53 and -195.66 ppm) compared to those for the corresponding *erythro* stereoisomer.

Figure 3.16. Synthesis of racemic methyl *threo*-9(10)-fluoro,10(9)-hydroxystearate (15).

Runname : 9t1264 Acquired Nominal data (Zero baseline). 5 Dec 95 9:39
Unsmoothed data only:

Figure 3.17. Gas chromatogram of racemic methyl *threo*-9(10)-fluoro,10(9)-hydroxystearate (15).

Figure 3.18. ^1H NMR spectrum of racemic methyl *threo*-9(10)-fluoro,10(9)-hydroxystearate (15).

Figure 3.19. ^1H -decoupled ^{19}F NMR spectrum of racemic methyl *threo*-9(10)-fluoro,10(9)-hydroxystearate (15). x denotes impurities.

Part 3: Searching for Hydroxylated Intermediates in Δ^9 Desaturation

Each fluorinated substrate (ca. 25 mg), methyl (*R,S*)-9-fluorostearate (12) and methyl (*R,S*)-10-fluorostearate (13), was incubated for 24 h with 150 mL growing cultures of *S. cerevisiae*.⁸⁹ The yeast cells grew out to a normal extent in each experiment and were collected by centrifugation. The cellular fatty acid fraction from each incubation experiment was isolated by a standard hydrolysis (1 M 50% KOH) (Chapter 5, 5.14.1)/methylation (CH_2N_2) (Chapter 5, 5.15) procedure⁸⁹ and analyzed by GC/MS. Examination of the fatty acid profiles revealed that uniformly high incorporation of the fluorinated substrates was achieved. Importantly, a single product (fluoroolefin) was produced in each case (Figures 3.20 - 3.23, Table 3.1). The proposed structure of the

fluoroalkenes was consistent with the MS data: m/z 314 (M^+); m/z 294 [$(M - HF)^+$] (methyl 9-fluoro-(*E*)-9-octadecenoate (16) (Figure 3.24) and methyl 10-fluoro-(*E*)-9-octadecenoate (17)) and 1H -decoupled ^{19}F NMR chemical shifts: δ -105.78 (methyl 9-fluoro-(*E*)-9-octadecenoate (16) (Figure 3.25)); -105.57 (methyl 10-fluoro-(*E*)-9-octadecenoate (17) (Figure 3.26)). The (*E*)-stereochemistry of the 9- and 10-fluoroolefinic products (16 and 17) was confirmed by comparison of the observed 1H -decoupled ^{19}F NMR chemical shifts with literature values obtained for (*E*)-7-fluoro-7-tetradecene (-105.6 ppm),¹²¹ (*Z*)-7-fluoro-7-tetradecene (-110.6 ppm)¹²¹ and (*E*)-4-fluoro-4-octene (-105.0 ppm).¹²²

It is known from past experience that if the product of desaturation is sufficiently nonpolar, it accumulates in the cell whereas if the products are sufficiently polar (i.e. oxygenated products), they are excreted as free acids into the culture medium.⁸⁹ However, examination of the methylated dichloromethane extracts of the culture medium obtained in each experiment revealed only the presence of the starting material. Thus, none (< 0.1% of the total fatty acids) of the possible oxygenated products arising from diverted desaturation (Figure 3.2) or their possible decomposition products/metabolites were detected by our analytical methods (GC/MS for non-fluorinated compounds or a combination of GC/MS and 1H -decoupled ^{19}F NMR for fluorinated products).

Figure 3.20. Biosynthesis of methyl 9-fluoro-(*E*)-9-octadecenoate (16) and methyl 10-fluoro-(*E*)-9-octadecenoate (17).

Runname : 9t2308 Acquired Nominal data (Zero baseline). 22 Jul 96 5:32
Unsmoothed data only:

Figure 3.21. Cellular fatty acid profile of control culture of *S. cerevisiae*.

Runname : 9t0551 Acquired Nominal data (Zero baseline). 12 Jun 95 7:31
Unsmoothed data only:

Figure 3.22. Cellular fatty acid profile of *S. cerevisiae*, grown in a medium supplemented with methyl (*R,S*)-9-fluorostearate (12).

Runname : 9f0552 Acquired Nominal data (Zero baseline). 12 Jun 95 8:01
Unsmoothed data only:

Figure 3.23. Cellular fatty acid profile of *S. cerevisiae*, grown in a medium supplemented with methyl (*R,S*)-10-fluorostearate (13).

Table 3.1. Incubation of methyl (*R,S*)-9-fluorostearate (12), methyl (*R,S*)-10-fluorostearate (13), using *S. cerevisiae*: Fatty acid profiles.

Exp.	Substrate Incubated	% Composition of Fatty Acids (FA) ^a						% Incorp. FA (F) ^b	% Desat. FA (F) ^c
		16:1	16:0	18:1	18:0	18:1 (F)	18:0 (F)		
Control	-	45	15	37	3	-	-	-	-
1	12	29	6	20	1	3	41	44	7 (7) ^d
2	13	38	7	16	1	8	30	38	21 (20) ^d

^a The relative amounts of each fatty acid component are expressed as a % of the total fatty acids; they were determined using GC/MS Total Ion Current (TIC) chromatograms. Each experiment was carried out at least twice and each entry represents an average value (standard deviation = 0.5%).

^b % Incorp. FA (F) refers to the ratio: $[(18:0 (F) + 18:1 (F) \times 100]_{\text{cell}} / (\text{Total FA})_{\text{cell}}$.

^c % Desat. FA (F) refers to the ratio: $[(18:1 (F))_{\text{cell}} \times 100] / [(18:0 (F) + 18:1 (F))_{\text{cell}}]$.

^d Values in brackets were obtained by integration of the relevant peaks in the ¹H-decoupled ¹⁹F NMR spectra of the fatty acid extracts.

9:0552 Scan 657 RT=15:18 100%=119980 mv 12 Jun 95 8:01
LRP +EI

Figure 3.24. Mass spectrum of methyl 9-fluoro-(*E*)-9-octadecenoate (16).

Figure 3.25. ^1H -decoupled ^{19}F NMR spectrum of cellular fatty acids of *S. cerevisiae*, grown in a medium supplemented with methyl (*R,S*)-9-fluorostearate (12).

Figure 3.26. ^1H -decoupled ^{19}F NMR spectrum of cellular fatty acids of *S. cerevisiae*, grown in a medium supplemented with methyl (*R,S*)-10-fluorostearate (13).

Failure to detect methyl 9-oxostearate (Figure 3.2) or its biological reduction product, methyl 9-hydroxystearate, in either the cells or the culture medium of the methyl 9-fluorostearate incubation, by GC/MS (appropriate synthetic standards were used to establish anticipated GC retention times), suggests that an alcohol intermediate is not involved in the desaturation reaction. This interpretation was substantiated by the results of the incubation experiment using methyl (*R,S*)-10-fluorostearate (13). If desaturases operated by a hydroxylation-dehydration route, then attempted desaturation of (*R*)-10-fluorostearate (13) should have given an *erythro*-1,2-fluorohydrin (Figure 3.2). This compound was not observed. In order to determine the stability of the 1,2-fluorohydrin under physiological and workup conditions, a trial experiment was performed: ca. 25 mg of the synthetic fluorohydrin was administered to 150 mL of the growing cultures. After 24 h growth at 30 °C, fluorohydrin was detected by GC/MS in the dichloromethane

extract of the culture medium (Chapter 5, 5.14.2). In addition, a synthetic sample of fluorohydrin was subjected to a hydrolysis (1 M 50% ethanolic KOH, (Chapter 5, 5.14.1))/methylation (CH_2N_2 , (Chapter 5, 5.15)) procedure.⁸⁹ The GC/MS analysis of the recovered material revealed the presence of the epoxide as the major decomposition product. However, only fluoroolefinic product was observed in the extract of 10-fluorostearate incubation. Thus, our results do not support Lewis acid-catalyzed dehydration of a hydroxylated intermediate as a route to olefin in the desaturation reaction.

This led us to consider whether the effects of fluorine substitution could be used to distinguish between two remaining alternatives: disproportionation and carbocation pathways (Figure 3.1, pathways b, c). It has been known that the electronegativity of fluorine (4 vs 2.1 for hydrogen) can have pronounced effects on the electron distribution within the molecule and lead to altered reactivity of the substrate.¹¹³ It is interesting to note in this regard that methyl 9-fluoro-(*E*)-9-octadecenoate (**16**) was produced ca. three times less efficiently than the methyl 10-fluoro-(*E*)-9-octadecenoate (**17**) (Table 3.1). This apparent reactivity ratio is consistent with results previously obtained in our group in which a mixture of methyl (*R,S*)-9-fluoropalmitate (**18**) and methyl (*R,S*)-10-fluoropalmitate (**19**) (1:1) (Figure 3.27) was incubated with *S. cerevisiae* and a mixture (2.6:1) of **21:20** was obtained ($k_{\text{FB}}/k_{\text{F}\alpha} = 2.6$).¹²³

Figure 3.27. Biosynthesis of methyl 9-fluoro-(*E*)-9-hexadecenoate (**20**) and methyl 10-fluoro-(*E*)-9-hexadecenoate (**21**).

In order to interpret this trend more precisely, we decided to determine directly the individual α - and β -fluoro substituent effects on desaturation. Previous work¹²³ had shown that the % desaturation of a remotely fluorinated analogue, methyl 16-fluoropalmitate (**22**) (80.6%) was similar to that of the endogenous methyl palmitate (85.8%). Thus the presence of a 16-fluoro fatty acid analogue did not appear to affect the overall rate of the Δ^9 desaturation process. We thus carried out a direct competition experiment between this compound and methyl (*R,S*)-10-fluoropalmitate (**19**) (Figure 3.28). A mixture of methyl (*R,S*)-10-fluoropalmitate (**19**) and methyl 16-fluoropalmitate (**22**) (1.4:1) was incubated with *S. cerevisiae* (150 mL), the cellular fatty acids extracted (1 M 50% ethanolic KOH, Chapter 5, 5.14.1), esterified (CH_2N_2 , Chapter 5, 5.15) and the amount of methyl 10-fluoro-(*E*)-9-hexadecenoate (**21**) and methyl 16-fluoropalmitooleate (**23**) (Figure 3.28) formed from each substrate in the cellular extract evaluated by ^1H -decoupled ^{19}F NMR (Figure 3.29). From this experiment, we estimated a $k_{\text{H}}/k_{\text{F}\beta} = (\text{am't } \mathbf{23} / \text{am't } \mathbf{21}) \div [(\text{am't } \mathbf{22}) / \text{am't } (\textit{S})\text{-}\mathbf{19}]_{\text{initial}} = (54 / 16) \div [(1 / (0.5)1.4)] = 2.4$. (The value for the amount of 10-fluoro substrate is divided by 2 since only 50% of this substrate can give olefin). Using this ratio, an α -fluorine substituent effect on desaturation can be calculated by combining the results of this competition experiment with that reported previously:¹²³ $k_{\text{H}}/k_{\text{F}\alpha} = k_{\text{H}}/k_{\text{F}\beta} \times k_{\text{F}\beta}/k_{\text{F}\alpha} = 2.4 \times 2.6 = 6.2$

Figure 3.28. Biosynthesis of methyl 10-fluoro-(*E*)-9-hexadecenoate (**21**) and methyl 16-fluoropalmitooleate (**23**).

Figure 3.29. ${}^1\text{H}$ -decoupled ${}^{19}\text{F}$ NMR spectrum of cell extract obtained from incubation of a 1.4/1 mixture of methyl (*R,S*)-10-fluoropalmitate (19)/methyl 16-fluoropalmitate (22). ${}^{19}\text{F}$ resonances due to 19 and 22 as well as the products 21 and 23 are assigned as indicated and the relative intensities are given in parentheses.

The significance of these results is that they are in qualitative agreement with what is known about the effect of fluorine substitution on the related biohydroxylation reaction- rate retardations are felt most strongly α to a fluorine substituent and fall off with increasing distance.^{114,124} Our estimate of the β -fluorine effect ($k_H/k_{F\beta} = 2.4$) on desaturation can easily be accounted for by an inductive effect on the initial hydrogen abstraction step by a strongly electrophilic agent¹²⁵ and is consistent with β -effects reported by Tedder *et al.*¹²⁶ for free-radical substitution of n-butylfluoride by a variety of electrophilic agents in gas phase at 25 °C. (No appropriate chemical model is available for precise mechanistic interpretation of the α -fluorine effects).

Assuming that: (a) the effects of (*S*)-9-fluoro and (*S*)-10-fluoro substitution on non-catalytic steps such as substrate binding are not dramatically different and that (b) fluorine substituent effects on possible carbocation formation are not completely masked by the effects of fluorine substitution on the initial hydrogen abstraction step - an event we know from our isotope effect studies to be kinetically important (Chapter 2),¹¹² then a 9-fluoro substituent should favour the formation of a carbocation at C-9 by overcoming σ -inductive withdrawal with 2p-2 π resonance donation while a 10-fluoro substituent could shut down neighboring carbocation formation by a strong electron withdrawing inductive effect (Figure 3.30).¹²⁷ Thus, if a carbocation was formed at C-9 during desaturation, a much less rate-retarding α -fluorine effect or even an acceleration might be expected since rate accelerations of $k_{F\alpha}/k_H = 71$ due to an α -fluorine effect on carbocation formation are known. Similarly, a far larger β -effect might be anticipated since rate retardations of $k_H/k_{F\beta} = 79$ per β -fluorine atom on the formation of allylic (delocalized) cations have been observed and effects on non-stabilized carbocations should be even greater.¹²⁷ Since these sorts of perturbations on the apparent α - and β -fluorine effects were not observed, we conclude that the intermediacy of the carbocation during desaturation is not supported by our data and disproportionation (Figure 3.1, pathway b) remains a viable alternative. It should be pointed out that if desaturation follows a disproportionation or carbocationic pathway (Figure 3.1 pathways b and c), (*R*)-10-fluoro substrates should be suicide inhibitors. That is, the initially formed radical intermediate at C-9 would be forced to attack the enzyme either directly or after

formation of the carbocation. Unfortunately our experimental approach relies on continuous *in vivo* production of enzyme and inactivation of the Δ^9 desaturase could go undetected under these conditions.

Figure 3.30. 9- and 10-Fluoro fatty acids as probes for a carbocationic intermediate.

Part 4: Attempt to Study Steric Effects of Fluorine Substitution on Δ^9 desaturation

It is of considerable interest from a mechanistic point of view to determine whether the rate of desaturation of racemic 9- and 10-fluoro substrates is affected by steric interactions of the fluorine substituent with the enzyme active site residues. The interpretation of our fluorine substituent effect studies is predicated on the notion that fluorine is a sterically unobtrusive substituent. A substantial apparent steric effect was previously observed in our group for methyl (*R,S*)-12-fluorostearate (**26**) (Figure 3.31, Table 3.2) which bears a fluorine at δ to the site of initial oxidation. It was also shown that both enantiomers of **26** are processed at comparable rates and thus the rate-retarding effect of 12-fluoro substitution is not stereospecific.¹²³ We decided to extend this study by employing methyl (*R,S*)-5-fluorostearate (**24**) and methyl (*R,S*)-7-fluorostearate (**25**) as substrates (Figure 3.31). We elected to use these substrates because they were readily prepared by DAST fluorination¹¹⁷ of the corresponding commercially available alcohols. All spectra and analytical data obtained for these compounds were in accord with their assigned structures. The mass spectral, ¹H NMR were similar to those obtained for methyl (*R,S*)-9-fluorostearate (**12**) (Figures 3.5 and 3.6, respectively). ¹H-decoupled ¹⁹F NMR: -181.26 ppm (methyl (*R,S*)-5-fluorostearate (**24**)) and -180.70 ppm (methyl (*R,S*)-7-fluorostearate (**25**)).

Each sample (ca. 25 mg) was incubated with growing cultures of *S. cerevisiae* (150 mL),⁸⁹ the cellular fatty acids were extracted and analyzed by GC/MS (Figures 3.32, 3.33 and Table 3.2). Substantial incorporation of the 5- and 7-fluorinated substrates was achieved and only fluoroolefin was obtained in each experiment. The MS spectral and ¹H-decoupled ¹⁹F NMR data were consistent with the assigned structure of the fluorinated olefinic products (Figure 3.32). MS: *m/z* 294 [(M - HF)⁺]; 262 [((M - HF) - MeOH)⁺] ((*R,S*)-5-fluorooleate (**27**) and (*R,S*)-7-fluorooleate (**28**)); and ¹H-decoupled ¹⁹F NMR: δ - 181.22 ((*R,S*)-5-fluorooleate (**27**)) and -179.78 ((*R,S*)-7-fluorooleate (**28**)).

Figure 3.31. Biosynthesis of methyl (R,S)-5-fluorooleate (27), methyl (R,S)-7-fluorooleate (28) and methyl (R,S)-12-fluorooleate (29).

Figure 3.32. Cellular fatty acid profile of *S. cerevisiae*, grown in a medium supplemented with methyl (R,S)-5-fluorostearate (24).

Runname : 9c2505 Acquired Nominal data (Zero baseline). 20 Aug 96 6:39
Unsmoothed data only:

Figure 3.33. Cellular fatty acid profile of *S. cerevisiae*, grown in a medium supplemented with methyl (*R,S*)-7-fluorostearate (**25**).

Table 3.2. Incubation of methyl (*R,S*)-5-fluorostearate (**24**), methyl (*R,S*)-7-fluorostearate (**25**) and methyl (*R,S*)-12-fluorostearate (**26**) using *S. cerevisiae*: Fatty acid profiles.

Exp.	Substrate Incubated	% Composition of Fatty Acids (FA) ^a						% Incorp. FA (F) ^b	% Desat. FA (F) ^c
		16:1	16:0	18:1	18:0	18:1 (F)	18:0 (F)		
Control	-	45	13	39	3	-	-	-	-
1	24	30	6	21	1	9	33	42	21
2	25	40	4	19	1	6	30	36	17 (20) ^d
3	26^e	30	8	25	1	2	34	36	6 (5) ^d

^a The relative amounts of each fatty acid component are expressed as a % of the total fatty acids; they were determined using GC/MS Total Ion Current (TIC) chromatograms. Each experiment was carried out at least twice and each entry represents an average value (standard deviation = 0.5%).

^b % Incorp. FA (F) refers to the ratio: $[18:0 (F) + 18:1 (F) \times 100]_{\text{cell}} / (\text{Total FA})_{\text{cell}}$.

^c % Desat. FA (F) refers to the ratio: $[(18:1 (F))_{\text{cell}} \times 100] / [(18:0 (F) + 18:1 (F))_{\text{cell}}]$.

^d Value in bracket was obtained by integration of the relevant peaks in the ¹H-decoupled ¹⁹F NMR spectrum of the fatty acid extracts.

^e Data is from a previous study in our group.¹²³

It is interesting to note that the value for % desaturation of the (*S*)-9-fluorostearate substrate (**12**) (ca. $7\% \div 0.5 = 14\%$) lies midway between that obtained for **24**, **25** and **26** (ca. 21%, 17% and 6%, respectively). (All values for % desaturation are significantly below those obtained for % desaturation of (*S*)-10-fluorostearate substrate (**13**) ($30\% \div 0.5 = 60\%$)). These long-range substituent effects (γ (7F), δ (12F), ϵ (5F)) clearly cannot be due to an electronic influence on the catalytic step but are probably due to unfavourable interactions of the fluorine substituent with the hydrophobic walls of the desaturase binding site. Our observations show that while monofluorination of fatty acid hydrocarbon chains may have minimal impact on the bulk biophysical properties of condensed monolayers,¹²⁸ the effects of the replacement of hydrogen by fluorine on enzyme-substrate interaction are often unexpected.¹²⁹

Summary: We have demonstrated that the use of fluorinated substrates shows considerable promise as a new probe to test the current mechanistic model for desaturation. Only (*E*)-fluoroolefinic products were obtained when racemic 9- and 10-fluoro fatty acids were processed by a *S. cerevisiae* Δ^9 desaturase as determined by GC/MS and ^1H -decoupled ^{19}F NMR analysis.^{123,130} No evidence for the intermediacy of an alcohol or carbocation intermediates was found. α - and β -Fluorine substituent effects on Δ^9 desaturation were measured to be 6.2 and 2.4, respectively.¹²³ Substantial rate-retardation was observed for monofluorinated substrates bearing the fluorine γ , δ and ϵ to initial oxidation.

Buist, P. H.; Behrouzian, B.; Alexopoulos, K. A.; Dawson, B.; Black, B. *J. Chem. Soc. Chem. Comm.* **1996**, 2671.

Buist, P. H.; Behrouzian, B.; Alexopoulos, K. A.; Dawson, B.; Black, B. *J. Chem. Soc. Perkin Trans. I* **1997**, 2617.

Future Work: Further work is required to extend and clarify our observations:

- (a) It would be interesting to determine the fate of methyl (*R*)-10-fluorostearate with an *in vitro* desaturase preparation. (b) Model studies for radical transfer reactions, involving fluoroalkanes would help to understand our findings. (c) Steric effect studies of fluorine substituents with amino acid residues at the yeast Δ^9 desaturase active site clearly require more systematic study using a complete set of fluorinated fatty acids. (d) It would be interesting to carry out analogous experiments using the purified plant Δ^9 desaturase enzyme system. (e) The use of fluorinated substrates can also be applied to other systems such as MMO and cytochrome P-450 in order to link mechanisms of heme- and non-heme-containing iron oxidants.

Chapter 4

Application of Kinetic Isotope Effects (KIEs) to Establish the Cryptoregiochemistry of Oleate Δ^{12} Desaturation

4.1 Experimental Approach

As discussed before (Chapter 1, 1.2), fatty acyl desaturases bear many superficial similarities to hydroxylases. Thus, many researchers have speculated that modification of the active site of fatty acid desaturases or hydroxylases could alter the outcome of the reaction, thereby converting a desaturase to a hydroxylase and vice versa.^{131,132} One example of an apparent diverted desaturation is evident in the biosynthesis of ricinoleic ((*R*)-12-hydroxyoleic) acid (**30**) (Figure 4.1)- a rare but important natural product which accumulates in the seed oil of the castor bean (*Ricinus communis*).¹³³ Ricinoleic acid (**30**) has been termed one of the world's most versatile natural products because it is used directly as a component of lubricants, soaps, adhesive, inks, comestics, etc. and also serves as an industrial feedstock for a variety of important materials such plasticizers, polyesters and polyurethanes.¹³⁴ Interest in the enzyme system responsible for the synthesis of this hydroxy acid has intensified recently in the context of efforts to broaden the scope of its agricultural production.¹³⁵

Figure 4.1. Structure of ricinoleic ((*R*)-12-hydroxyoleic) acid (**30**).

It has been shown that ricinoleate (**32**) (Figure 4.2) is synthesized by direct (*R*)-12-hydroxylation of oleate (**31**) esterified to phosphatidylcholine (PC).^{42,132} The membrane-bound oleate 12-hydroxylase enzyme is similar in many of its biochemical properties to the membrane-bound Δ^{12} desaturase which is responsible for the desaturation of the oleate (**31**) to linoleate (**33**) (Figure 4.2). These similarities include: (a) substrate requirements: oleate esterified to PC;^{42,132} (b) regio- and stereo-selectivity of the reactions: removal of *pro-R*-hydrogen(s) from C-12(13);⁴² (c) b_5 electron-transport system;¹³⁵ (d) inhibition with CN^- but not with CO_2 ;^{133,134} and (e) homology in amino acid sequence.¹³¹ Thus, it has been suggested that the oleate 12-hydroxylase is an example of a modified oleate Δ^{12} desaturase.¹³¹

Figure 4.2. Biosynthesis of ricinoleate (**32**) and linoleate (**33**).

On the basis of Figure 1.29 (Chapter 1), one would predict that if a desaturase could be induced to act as an oxygenase, the regioselectivity of oxygenation should match that of the hydrogen abstraction step involved in the corresponding desaturation reaction.

We demonstrated that this is indeed the case using an *in vivo*, *S. cerevisiae* Δ^9 desaturase using a kinetic isotope effect (KIE) approach (Chapter 2).¹¹² Thus, a critical test of the hypothesis that ricinoleic acid (**30**) is produced by a subtly-modified oleate Δ^{12} desaturase would be to demonstrate that the oleate Δ^{12} desaturase initiates the oxidation of substrate at C-12. We have shown that this is indeed the case by applying the methodology described in the study of the cryptoregiochemistry of the yeast Δ^9 desaturase (Chapter 2, 2.1).

The selected source of the oleate Δ^{12} desaturase enzyme was a pDR₁-transformed strain of *S. cerevisiae* which contains a functional plant oleate Δ^{12} desaturase from *Arabidopsis thaliana* in addition to its own native Δ^9 desaturase.¹³⁶ The availability of this organism provided an extremely convenient opportunity to measure the primary deuterium kinetic isotope effects (KIEs) on the two C–H bond cleavages involved in the Δ^{12} desaturation process since the Δ^{12} desaturase is unstable and has resisted all attempts at purification. Our approach was as follows: the d_1/d_0 ratio of thialinoleate products derived from a ca. 1:1 mixture of methyl 7-thiastearate (**10**) and methyl [12,12-²H₂]-7-thiastearate ([12,12-²H₂]-**10**) was compared to that derived from a ca. 1:1 mixture of methyl 7-thiastearate and methyl [13,13-²H₂]-7-thiastearate ([13,13-²H₂]-**10**). This approach avoids the more complicated synthesis of the dideuterated thiaoleates and assumes, as is reasonable, that Δ^9 desaturation of methyl [12,12-²H₂]-7-thiastearate ([12,12-²H₂]-**10**) and methyl [13,13-²H₂]-7-thiastearate ([13,13-²H₂]-**10**) occurs at equal rates.

4.2 Results and Discussion

Part 1: Synthesis of Substrates

4.2.1 Methyl 7-thiastearate (**10**)

The synthesis of methyl 7-thiastearate (**10**) and its chromatographic (GC) and spectral properties (MS, ^1H and ^{13}C NMR) are described in Chapter 2 (2.2.1).

4.2.2 Methyl [12,12- $^2\text{H}_2$]-7-thiastearate ([12,12- $^2\text{H}_2$]-**10**)

Methyl [12,12- $^2\text{H}_2$]-7-thiastearate ([12,12- $^2\text{H}_2$]-**10**) was synthesized in 4% overall est. yield as outlined in Figure 4.3. Reduction of the heptanoic acid using LiAlD_4 ,¹⁰³ followed by bromination¹³⁶ yielded [1,1- $^2\text{H}_2$]-1-bromoheptane. Cu(I)-Grignard coupling of 5-bromo-1-pentene with this compound in THF at 0 °C gave [6,6- $^2\text{H}_2$]-1-dodecene.¹³⁷ Oxidative cleavage¹⁰⁶ of the [6,6- $^2\text{H}_2$]-1-dodecene yielded [5,5- $^2\text{H}_2$]-undecanoic acid which in turn was reduced¹⁰³ to provide [5,5- $^2\text{H}_2$]-undecan-1-ol. The corresponding tosylate,¹⁰³ [5,5- $^2\text{H}_2$]-1-*p*-toluenesulfonyloxyundecane, was used to S-alkylate the dilithio salt of 6-mercaptohexanoic acid.¹⁰⁰ Methylation¹⁰¹ of the [12,12- $^2\text{H}_2$]-7-thiastearic acid followed by purification (silica gel, 4% EtOAc/hexanes) yielded methyl [12,12- $^2\text{H}_2$]-7-thiastearate ([12,12- $^2\text{H}_2$]-**10**). This compound was essentially pure as determined by GC (Figure 4.4).

Figure 4.3. Synthesis of methyl [12,12- $^2\text{H}_2$]-7-thiastearate ([12,12- $^2\text{H}_2$]-10).

The key step in this synthesis was the Grignard-coupling reaction. A trial Grignard reaction of 5-bromo-1-pentene with nondeuterated 1-bromoheptane and 1-*p*-toluenesulfonyloxyheptane under four different conditions (temperature: 25 °C, 0 °C, solvents: THF and Et₂O) revealed that 1,9-decadiene and tetradecane, obtained from homo-coupling, were the major products. It has been reported that a high yield of cross-coupling product (1-dodecene) can be obtained by performing the reaction in THF solution, at 0 °C or lower temperatures, and in the presence of Cu(I) as the catalysis.¹³⁸ Thus, Grignard coupling of the 5-bromo-1-pentene with nondeuterated 1-bromoheptane under these conditions succeeded; the major impurity was unreacted 1-bromoheptane (estimated to be ca. 20% by GC/MS and ¹H NMR). Trial runs of the subsequent reactions using unlabelled material showed that oxidation and reduction steps were not affected by the presence of unreacted starting compound (1-bromoheptane). After the reduction step the 1-bromoheptane was no longer present as judged by ¹H NMR and GC/MS. Thus, in the synthesis of labelled compound, the crude [6,6-²H₂]-1-dodecene was used in the next step without further purification.

The desired compound ([12,12-²H₂]-**10**) was obtained with high deuterium content (essentially 100 % within experimental error) as determined by GC/MS. The MS, ¹H and ¹³C NMR data were in accord with our assigned structure. The MS and ¹H NMR data were similar to those obtained for methyl [9,9-²H₂]-7-thiastearate ([9,9-²H₂]-**10**) (Chapter 2, Figure 2.8) and methyl 7-thiastearate (**10**) (Chapter 2, Figure 2.4), respectively. The ¹³C NMR spectrum (Figure 4.5 and Appendix B) confirmed the position of the deuterium label: the signal assigned to the C-12 at 29.57 ppm, present in the unlabelled analogous (**10**) (Chapter 2, Figure 2.5 and Appendix B) had disappeared. An upfield β-isotope shift (0.21 ppm) at C-11 and C-13 and an upfield γ-isotope shift (0.06 ppm) at C-10 and C-14 were observed.

Figure 4.4. Gas chromatogram of methyl [12,12-²H₂]-7-thiastearate ([12,12-²H₂]-10).

Figure 4.5. Partial ^{13}C NMR spectrum of methyl $[12,12\text{-}^2\text{H}_2]\text{-7-thiastearate}$ ($[12,12\text{-}^2\text{H}_2]\text{-10}$).

4.2.3 Methyl [13,13-²H₂]-7-thiastearate ([13,13-²H₂]-10)

The title compound was synthesized using the pathway similar to that described for the synthesis of methyl [12,12-²H₂]-7-thiastearate ([12,12-²H₂]-10) (Figure 4.6): [1,1-²H₂]-1-bromohexane, obtained via the LiAlD₄ reduction¹⁰² of the hexanoic acid followed by bromination,¹³⁶ was coupled with 6-bromo-1-hexene to give [7,7-²H₂]-1-dodecene. Oxidation¹⁰⁶ of this compound yielded [7,7-²H₂]-undecanoic acid which in turn was reduced¹⁰³ using LiAlH₄ to yield [6,6-²H₂]-undecan-1-ol. S-alkylation¹⁰⁰ of the dilithio salt of the 6-mercaptohexanoic acid using the corresponding tosylate,¹⁰⁴ [6,6-²H₂]-1-*p*-toluenesulfonyloxyundecane, followed by methylation¹⁰¹ and purification (silica gel, 4% EtOAc/hexanes) gave methyl [13,13-²H₂]-7-thiastearate ([13,13-²H₂]-10) in 6% est. overall yield. This compound was essentially pure as determined by GC (Figure 4.7).

The deuterated substrate consisted entirely of dideuterated species (within experimental error) as determined by GC/MS. The MS, ¹H and ¹³C NMR data were in accord with our assigned structure. The mass and ¹H NMR spectra were similar to those obtained for methyl [9,9-²H₂]-7-thiastearate ([9,9-²H₂]-10) (Chapter 2, Figure 2.8) and methyl 7-thiastearate (10) (Chapter 2, Figure 2.4), respectively. The ¹³C NMR spectrum (Figure 4.8 and Appendix B) was used to confirm the position of the deuterium label: the signal assigned to the C-13 at 29.64 ppm, in the unlabelled compound (10) (Chapter 2, Figure 2.5 and Appendix B) had disappeared. A β-isotope shift was observed at C-12 (0.23 ppm) and C-14 (0.21 ppm) as well as a γ-isotope shift at C-11 (0.06 ppm) and C-15 (0.07 ppm).

Figure 4.6. Synthesis of methyl [13,13- $^2\text{H}_2$]-7-thiastearate ([13,13- $^2\text{H}_2$]-10).

Runname : 1s0023 Acquired Nominal data (Zero baseline). 7 Feb 97 3:07
Unsmoothed data only:

Figure 4.7. Gas chromatogram of methyl $[13,13\text{-}^2\text{H}_2]$ -7-thiastearate ($[13,13\text{-}^2\text{H}_2]$ -10).

Figure 4.8. Partial ^{13}C NMR spectrum of methyl $[13,13\text{-}^2\text{H}_2]$ -7-thiastearate ($[13,13\text{-}^2\text{H}_2]$ -10).

Part 2: Trial Incubation Experiments

The trial experiments were performed: (a) to show the accumulation of linoleic acid ($18:2\Delta^{9,12}$) that is not normally present in the wild-type yeast; (b) to measure the percent incorporation of methyl 7-thiastearate (**10**); and (c) to examine the bioformation of 7-thialinoleic acid (**34**) (Figure 4.9). Thus, ca. 25 mg of methyl 7-thiastearate (**10**) was added as an ethanolic 5% w/v solution to 150 mL of growing cultures of pDR₁-transformed strain of *S. cerevisiae* (Chapter 5, 5.13.2).¹³⁹ The inoculated culture was then incubated at 25 °C for 3 days. After growing the yeast cells to a normal extent, they were collected by centrifugation. The GC/MS of the methylated dichloromethane extracts of the culture medium revealed only the presence of substrate (**10**) (Chapter 5, 5.14.2). The cellular fatty acid fraction (ca. 30 mg) was then isolated by a standard hydrolysis (1 M 50% ethanolic KOH solution, (Chapter 5, 5.14.1))/methylation (CH_2N_2 , (Chapter 5, 5.15))⁸⁹ procedure and analyzed by GC/MS (Figures 4.10, 4.11 and Table 4.1).

The accumulation of cellular methyl linoleate ($18:2\Delta^{9,12}$) was measured to be ca. 2% of the total cellular fatty acids (Figures 4.10, 4.11 and Table 4.1). The mass spectrum of methyl linoleate ($18:2\Delta^{9,12}$) (Figures 4.12) was in accord with its structure: m/z 294 (M^+); m/z 263 [$(\text{M} - \text{OMe})^+$], m/z 150 [$(\text{CH}_3(\text{CH}_2)_4\text{CH}=\text{CHCH}=\text{CHCH}=\text{CH}_2)^+$]. The methyl 7-thiastearate was incorporated into the cells to a significant extent. The methyl 7-thiaoleate (**11**) was identified and its mass spectrum was similar to that shown in Figure 2.19 (Chapter 2). However, no methyl 7-thialinoleate (**34**) was detected in this trial experiment.

Figure 4.9. Biosynthesis of methyl 7-thiaoleate (11) and methyl 7-thialinoleate (34) in *S. cerevisiae* (pDR1 strain).

Runname : 9t2691 Acquired Nominal data (Zero baseline). 2 Oct 96 4:55
Unsmoothed data only:

Figure 4.10. Cellular fatty acid profile of *S. cerevisiae* (pDR1 strain), grown at 25 °C for 3 days.

Runname : 9t2692 Acquired Nominal data (Zero baseline). 2 Oct 96 5:23
Unsmoothed data only:

Figure 4.11. Cellular fatty acid profile of *S. cerevisiae* (pDR1 strain), grown in a medium supplemented with methyl 7-thiastearate (10) at 25 °C for 3 days.

Table 4.1. Incubation of methyl 7-thiastearate (10) using *S. cerevisiae* (pDR1 strain) grown at 25 °C for 3 days: Fatty acid profiles.

Exp.	Substrate Incubated	% Composition of Fatty Acids (FA) ^a								% Incorp. Thia FA ^b
		16:1	16:0	18:2	18:1	18:0	Thia-18:2	Thia-18:1	Thia-18:0	
Control	-	22	18	2	40	18	-	-	-	-
1	10	28	17	2	29	11	0	4	9	13

^a The relative amounts of each fatty acid component are expressed as a % of the total fatty acids; they were determined using GC/MS Total Ion Current (TIC) chromatograms. Each entry represents an average value based on two to three GC/MS runs (standard deviation = 0.5%).

^b % Incorp. Thia FA refers to the ratio: $[(\text{Thia-18:0} + \text{Thia-18:1} + \text{Thia-18:2})_{\text{cell}} \times 100] / (\text{Total FA})_{\text{cell}}$.

92849 Scan 660 RT=14:58 100%=65529 mv 8 Nov 96 6:07
LRP +EI control

Figure 4.12. Mass spectrum of methyl linoleate (18:2Δ^{9,12}).

It had been reported¹³⁶ that when pDR₁ transformants are grown at lower temperatures there is a greater accumulation of the linoleic acid (18:2 $\Delta^{9,12}$): 28 °C (0.6%), 22 °C (6.4%) and 15 °C (9.2%). Thus, we decided to repeat the trial incubation experiment at 15 °C using methyl nondeuterated and dideuterated 7-thia fatty acid analogues (**10**, [12,12-²H₂]-**10** and [13,13-²H₂]-**10**). The dideuterated substrates were incubated to determine the extent of incorporation of these materials and to examine the bioformation of methyl [12-²H₁]-7-thialinoleate ([12-²H₁]-**34**) and [13-²H₁]-7-thialinoleate ([13-²H₁]-**34**) (Figure 4.13). Thus, ca. 25 mg of each substrate (**10**, [12,12-²H₂]-**10** and [13,13-²H₂]-**10**) was added as a 5% w/v ethanolic solution to 150 mL of growing cultures of *S. cerevisiae* (pDR₁ strain)¹³⁹ at 15 °C. After 1 wk, the yeast cells (pDR₁ strain) in the control culture and medium supplemented with **10** and [13,13-²H₂]-**10** were grown to a normal extent. No growth was observed in the medium incubated with [12,12-²H₂]-**10** as it appeared that the yeast cells had occluded the substrate particles present in the medium.

The yeast cells, obtained from incubation of **10** and [13,13-²H₂]-**10**, were collected by centrifugation and hydrolyzed using 1 M 50% ethanolic KOH solution (Chapter 5, 5.14.1).⁸⁹ The cellular fatty acids were then extracted, converted to their methyl esters (CH₂N₂, (Chapter 5, 5.15)) via standard procedures and analyzed by GC/MS (Figures 4.14-4.16 and Table 4.2). The methyl linoleate (18:2 $\Delta^{9,12}$) accumulation was increased to ca. 9% of the total cellular fatty acids. Importantly, methyl 7-thialinoleate (**34**) (Figure 4.15) and methyl [13-²H₁]-7-thialinoleate ([13-²H₁]-**34**) (Figure 4.16) were also detected. The mass spectra (fragmentation pattern) of these compounds was in accord with their proposed structures: for unlabelled methyl 7-thialinoleate (**34**) (Figure 4.17): m/z 281 [(M - OMe)⁺], m/z 150 [(CH₃(CH₂)₄CH=CHCH=CHCH=CH₂)⁺], and for labelled methyl 7-thialinoleate ([13-²H₁]-**34**) (Figure 4.18): m/z 282 [(M - OMe)⁺], m/z 151 [(CH₃(CH₂)₄CD=CHCH=CHCH=CH₂)⁺].

Figure 4.13. Biosynthesis of methyl [12-²H₁]-7-thialinoleate ([12-²H₁]-34) and methyl [13-²H₁]-7-thialinoleate ([13-²H₁]-34).

Figure 4.14. Cellular fatty acid profile of *S. cerevisiae* (pDR1 strain), grown at 15 °C for 1 wk.

Figure 4.15. Cellular fatty acid profile of *S. cerevisiae* (pDR1 strain), grown in a medium supplemented with methyl 7-thiastearate (10) at 15 °C for 1 wk.

Figure 4.16. Cellular fatty acid profile of *S. cerevisiae* (pDR1 strain), grown in a medium supplemented with methyl [13,13- $^2\text{H}_2$]-7-thiastearate ([13,13- $^2\text{H}_2$]-10) at 15 °C for 1 wk.

Table 4.2. Incubation of methyl 7-thiastearate (10) and methyl [13,13-²H₂]-7-thiastearate ([13,13-²H₂]-10) using *S. cerevisiae* (pDR1 strain) grown in a medium at 15 °C for 1 wk: Fatty acid profiles.

Exp.	Substrate Incubated	% Composition of Fatty Acids (FA) ^a								% Incorp. Thia FA ^b
		16:1	16:0	18:2	18:1	18:0	Thia-18:2	Thia-18:1	Thia-18:0	
Control	-	26	30	9	16	19	-	-	-	-
1	10	23	13	9	19	10	1	6	19	26
2	[13,13- ² H ₂]-10	25	18	8	15	12	1	4	17	22

^a The relative amounts of each fatty acid component are expressed as a % of the total fatty acids; they were determined using GC/MS Total Ion Current (TIC) chromatograms. Each entry represents an average value based on two to three GC/MS runs (standard deviation = 0.5%).

^b % Incorp. Thia FA refers to the ratio: $[(\text{Thia-18:0} + \text{Thia-18:1} + \text{Thia-18:2})_{\text{cell}} \times 100] / (\text{Total FA})_{\text{cell}}$.

9:2351 Scan 752 RT=16:42 100%=6631 mv 8 Nov 96 7:09
LRP +EI

Figure 4.17. Mass spectrum of methyl 7-thialinoleate (34).

2a0178 Scan 290 RT=17.21 100%=91614 mv 11 Mar 97 9:38
LRP +EI cd0

Figure 4.18. Mass spectrum of methyl $[13-^2\text{H}_1]$ -7-thialinoleate ($[13-^2\text{H}_1]$ -34).

Part 3: Intermolecular Kinetic Isotope Effect (KIE) Studies

The intermolecular competitive isotope effect study was performed as described in chapter 2 (Part 3): a ca. 1:1 mixture of each deuterated and nondeuterated substrate (ca. 25 mg) was prepared. Each incubation was carried out two times and the four starting mixtures were individually prepared. The d_0/d_2 ratio of each sample was first determined by GC/MS using the following ions: m/z 316 (d_0), M^+ (methyl 7-thiastearate) and m/z 318 (d_2), M^+ (methyl dideuterated 7-thiastearate). Each mixture was then administered as a 5% w/v ethanolic solution to growing cultures (150 mL) of the *S. cerevisiae* transformant at 15 °C.¹³⁸ The growth of yeast cells (pDR1 strain) was observed for all incubations. After 1 wk of growth, the cells were collected by centrifugation and hydrolyzed in base (1 M 50% ethanolic KOH, (Chapter 5, 5.14.1)).⁸⁹ The cellular fatty acids were extracted, methylated (CH_2N_2 , (Chapter 5, 5.16)) and analyzed by GC/MS. The fatty acid profile from incubation of each mixture is shown in Figures 4.19, 4.20

and Table 4.3. The deuterium content of methyl 7-thialinoleate products (d_0/d_1) was assessed via the following ions: m/z 150 (d_0) $[(CH_3(CH_2)_4CH=CHCH=CHCH=CH_2)^+]$ (methyl 7-thialinoleate) and m/z 151 (d_1) (monodeuterated methyl 7-thialinoleate).

Figure 4.19. Cellular fatty acid profile of *S. cerevisiae* (pDR1 strain), grown in a medium supplemented with a mixture of methyl 7-thiastearate (10)/methyl $[12,12-^2H_2]$ -7-thiastearate ($[12,12-^2H_2]$ -10) at 15 °C for 1 wk.

Runname : 1a0297 Acquired Nominal data (Zero baseline). 27 Mar 97 2:51
Unsmoothed data only:

Figure 4.20. Cellular fatty acid profile of *S. cerevisiae* (pDR1 strain), grown in a medium supplemented with a mixture of methyl 7-thiastearate (10)/methyl [13,13-²H₂]-7-thiastearate ([13,13-²H₂]-10) at 15 °C for 1 wk.

Table 4.3. Incubation of a mixture of methyl 7-thiastearate (10)/methyl [12,12-²H₂]-7-thiastearate ([12,12-²H₂]-10) and methyl 7-thiastearate (10)/methyl [13,13-²H₂]-7-thiastearate ([13,13-²H₂]-10) using *S. cerevisiae* (pDR1 strain), grown at 15 °C for 1 wk: Fatty acid profiles.

Exp.	Mixture Incubated	% Composition of Fatty Acids (FA) ^a								% Incorp. Thia FA ^b
		16:1	16:0	18:2	18:1	18:0	Thia-18:2	Thia-18:1	Thia-18:0	
1	10, [12,12- ² H ₂]-10	25	13	9	20	11	1	5	16	22
2	10, [12,12- ² H ₂]-10	25	12	9	21	13	1	5	14	20
1	10, [13,13- ² H ₂]-10	25	13	9	18	12	2	5	16	23
2	10, [13,13- ² H ₂]-10	24	12	9	21	12	2	5	15	22

^a The relative amounts of each fatty acid component are expressed as a % of the total fatty acids; they were determined using GC/MS Total Ion Current (TIC) chromatograms. Each entry represents an average value based on two to three GC/MS runs (standard deviation = 0.5%).

^b % Incorp. Thia FA refers to the ratio: [Thia-18:0 + Thia-18:1 + Thia-18:2] × 100]_{cell} / (Total FA)_{cell}.

Since the overall percent conversion of incubated mixture to products was clearly less than 10% for each incubation, the isotope effects (k_H/k_D) were calculated using the following ratio (Appendix C):⁹⁹

$$\frac{k_H}{k_D} = \frac{P_H / P_D}{S_H^0 / S_D^0}$$

where, P_H / P_D is the ratio of nondeuterated to monodeuterated methyl 7-thialinoleate and S_H^0 / S_D^0 is the ratio of the nondeuterated to dideuterated methyl 7-thiastearates. Thus,

$$\frac{k_H}{k_D} = \frac{(\%d_0)_P}{(\%d_1)_P} / \frac{(\%d_0)_S}{(\%d_2)_S}$$

This calculation was based on the assumption that the d_0/d_2 ratio of the intermediate biosynthetic methyl 7-thiaoleates was essentially the same as that of the starting materials (methyl 7-thiastearates). Mass spectrometric examination of the thiaoleate fraction confirmed that this was indeed the case within experimental error using the following ions: m/z 152 (d_0), $[(CH_3(CH_2)_6CH=CHCH=CH_2)^+]$ (methyl 7-thiaoleate); and m/z 154 (d_2), (methyl dideuterated 7-thiaoleate) (Table 4.4).

The analytical data is shown in Table 4.4. A large primary deuterium isotope effect (7.3 ± 0.4 , average of 2 experiments $\pm$ standard deviation) was determined for the carbon-hydrogen bond cleavage at C-12, while the C-13-H bond-breaking step was shown to be insensitive to deuterium substitution ($KIE = 1.05 \pm 0.04$, average of 2 experiments $\pm$ standard deviation).

Our observed isotope effect at C-12 is close to the theoretical maximum value for a primary deuterium isotope effect at 15 °C ($k_H/k_D = 7.4$). However, as it mentioned in Chapter 2 (Part 3): (a) a small proportion (<10%) of the observed isotope effect may be due to an α -secondary isotope effect;¹⁰⁹ (b) the magnitude of the primary deuterium isotope effect at C-12 must be regarded as a lower limit for the “intrinsic” kinetic isotope

effect since our value is derived via an intermolecular competition experiment and is therefore subject to partial masking by other enzymatic steps.¹⁰⁹

Table 4.4. Intermolecular isotopic discrimination in Δ^{12} desaturation of [12,12- $^2\text{H}_2$]-7-thiaoleate and [13,13- $^2\text{H}_2$]-7-thiaoleate derived from the corresponding methyl stearates ([12,12- $^2\text{H}_2$]-10 and [13,13- $^2\text{H}_2$]-10).

Exp.	Isotopic Composition (% mol) ^a						KIE
	Substrates ^b			Products			
	d ₀	12-d ₂	13-d ₂	d ₀	12-d ₁	13-d ₁	
1	50.4 ± 1.0 (49.7 ± 1.2) ^c	49.6 ± 1.0 (50.3 ± 1.2) ^c		87.6 ± 1.0	12.4 ± 1.0		7.0 ± 0.6
2	51.8 ± 0.9 (49.8 ± 0.8) ^c	48.2 ± 0.9 (50.2 ± 0.8) ^c		89.1 ± 0.7	10.9 ± 0.7		7.6 ± 0.6
							(7.3 ± 0.4) ^d
1	49.6 ± 0.8 (52.7 ± 1.3) ^c		50.4 ± 0.8 (47.4 ± 1.3) ^c	51.3 ± 1.5		48.7 ± 1.5	1.07 ± 0.05
2	51.8 ± 1.3 (51.7 ± 1.8) ^c		48.2 ± 1.3 (48.3 ± 1.8) ^c	52.3 ± 1.2		47.7 ± 1.2	1.02 ± 0.05
							(1.05 ± 0.04) ^e

^a The % mol of each isotopic species is given as an average value based on two to three GC/MS runs. ^b The four starting mixtures were individually prepared. ^c Isotopic species of the biosynthetic 7-thiaoleates. ^d The average KIE at C-12 ± standard deviation. ^e The average KIE at C-13 ± standard deviation.

These results are consistent with the notion that Δ^{12} desaturase initiates oxidation at C-12 in a relatively slow, isotopically sensitive step in accordance with our mechanistic model. The second C–H cleavage at C-13 appears to be fast as might be expected for the collapse of a radical intermediate or a carbocation (Chapter 1, Figure 1.29). Thus, Δ^{12} desaturation involves stepwise, not synchronous removal of the two hydrogens. That the initial oxidation for *A. thaliana* Δ^{12} desaturation appears to be at C-12 is in accord with the postulate that ricinoleic acid (**30**) is produced by an altered Δ^{12} plant desaturase which initiates C–H bond cleavage at C-12 but because of an as yet undetermined reason cannot prevent alcohol formation.¹³¹ Importantly, very recently Shanklin *et al.* has

succeeded in converting the oleate Δ^{12} desaturase to a 12-hydroxylase through appropriate site-directed mutagenesis experiments.¹⁴⁰ However, the regiochemical and stereochemical purity of the hydroxyl product are not yet known.

It is also interestingly to note that the pattern of primary isotope effects (one large ($k_H/k_D = 7.3$, C-12) and one small ($k_H/k_D = 1.05$, C-13)) which we have documented in this work is remarkably similar to that obtained previously for other desaturases: Δ^9 desaturase of *S. cerevisiae* (Chapter 2);¹¹² Δ^9 desaturase of *C. diptheriae*;^{24,37} Δ^6 desaturase of *Tetrahymena*;¹¹⁰ and cytochrome P-450-mediated 4,5-dehydrogenation of valproic acid.¹¹¹ It should be pointed out that a kinetic isotope effect at only one position is consistent with the results obtained by Morris *et al.* in the biosynthesis of linoleate ($18:2\Delta^{9,12}$) using *C. vulgaris*.⁴² However, as discussed in Chapter 2 (Part 3), the results obtained from this laboratory are controversial.

Summary: We were the first to determine the intermolecular primary deuterium isotope effects involved in oleate Δ^{12} desaturation.¹⁴¹ Our results strongly suggest that the initial oxidative attack for Δ^{12} desaturation is at C-12 and that the production of ricinoleic acid (**30**) in the castor plant is indeed carried out by a modified Δ^{12} desaturase.

Buist, P. H.; Behrouzian, B. *J. Am. Chem. Soc.* **1997**, *in press*.

Chapter 5

Experimental

Part 1: Materials and Methods

^1H NMR (200 and 400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on Varian XL 200 and Bruker AMX 400 spectrometers using dilute CDCl_3 solutions. Chemical shifts for ^1H and ^{13}C NMR spectra are reported relative to tetramethylsilane (TMS). CDCl_3 was used as the NMR solvent, and the chemical shifts were referenced to natural abundance CHCl_3 at 7.24 ppm. ^{19}F NMR spectra were recorded on a Bruker AM 400 MHz spectrometer (Health Canada) operating at 376.5 MHz using CDCl_3 as solvent. Fluorotrichloromethane (CFCl_3) was used as the reference material. Abbreviations used in assigning peaks are the following: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. All chemical shifts are expressed in ppm (δ) and J values are reported in Hertz (Hz).

The GC/MS data were collected at the University of Ottawa using a Kratos Concept 1H mass spectrometer at 70 eV interfaced with a J. & W. 30 m $\times$ 0.21 mm, DB-5 capillary column which was temp. programmed from 120 $^\circ\text{C}$, (held, 2 min) to 320 $^\circ\text{C}$ at 10 $^\circ\text{C}/\text{min}$. Deuterium content was estimated using a MS scan rate of 0.5/decade which resulted in 5-8 scans per GC peak; the integrated intensities of the individual ions in the molecular ion cluster were recorded a SUN SPARC I workstation equipped with Kratos Mach 3 software and have been corrected for natural isotopic abundance. MS spectra of carboxylic acids were obtained using a direct probe.

The elemental analyses were obtained on a Perkin Elmer Elemental Analyzer 240C operated at the Institute of Biological Sciences at N.R.C. Melting point determinations were performed on a Fisher-Johns melting point apparatus and are uncorrected.

Fatty acid substrates were purified by flash chromatography using Merck silica gel 60 (230-400 mesh) employing an air pressure of ca. 7 psi. Analytical thin layer chromatography (TLC) was performed using Merck-coated silica gel 60 F-254 glass plates. Visualization of UV-inactive materials was accomplished by using a combination of I₂ vapor followed by a water spray.

All reagents and starting materials were purchased from Aldrich Chemical Company and used without purification. With the exception of oxidation processes, all reactions were performed under N₂. A Büchi RE 111 Rotovapor was utilized for the removal of solvents in *vacuo*. Solvents were distilled before used, or were of “absolute” or “spectral grade” quality. Anhydrous diethyl ether (Et₂O) and tetrahydrofuran (THF) were freshly distilled as required from LiAlH₄ and collected in a septum-sealed, 3-necked, round-bottomed flask containing molecular sieves. Dimethyl sulfoxide (DMSO) was distilled in *vacuo* from CaH₂ and stored over molecular sieves under N₂.

Part 2: Synthesis of Substrates

5.1 Methyl 7-thiastearate

5.1.1 6-Mercaptohexanoic acid

A solution of 6-bromohexanoic acid (4.33 g, 22.2 mmol) and a slight excess of thiourea (72.5 g, 23.7 mmol) in 50 mL of absolute EtOH was refluxed for 2.5 h in a 500

mL, round-bottomed flask. The pale yellowish solution was cooled to room temperature. 200 mL of a 2 M NaOH solution (EtOH/dH₂O, 1:1) was then added, resulting in a white cloudy mixture which clarified upon reheating. The basic solution was refluxed for 1.5 h, then diluted with 100 mL of dH₂O, transferred to a separatory funnel, and slowly added to a stirred mixture of sulfuric acid (6 M, 45 mL) and CH₂Cl₂ (100 mL) in a 1 L Erlenmeyer flask. The organic layer was removed, and the resultant aqueous layer was further extracted with CH₂Cl₂ (3 × 30 mL). The organic layers were combined, washed with acidified saturated NaCl solution (5 drops of 6 M H₂SO₄ in 25 mL NaCl), dried over Na₂SO₄, and concentrated in *vacuo* to give 3.0 g (91% est. yield) of a white solid.¹⁰⁰

R_f 0.33 (60% EtOAc/hexanes)

¹H NMR (200 MHz) δ 2.54 (m, 2H, HSCH₂CH₂-), 2.39 (t, 2H, -CH₂CH₂C(O)OH), 1.64 (br m, 4H, HSCH₂CH₂CH₂CH₂CH₂-), 1.45 (m, 2H, HSCH₂CH₂CH₂CH₂-), 1.22 (t 1H, HSCH₂-)

5.1.2 7-Thiastearic acid

A 100 mL 3-necked, round-bottomed flask equipped with a condenser, a stirring bar, a rubber septum and a glass stopper was charged with 16 mL of anhydrous THF/HMPA (3:1). 6-Mercaptohexanoic acid (0.7 g, ca. 7 mmol) was transferred to the flask and the solution cooled to 0°C (ice-bath). Approximately 2 equivalents of a 1.6 M solution of n-butyllithium in hexanes (12 mL, 20 mmol) was added slowly via a syringe.

The solution was stirred for 30 min at 0 °C after which 1-bromoundecane (1.8 g, 7.7 mmol) was added dropwise. The clear yellow solution was stirred overnight at room temperature and then quenched with 20 mL of dH₂O. The mixture was acidified to below pH 2 by addition of 20 mL of 3 M HCl. The acidic aqueous solution was transferred to a separatory funnel and extracted with hexanes (4 × 25 mL). The combined organic layers were dried over Na₂SO₄ and concentrated in *vacuo* to yield 1.6 g of the crude 7-thiastearic acid.¹⁰⁰

R_f 0.32 (50% EtOAc/hexanes)

MS (EI, 70 eV) m/z 302 (M⁺)

¹H NMR (200 MHz) δ 2.51 (two overlapping t, 4H, -CH₂CH₂SCH₂CH₂-), 2.34 (t, 2H, -CH₂CH₂C(O)OH), 1.52 (br m, 4H, -CH₂CH₂CH₂SCH₂CH₂CH₂-), 1.29 (br s, 20H, CH₃(CH₂)₈CH₂CH₂SCH₂CH₂(CH₂)₂CH₂-), 0.87 (t, 3H, CH₃CH₂-)

5.1.3 Methyl 7-thiastearate

A solution of the crude 7-thiastearic acid (0.67 g) in 50 mL of 10% BF₃/MeOH solution (v/v) was refluxed for 1.5 h. The solution was then cooled, diluted with 50 mL of dH₂O, transferred to a separatory funnel, and extracted with CH₂Cl₂ (4 × 25 mL). The combined organic layers were washed with saturated NaCl, dried over Na₂SO₄, and concentrated in *vacuo*.¹⁰¹ Purification by flash chromatography (4% EtOAc/hexanes) yielded 357 mg (1.13 mmol, 37% over two steps) of a colourless oil at room temperature.

mp 23-26 °C

R_f 0.34 (10% EtOAc/hexanes)

MS (EI, 70 eV) m/z 316 (M⁺), 285 [(M - OCH₃)⁺], 187 [(CH₃(CH₂)₁₀S)⁺], 129 [(M - CH₃(CH₂)₁₀S)⁺]

Anal. Calcd for C₁₈H₃₆O₂S: C, 68.30; H, 11.46; S, 10.13. Found: C, 68.29; H, 11.45; S, 9.93

¹H NMR (200 MHz) δ 3.63 (s, 3H, -CH₂C(O)OCH₃) 2.47 (two overlapping t, 4H, -CH₂CH₂SCH₂CH₂-), 2.31 (t, 2H, -CH₂CH₂C(O)OCH₃), 1.60 (br m, 4H, -CH₂CH₂-CH₂SCH₂CH₂CH₂-), 1.29 (br s, 20H, CH₃(CH₂)₈CH₂CH₂SCH₂CH₂(CH₂)₂CH₂-), 0.89 (t, 3H, CH₃CH₂-)

¹³C NMR (100 MHz) δ 174.09 (C-1), 51.49 (CH₃O), 33.98 (C-2), 32.26 (C-8), 31.98 (C-6 or C-16), 31.94 (C-6 or C-16), 29.77 (C-9), 29.64 (C-13), 29.64 (C-14), 29.57 (C-12), 29.37 (C-4 or C-5), 29.37 (C-15), 29.30 (C-11), 28.99 (C-10), 28.43 (C-4 or C-5), 24.60 (C-3), 22.71 (C-17), 14.12 (C-18)

5.2 Methyl [9,9-²H₂]-7-thiastearate

5.2.1 [1,1-²H₂]-Decan-1-ol

LiAlD₄ (1.52 g, 36.2 mmol) was added to a pre-cooled (ice-bath), 250 mL, 3-necked, round-bottomed flask which was fitted with a reflux condenser. 9 mL of anhydrous Et₂O was slowly added via a syringe. A solution of decanoic acid (4.94 g,

28.7 mmol) in anhydrous Et₂O was added dropwise to the vessel. The mixture was stirred for 4 h, then diluted with 5 mL of dH₂O and 10 mL of 3 M HCl. After addition of 20 mL of CH₂Cl₂, the mixture was transferred to a separatory funnel. The emulsion was partially clarified by the addition of 30 mL of 3 M HCl. The CH₂Cl₂ was removed and the aqueous layer was further extracted with CH₂Cl₂ (4 × 20 mL). The combined organic layers were washed with 40 mL of 3 M HCl, dried over Na₂SO₄, and concentrated in *vacuo* to give 4.1 g (90% est. yield) of a colourless oil.¹⁰³

R_f 0.32 (20% EtOAc/hexanes)

¹H NMR (200 MHz) δ 1.56 (br t, 2H, -CH₂CH₂CD₂OH), 1.30 (br s, 14H, CH₃(CH₂)₇CH₂-), 0.89 (t, 3H, CH₃CH₂-)

5.2.2 [1,1-²H₂]-1-*p*-Toluenesulfonyloxydecane

p-Toluenesulfonyl chloride (5.1 g, 27 mmol) was added to a solution of [1,1-²H₂]-decan-1-ol (4.13 g, ca. 25.8 mmol) in 21 mL of dry pyridine at 0 °C (ice-bath). The reaction mixture was stirred for 1 h at 0 °C and then kept at 4 °C overnight. The mixture was diluted with H₂O (100 mL) and extracted with Et₂O (3 × 70 mL). The combined ethereal extracts were washed with saturated aqueous copper sulfate (4 × 40 mL), dH₂O (2 × 50 mL), and Na₂CO₃ solution (5%, 2 × 50 mL). The extracts were dried over Na₂SO₄, and concentrated in *vacuo* to yield 7.1 g (88% est. yield) of the [1,1-²H₂]-1-*p*-toluenesulfonyloxydecane as a yellowish oil.¹⁰⁴

MS (EI, 70 eV) m/z 159 $[(M - Ts)^+]$

^1H NMR (200 MHz) δ 7.80 (d, 2H, Ar-**H**), 7.35 (d, 2H, Ar-**H**), 2.46 (s, 3H, Ar-**CH**₃), 1.65 (br t, 2H, -CH₂CH₂CD₂-), 1.27 (br s, 14H, CH₃(CH₂)₇CH₂-), 0.89 (t, 3H, CH₃CH₂-)

5.2.3 [3,3-²H₂]-Dodec-1-yne

Lithium acetylide-ethylenediamine complex (5.29 g, 57.5 mmol) was placed in a 250 mL, round-bottomed flask equipped with a stirrer bar and a N₂ inlet adaptor. Anhydrous DMSO (15 mL) was added dropwise to the flask via a syringe. A mixture of [1,1-²H₂]-1-*p*-toluenesulfonyloxydecane (7.16 g, ca. 22.8 mmol) in anhydrous DMSO (20 mL) was slowly added to the reaction vessel. The mixture was stirred vigorously at room temperature for 4 h. The brown slurry was poured on ice (250 g) and the resultant basic solution was neutralized with 25 mL of 3 M HCl. The aqueous layer was extracted with Et₂O (4 × 150 mL). The combined ethereal layers were washed with dH₂O, saturated NaCl (150 mL), dried over Na₂SO₄, and concentrated in *vacuo* to give 3.4 g (89% est. yield) of a golden liquid.^{103,105}

^1H NMR (200 MHz) δ 1.91 (s, 1H, -CD₂-C≡C-**H**), 1.50 (br t, 2H, -CH₂CH₂CD₂-), 1.30 (br s, 14H, CH₃(CH₂)₇CH₂-), 0.88 (t, 3H, CH₃CH₂-)

5.2.4 [2,2-²H₂]-Undecanoic acid

A 1 L round-bottomed flask was charged with KMnO_4 (31.6 g, 97.5 mmol) and 100 mL of dH_2O . The flask was cooled in an ice-bath and the mixture stirred vigorously using a magnetic bar to yield a deep purple solution. A solution of [3,3-²H₂]-dodec-1-yne (3.28 g, ca. 19.5 mmol) and 264 mg of the phase transfer agent, hexadecyltributyl phosphonium bromide, in 125 mL of hexanes/glacial acetic acid (4:1) was added in one portion. The solution was stirred and allowed to come to room temperature overnight. Sulfurous acid (sodium sulfite (Na_2SO_3) in dilute H_2SO_4) was added to the cooled reaction mixture until the solution turned colourless. Two clear layers were visible. The aqueous layer was extracted with hexanes (3×50 mL) and the combined organic layers were washed with 50 mL of dH_2O , dried over Na_2SO_4 , and concentrated in *vacuo* to provide 2.8 g (76% est. yield) of a white solid as a product.¹⁰⁶

R_f 0.42 (40% EtOAc/hexanes)

MS (EI, 70 eV) m/z 188 (M^+)

¹H NMR (200 MHz) δ 1.62 (br t, 2H, $-\text{CH}_2\text{CH}_2\text{CD}_2-$), 1.32 (br s, 16H, $\text{CH}_3(\text{CH}_2)_7\text{CH}_2-$), 0.89 (t, 3H, CH_3CH_2-)

5.2.5 [2,2-²H₂]-Undecan-1-ol

The reaction was performed following the procedure described in 5.2.1. [2,2-²H₂]-Undecanoic acid (2.11 g, ca. 11.2 mmol) in 10 mL of anhydrous Et₂O was added to LiAlH₄ (0.68 g, 17 mmol) in 4 mL of anhydrous Et₂O. Work-up yielded 1.0 g (53% est. yield) of the [2,2-²H₂]-undecan-1-ol as an oil.¹⁰³

R_f 0.33 (20% EtOAc/hexanes)

¹H NMR (200 MHz) δ 1.59 (br t, 2H, -CH₂CH₂CD₂OH), 1.33 (br s, 16H, CH₃(CH₂)₈CH₂-), 0.92 (t, 3H, CH₃CH₂-)

5.2.6 [2,2-²H₂]-1-*p*-Toluenesulfonyloxyundecane

The reaction was performed as previously described (5.2.2). [2,2-²H₂]-Undecan-1-ol (1.1 g, ca. 6.9 mmol) was converted to the corresponding tosylate (1.6 g, 72% est. yield) using *p*-toluenesulfonyl chloride (1.6 g, 4.9 mmol).¹⁰⁴

MS (EI, 70 eV) m/z 173 $[(M - Ts)^+]$

1H NMR (200 MHz) δ 7.80 (d, 2H, Ar-H), 7.35 (d, 2H, Ar-H), 3.99 (br s, 2H, $-CD_2CH_2O-$), 2.43 (s, 3H, Ar- CH_3), 1.62 (br t, 2H, $-CH_2CH_2CD_2-$), 1.24 (br s, 14H, $CH_3(CH_2)_7CH_2-$), 0.88 (t, 3H, CH_3CH_2-)

5.2.7 [9,9- 2H_2]-7-Thiastearic acid

A solution of 7-mercaptohexanoic acid (0.3 g, 2 mmol) in 15 mL of THF/HMPA (3:1) was transferred to a 100 mL 3-necked, round-bottomed flask and cooled in ice-bath. Approximately 2 equivalents of 1.6 M *n*-butyllithium in hexanes (4 mL, 5 mmol) was then added slowly with stirring at 0 °C. The solution was stirred for a further 30 min at 0 °C. [2,2- 2H_2]-1-*p*-Toluenesulfonyloxyundecane (886 mg, ca. 2.70 mmol) was added dropwise to give a reddish-brown solution, stirring was continued overnight at room temperature and then quenched with 50 mL of dH₂O. The mixture was acidified to below pH 2 by addition of ca. 14 mL of 3 M HCl. The aqueous phase was extracted with hexanes (4 × 15 mL). The combined organic layers were dried over Na₂SO₄ and concentrated in *vacuo* to yield 0.7 g of the crude product.¹⁰⁰ The chromatographic and spectral properties were similar to those of unlabelled material except for the following:

MS (EI, 70 eV) m/z 304 (M^+)

^1H NMR (200 MHz) δ 2.46 (br s, 2H $-\text{CD}_2\text{CH}_2\text{S}-$), 2.48 (t, 2H, $-\text{SCH}_2\text{CH}_2-$), 1.65 (br m, 2H, $-\text{SCH}_2\text{CH}_2\text{CH}_2-$)

5.2.8 Methyl [9,9- $^2\text{H}_2$]-7-thiastearate

Crude 7-thiastearic acid (0.67 g) was methylated following the procedure described in 5.1.3. Purification by flash chromatography (4% EtOAc/hexanes) yielded 357 mg (1.12 mmol, 8% est. overall yield) of a colourless oil at room temperature.¹⁰¹ The physical, chromatographic and spectral properties were similar to those of unlabelled material except for the following:

MS (EI, 70 eV) m/z 318 (M^+), 287 [$(\text{M} - \text{OCH}_3)^+$], 189 [$(\text{CH}_3(\text{CH}_2)_8\text{CD}_2\text{CH}_2\text{S})^+$], 129 [$(\text{M} - \text{CH}_3(\text{CH}_2)_8\text{CD}_2\text{CH}_2\text{S})^+$]

^1H NMR (200 MHz) δ 2.52 (t, 2H, $-\text{SCH}_2\text{CH}_2-$), 2.48 (br s, 2H $-\text{CD}_2\text{CH}_2\text{S}-$), 1.65 (br m, 2H, $-\text{SCH}_2\text{CH}_2\text{CH}_2-$)

^{13}C NMR (100 MHz) δ 32.07 (C-8, upfield β -isotope shift (0.19 ppm)), 29.77 (C-9, absent), 29.24 (C-11, upfield γ -isotope shift (0.06 ppm)), 28.80 (C-10 upfield β -isotope shift (0.19 ppm))

5.3 Methyl [10,10-²H₂]-7-thiastreate

5.3.1 [1,1-²H₂]-Nonan-1-ol

Following the procedure described in 5.2.1, nonanoic acid (12.7 g, 80.3 mmol) was reduced to [1,1-²H₂]-nonan-1-ol (11.5 g, 98% est. yield) using LiAlD₄ (4.62 g, 110 mmol).¹⁰³

R_f 0.33 (20% EtOAc/hexanes)

¹H NMR (200 MHz) δ 1.56 (br t, 2H, -CH₂CH₂CD₂OH), 1.31 (br s, 12H, CH₃(CH₂)₆CH₂-), 0.91 (t, 3H, CH₃CH₂-)

5.3.2 [1,1-²H₂]-1-Bromononane

A solution of [1,1-²H₂]-nonan-1-ol (11.5 g, ca. 78.8 mmol) in 30 mL of anhydrous Et₂O was treated with PBr₃ (4.8 mL, 50 mmol) at 0 °C with stirring. The mixture was

allowed to warm up to room temperature over a 3 h period and then refluxed for 1.5 h, cooled on ice, and quenched by the dropwise addition of 10 mL of dH₂O. The solution was transferred to a separatory funnel, the initial ethereal layer was removed, and the aqueous layer was extracted with Et₂O (2 × 15 mL). The combined organic extracts were dried over Na₂SO₄ and concentrated in *vacuo* to yield [1,1-²H₂]-1-bromononane (16 g, 99% est. yield).¹⁰⁸

R_f 0.43 (6% EtOAc/hexanes)

¹H NMR (200 MHz): δ 1.83 (br t, 2H, -CH₂CH₂CD₂Br), 1.35 (br s, 12H, CH₃-(CH₂)₆CH₂-), 0.83 (t, 3H, CH₃CH₂-)

5.3.3 [3,3-²H₂]-Undecanoic acid

To a stirred solution of sodium ethoxide (0.324 g, 14.1 mmol sodium reacted in 10 mL of absolute EtOH) was added diethyl malonate (2.50 g, 15.5 mmol). The solution was heated to boiling, after which [1,1-²H₂]-1-bromononane (2.20 g, ca. 10.5 mmol) was added via a syringe. The mixture was refluxed for 1.5 h. Most of the EtOH was removed using a rotary evaporator. The residue was hydrolyzed by adding 100 mL of 5% KOH in 80% aqueous EtOH and refluxing for 1 h at 80 °C. Approximately 80 mL of 2 M H₂SO₄ were added dropwise to the cooled solution and stirring was continued for 0.5 h. EtOH was evaporated in *vacuo* using a rotary evaporator and 30 mL of dH₂O was added. The solution was transferred to a separatory funnel, and the aqueous layer was then extracted

with Et₂O (3 × 50 mL). The combined ethereal phases were washed with dH₂O until the washings were neutral. After drying over Na₂SO₄, the solution was concentrated in *vacuo* to yield [3,3-²H₂]-2-carboxyundecanoic acid as a white solid. This material was decarboxylated by heating the flask in a silicone bath at 165 °C (temp. initial, ca. 125 °C). After cooling, [3,3-²H₂]-undecanoic acid (1.2 g, 59% est. yield) was formed as a white solid.¹⁰⁷

R_f 0.21 (20% EtOAc/hexanes)

MS (EI, 70 eV) m/z 188 (M⁺)

¹H NMR (200 MHz) δ 2.26 (br t, 2H, -CD₂CH₂C(O)OH), 1.26 (br s, 14H, CH₃(CH₂)₇-CD₂-), 0.87 (t, 3H, CH₃CH₂-)

5.3.4 [3,3-²H₂]-Undecan-1-ol

[3,3-²H₂]-Undecanoic acid (1.2 g, ca. 6.2 mmol) was reduced to [3,3-²H₂]-undecan-1-ol (870 mg, 81% est. yield) using LiAlH₄ (0.3 g, 8 mmol). A procedure similar to that reported in 5.2.1 was employed.¹⁰³

R_f 0.33 (20% EtOAc/hexanes)

¹H NMR (200 MHz) δ 3.65 (br t, 2H, -CH₂CH₂OH), 1.56 (br t, 2H, -CD₂CH₂CH₂OH), 1.30 (br s, 14H, CH₃(CH₂)₇CD₂-), 0.92 (t, 3H, CH₃CH₂-)

5.3.5 [3,3-²H₂]-1-*p*-Toluenesulfonyloxyundecane

Using the procedure reported in 5.2.2, [3,3-²H₂]-undecan-1-ol (512 mg, ca. 2.94 mmol) was converted to the corresponding tosylate (750 mg, 77% est. yield) using *p*-toluenesulfonyl chloride (0.72 g, 3.8 mmol).¹⁰⁴

MS (EI, 70 eV) m/z 173 $[(M - Ts)^+]$

¹H NMR (200 MHz) δ 7.80 (d, 2H, Ar-H), 7.34 (d, 2H, Ar-H), 4.01 (t, 2H, -CH₂-CH₂O-), 2.45 (s, 3H, Ar-CH₃), 1.65 (br t, 2H, -CD₂CH₂CH₂O-), 1.27 (br s, 14H, CH₃(CH₂)₇CH₂-), 0.88 (t, 3H, CH₃CH₂-)

5.3.6 [10,10-²H₂]-7-Thiastearic acid

A procedure similar to that described in 5.2.7 was employed. Crude [10,10-²H₂] 7-thiastearic acid (745 mg) was prepared via S-alkylation of the dilithio salt of 7-mercaptohexanoic acid (0.3 g, 2 mmol) using [3,3-²H₂]-1-*p*-toluenesulfonyloxyundecane (721 mg, ca. 2.20 mmol).¹⁰⁰ The chromatographic and spectral properties were similar to those of unlabelled material except for the following:

MS (EI, 70 eV) *m/z* 304 (*M*⁺)

5.3.7 Methyl [10,10-²H₂]-7- thiastearate

Methylation of the crude [10,10-²H₂]-7-thiastearic acid (745 mg) using BF₃/MeOH was performed employing the procedure described in 5.1.3.¹⁰¹ Purification of the product by flash chromatography (4% EtOAc/hexanes) gave 147 mg (0.462 mmol, 7.4% est. overall yield) of the methyl [10,10-²H₂]-7-thiastearate as a colourless oil at room temperature. The physical, chromatographic and spectral properties were similar to those of unlabelled material except for the following:

MS (EI, 70 eV) *m/z* 318 (*M*⁺), 287 [*M* - OCH₃]⁺, 189 [(CH₃(CH₂)₇CD₂(CH₂)₂S)⁺], 129 [*M* - CH₃(CH₂)₇CD₂(CH₂)₂S]⁺,

^{13}C NMR (100 MHz) δ 32.17 (C-8, upfield γ -isotope shift (0.09 ppm)), 29.51 (C-12 upfield γ -isotope shift (0.06 ppm)), 29.51 (C-9, upfield β -isotope shift (0.26 ppm)), 29.08 (C-11, upfield β -isotope shift (0.22 ppm)), 28.99 (C-10, absent)

5.4 Methyl (*R,S*)-5-fluorostearate

5.4.1 Methyl (*R,S*)-5-hydroxystearate

To a solution of NaBH_4 (228 mg, 6.00 mmol) in 5 mL of absolute MeOH, was added a solution of methyl 12-oxostearate (456 mg, 1.46 mmol) in 7 mL of absolute MeOH. The mixture was stirred for 2 h and then diluted with 25 mL of dH_2O followed by a few drops of dilute HCl to destroy any excess NaBH_4 . The solution was transferred to a separatory funnel, and the aqueous layer was extracted with CH_2Cl_2 (3×30 mL). The combined organic extracts were washed with dH_2O , dried over Na_2SO_4 , and concentrated in *vacuo* to constant weight. The yield was 428 mg (93% est. yield) of a white solid.¹⁴²

mp 37-39 °C

R_f 0.45 (20% EtOAc/hexanes)

MS (EI, 70 eV) m/z 296 $[(M - \text{H}_2\text{O})^+]$

^1H NMR (200 MHz) δ 3.62 (s, 3H, $-\text{C}(\text{O})\text{OCH}_3$), 3.61 (m, 1H, $-\text{CH}_2\text{CHOHCH}_2-$), 2.38 (t, 2H, $-\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OCH}_3$), 1.65 (m, 4H, $-\text{CH}_2\text{CH}_2\text{CHOHCH}_2\text{CH}_2-$), 1.38 (br s, 24H, $\text{CH}_3(\text{CH}_2)_{11}\text{CH}_2\text{CHOHCH}_2\text{CH}_2\text{CH}_2-$), 0.88 (t, 3H, $-\text{CH}_2\text{CH}_3$)

5.4.2 Methyl (*R,S*)-5-fluorostearate

A 50 mL, 2-necked, round-bottomed flask was cooled to ca. $-50\text{ }^{\circ}\text{C}$ in a dry-ice bath and CH_2Cl_2 (5 mL) followed by diethylaminosulfur trifluoride (DAST) (238 μL , 1.80 mmol) was added via a syringe. Methyl (*R,S*)-5-hydroxystearate (0.50 g, ca. 1.6 mmol), dissolved in 5 mL of CH_2Cl_2 , was added dropwise to the reaction vessel. The solution was a clear yellow colour. Once the addition was complete, the solution was allowed to warm up to room temperature and stirred for a further 4 h to give a deep yellow solution. The reaction was quenched with 20 mL of dH_2O and transferred to a separatory funnel. The organic layer was removed and the aqueous phase was extracted with CH_2Cl_2 ($3 \times 20\text{ mL}$). The combined organic layers were washed with saturated NaHCO_3 (15 mL), saturated NaCl (15 mL), dried over Na_2SO_4 , and concentrated in *vacuo* to yield 356 mg of a yellow oil.¹¹⁷ As judged by the integration of additional ^1H NMR signals at δ 5.38, this material consisted of a 3:1 mixture of fluoride/olefinic by-product. The product was purified by flash chromatography (2% EtOAc/hexanes) to give 134 mg (0.423 mmol, 24% yield) of a white crystalline solid.

mp $39\text{--}41\text{ }^{\circ}\text{C}$

R_f 0.33 (10% EtOAc/hexanes)

MS (EI, 70 eV) m/z 296 $[(M - HF)^+]$, 264 $[(M - HF) - MeOH]^+$

Anal. Calcd for $C_{19}H_{37}FO_2$: C, 72.10; H, 11.78. Found: C, 72.31; H, 11.81

^{19}F NMR (376.5 MHz) δ -181.26

1H NMR (200 MHz) δ 4.46 (d of m, 1H, $J_{HF} = 48$ Hz, $-CH_2CHFCH_2-$), 3.69 (s, 3H, $-C(O)OCH_3$), 2.34 (t, 2H, $-CH_2CH_2C(O)OCH_3$), 1.69 (m, 4H, $-CH_2CH_2CHFCH_2CH_2-$), 1.35 (br s, 24H, $CH_3(CH_2)_{11}CH_2CHFCH_2CH_2CH_2-$), 0.88 (t, 3H, CH_3CH_2-)

5.5 Methyl (*R,S*)-7-fluorostearate

5.5.1 Methyl (*R,S*)-7-hydroxystearate

Using the procedure described in 5.4.1, methyl 7-oxostearate (1.1 g, 3.5 mmol) was reduced with $NaBH_4$ (730 mg, 19.2 mmol) to give methyl (*R,S*)-7-hydroxystearate (1.1 g, 91% est. yield) as a white crystalline solid.¹⁴²

mp 53-56 °C

R_f 0.42 (25% EtOAc/hexanes)

MS (EI, 70 eV) m/z 296 $[(M - H_2O)^+]$

1H NMR (200 MHz) δ 3.61 (s, 3H, $-C(O)OCH_3$), 3.61 (m, 1H, $-CH_2CHOHCH_2-$), 2.32 (t, 2H, $-CH_2CH_2C(O)OCH_3$), 1.65 (m, 4H, $-CH_2CH_2CHOHCH_2CH_2-$), 1.47 (br s, 24H, $CH_3(CH_2)_9CH_2CHOHCH_2(CH_2)_3CH_2-$), 0.89 (t, 3H, CH_3CH_2-)

5.5.2 Methyl (*R,S*)-7-fluorostearate

Following the procedure reported in 5.4.2, 0.9 g of methyl (*R,S*)-7-fluorostearate was prepared by fluorinating methyl (*R,S*)-7-hydroxystearate (1.1 g, ca. 3.2 mmol) using DAST (520 μ L, 3.93 mmol).¹¹⁷ Purification by flash chromatography (2% EtOAc/hexanes) yielded 350 mg (1.11 mmol, 34% yield) of the product as a colourless crystalline solid.

mp 30-32 °C (lit., 38.5-39.9 °C (from MeOH))

R_f 0.35 (10% EtOAc/hexanes)

MS (EI, 70 eV) m/z 296 [(M - HF) $^+$], 264 [(M - HF) - MeOH] $^+$

Anal. Calcd for $C_{19}H_{37}FO_2$: C, 72.10; H, 11.78. Found: C, 71.88; H, 12.01

^{19}F NMR (376.5) δ -180.70

1H NMR (200 MHz) δ 4.45 (d of m, 1H, J_{HF} = 48 Hz, -CH₂CHFCH₂-), 3.66 (s, 3H, -C(O)OCH₃), 2.30 (t, 2H, -CH₂CH₂C(O)OCH₃), 1.62 (m, 4H, -CH₂CH₂CHFCH₂CH₂-), 1.36 (br s, 24H, CH₃(CH₂)₉CH₂CHOHCH₂(CH₂)₃CH₂-), 0.88 (t, 3H, CH₃CH₂-)

5.6 Methyl (*R,S*)-9-fluorostearate

5.6.1 9-Decenal

A solution of oxalyl chloride (4.17 mL, 47.8 mmol) in CH_2Cl_2 (50 mL) was added to a 250 mL, 3-necked, round-bottomed flask equipped with a magnetic stirring bar, a thermometer, and two dropping funnels- one containing a solution of DMSO (6.23 mL, 87.9 mmol) in CH_2Cl_2 (30 mL) and the other a solution of 9-decen-1-ol (6.2 mL, ca. 40 mmol) in 50 mL CH_2Cl_2 . The DMSO-containing solution was added to the oxalyl chloride at ca. $-50\text{ }^\circ\text{C}$ with stirring and the reaction mixture was stirred for a further 2 min. The alcohol-containing solution was then added and stirring was continued for an additional 15 min. 40.7 mL (292 mmol) of triethyl amine (TEA) was added and the reaction mixture was stirred for 5 min and then allowed to warm up to room temperature. 150 mL of dH_2O was added and aqueous layer was extracted with additional CH_2Cl_2 ($3 \times 100\text{ mL}$). The organic layers were combined, washed with dilute HCl (1%), dH_2O , dilute Na_2CO_3 (5%), dH_2O , dried over Na_2SO_4 , and concentrated in *vacuo* to give a slightly yellow oil (5.6 g, 98.5% est. yield) as a product.¹¹⁶

^1H NMR (200 MHz) δ 9.72 (t, 1H, $-\text{CH}_2\text{CHO}$), 5.73 (m, 1H, $\text{CH}_2=\text{CHCH}_2-$), 4.91 (m, 2H, $\text{CH}_2=\text{CH}-$), 2.39 (t of d, 2H, $-\text{CH}_2\text{CH}_2\text{CHO}$), 2.11 (m, 2H, $\text{CH}_2=\text{CH}_2\text{CH}_2\text{CH}_2-$), 1.61 (m, 2H, $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CHO}$), 1.35 (br s, 10H, $-\text{CH}_2(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{CHO}$)

5.6.2 (*R,S*)-1-Nonadecen-10-ol

Magnesium turnings (1.04 g, 42.9 mmol, previously washed with anhydrous Et₂O and dried in an oven overnight) were added to a 250 mL, 3-necked, dry, round-bottomed flask equipped with a stirring bar, condenser, and two dropping funnels. 6 mL of anhydrous Et₂O followed by 1-bromononane (7.82 mL, 40.9 mmol) in 10 mL of anhydrous Et₂O was added using one of the dropping funnels. The mixture was heated in a water bath until the Et₂O was refluxing gently. Upon the onset of the exothermic formation of Grignard reagent, the hot water bath was removed and the stirrer was set in motion. The solution was alternately heated and cooled until the reaction was complete (30 min) as judged by the almost complete disappearance of the magnesium turnings and the formation of a clear brown solution. The Grignard reagent was cooled to ca. -10 °C in an ice-CaCl₂ bath and 9-decenal (5.96 g, ca. 38.7 mmol) in 15 mL of anhydrous Et₂O was added dropwise to the flask over a period of 30 min using the second dropping funnel. The reaction product was then poured on 25 g of crushed ice and H₂SO₄ (3 M, 50 mL). The mixture was transferred to a separatory funnel, the organic layer removed, and the aqueous layer extracted with Et₂O (3 × 25 mL). The combined organic layers were washed with H₂SO₄ (3 M, 40 mL), saturated NaCl (40 mL), dried over Na₂SO₄, and concentrated in *vacuo* to provide 10.7 g (98.7% est. yield) of the product as a white solid.¹¹⁵

mp 50-53 °C

MS (EI, 70 eV) *m/z* 264 [(M - H₂O)⁺]

^1H NMR (200 MHz) δ 5.75 (m, 1H, $-\text{CH}_2\text{CH}=\text{CH}_2$), 4.88 (m, 2H, $-\text{CH}=\text{CH}_2$), 3.56 (m, 1H, $-\text{CH}_2\text{CHOHCH}_2-$), 2.13 (m, 2H, $-\text{CH}_2\text{CH}_2\text{CH}=\text{CH}_2$), 1.61 (m, 2H, $-\text{CH}_2\text{CH}_2\text{COHCH}_2\text{CH}_2-$), 1.36 (br s, 22H, $\text{CH}_3(\text{CH}_2)_6\text{CH}_2\text{CHOHCH}_2(\text{CH}_2)_5\text{CH}_2-$), 0.89 (t, 3H, CH_3CH_2-)

5.6.3 (*R,S*)-10-Fluoro-1-nonadecene

The reaction was performed as described previously (5.4.2). Fluorination of the (*R,S*)-1-nonadecen-10-ol (1.8 g, ca. 6.4 mmol) using DAST (930 μL , 7.04 mmol) yielded crude (*R,S*)-10-fluoro-1-nonadecene (1.6 g).¹¹⁷ The major impurity was the elimination byproduct. This intermediate was used in the next step without further purification.

^1H NMR (200 MHz) δ 5.76 (m, 1H, $-\text{CH}_2\text{CH}=\text{CH}_2$), 5.38 (m, 2H, $-\text{CH}_2\text{CH}=\text{CHCH}_2-$ elim. byprod.), 4.92 (m, 2H, $-\text{CH}=\text{CH}_2$), 4.43 (d of m, 1H, $J_{\text{HF}} = 48$ Hz, $-\text{CH}_2\text{CHFCH}_2-$), 2.03 (m, 2H, $-\text{CH}_2\text{CH}_2\text{CH}=\text{CH}_2$), 1.62 (m, 4H, $-\text{CH}_2\text{CH}_2\text{CFCH}_2\text{CH}_2-$), 1.35 (br s, 22H, $\text{CH}_3(\text{CH}_2)_6\text{CH}_2\text{CHOHCH}_2(\text{CH}_2)_5\text{CH}_2-$), 0.89 (t, 3H, CH_3CH_2-)

acid (1.2 g). Purification by flash chromatography (2% EtOAc/hexanes) yielded 579 mg (1.83 mmol, 28% over three steps, 27% est. overall yield) of the product as a white crystalline solid.¹⁰¹

mp 37-39 °C

R_f 0.44 (20% EtOAc/hexanes)

MS (EI, 70 eV) m/z 296 [(M - HF)⁺], 264 [(M - HF) - MeOH)⁺]

Anal. Calcd for C₁₉H₃₇FO₂: C, 72.10; H, 11.78; Found: C, 72.08; H, 11.79

¹⁹F NMR (376.5) δ -180.50

¹H NMR (200 MHz) δ 4.45 (d of m, 1H, J_{HF} = 48 Hz, -CH₂CHFCH₂-), 3.66 (s, 3H, -C(O)OCH₃), 2.37 (t, 2H, -CH₂CH₂C(O)OCH₃), 1.63 (m, 4H, -CH₂CH₂CHFCH₂CH₂-), 1.35 (br s, 24H, CH₃(CH₂)₇CH₂CHFCH₂(CH₂)₅CH₂-), 0.89 (t, 3H, CH₃CH₂-)

5.7 Methyl (*R,S*)-10-fluorostearate

5.7.1 (*R,S*)-1-Nonadecen-11-ol

22.5 mL of octylmagnesium chloride (45.1 mmol, Grignard reagent, 2 M solution in Et₂O) was added via a syringe to a dry, round-bottomed flask equipped with a 50 mL dropping funnel and a stirring bar. After cooling the flask to ca. -10 °C in an ice-CaCl₂ bath, 10-undecenal (5.19 mL, 44.6 mmol) in 15 mL of anhydrous Et₂O was added slowly

over 45 min via the dropping funnel. The reaction mixture was then poured into a beaker containing 37.5 g of crushed ice and 70 mL of 3 M H_2SO_4 . The mixture was transferred to a separatory funnel and the aqueous phase was extracted with Et_2O (3×50 mL). The combined ethereal layers were washed with H_2SO_4 (3 M, 50 mL), saturated NaCl (50 mL), dried over Na_2SO_4 , and concentrated in *vacuo* to yield (*R,S*)-1-nonadecen-11-ol (7.7 g, 61% est. yield) as a white crystalline solid.¹¹⁵

mp 38-41 °C

MS (EI, 70 eV) m/z 264 $[(M - \text{H}_2\text{O})^+]$

^1H NMR (200 MHz): δ 5.83 (m, 1H, $-\text{CH}_2\text{CH}=\text{CH}_2$), 4.97 (m, 2H, $-\text{CH}=\text{CH}_2$), 3.56 (m, 1H, $-\text{CH}_2\text{CHOHCH}_2-$), 2.11 (m, 2H, $-\text{CH}_2\text{CH}_2\text{CH}=\text{CH}_2$), 1.58 (m, 4H, $-\text{CH}_2\text{CH}_2\text{COHCH}_2\text{CH}_2-$), 1.35 (br s, 24H, $\text{CH}_3(\text{CH}_2)_6\text{CH}_2\text{CHOHCH}_2(\text{CH}_2)_6\text{CH}_2-$), 0.89 (t, 3H, CH_3CH_2-)

5.7.2 (*R,S*)-11-Fluoro-1-nonadecene

(*R,S*)-1-Nonadecen-11-ol (1.5 g, ca. 5.3 mmol) in 5 mL CH_2Cl_2 was treated with DAST (750 μL , 5.68 mmol) in 5 mL CH_2Cl_2 to give 1.3 g of crude (*R,S*)-11-fluoro-1-nonadecene as a yellow oil using the procedure described in 5.4.2.¹¹⁷ The major impurity was the elimination byproduct and it was not necessary to separate this material prior to the next step.

^1H NMR (200 MHz) δ 5.83 (m, 1H, $-\text{CH}_2\text{CH}=\text{CH}_2$), 5.38 (m, 2H, $-\text{CH}_2\text{CH}=\text{CHCH}_2-$ elim. byprod.), 4.92 (m, 2H, $-\text{CH}=\text{CH}_2$), 4.47 (d of m, 1H, $J_{\text{HF}} = 47$ Hz, $-\text{CH}_2\text{CHFCH}_2-$), 2.03 (m, 2H, $-\text{CH}_2\text{CH}_2\text{CH}=\text{CH}_2-$), 1.61 (m, 4H, $-\text{CH}_2\text{CH}_2\text{CFCH}_2\text{CH}_2-$), 1.36 (br s, 24H, $\text{CH}_3(\text{CH}_2)_6\text{H}_2\text{CHOHCH}_2(\text{CH}_2)_6\text{CH}_2\text{CH}=\text{CH}_2$), 0.89 (t, 3H, CH_3CH_2-)

5.7.3 (*R,S*)-10-Fluorostearic acid

The reaction was performed as previously described (5.2.4). Oxidation of (*R,S*)-11-fluoro-1-nonadecene (1.3 g) using a 2 M KMnO_4 solution yielded 1.1 g of the crude (*R,S*)-10-fluorostearic acid.¹⁰⁶

^1H NMR (200 MHz) δ 4.45 (d of m, 1H, $J_{\text{HF}} = 48$ Hz, $-\text{CH}_2\text{CHFCH}_2-$), 2.32 (t, 2H, $-\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.62 (m, 4H, $-\text{CH}_2\text{CH}_2\text{CHFCH}_2\text{CH}_2-$), 1.36 (br s, 24H, $\text{CH}_3(\text{CH}_2)_6\text{CH}_2\text{CHFCH}_2(\text{CH}_2)_6\text{CH}_2-$), 0.89 (t, 3H, CH_3CH_2-)

5.7.4 Methyl (*R,S*)-10-fluorostearate

Following the procedure described in 5.1.3, 1.1 g of the crude (*R,S*)-10-fluorostearic acid was treated with BF_3/MeOH to yield 970 mg of the crude methyl (*R,S*)-10-fluorostearate. Purification by flash chromatography (2% EtOAc/hexanes) gave 548 mg (1.73 mmol, 32% over three steps, 19.5% est. overall yield) of the product as a white crystalline solid.¹⁰¹

mp 34-36 °C

R_f 0.36 (10% EtOAc/hexanes)

MS (EI, 70 eV) m/z 296 $[(M - \text{HF})^-]$, 264 $[(M - \text{HF}) - \text{MeOH}]^+$

Anal. Calcd. for $\text{C}_{19}\text{H}_{37}\text{FO}_2$: C, 72.10; H, 11.78; Found: C, 72.03; H, 11.98

^{19}F NMR (376.5) δ -180.46

^1H NMR (200 MHz) δ 4.45 (d of m, 1H, $J_{\text{HF}} = 48$ Hz, $-\text{CH}_2\text{CHFCH}_2-$), 3.67 (s, 3H, $-\text{C}(\text{O})\text{OCH}_3$), 2.37 (t, 2H, $-\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OCH}_3$), 1.63 (m, 4H, $-\text{CH}_2\text{CH}_2\text{CHFCH}_2\text{CH}_2-$), 1.36 (br s, 24H, $\text{CH}_3(\text{CH}_2)_6\text{CH}_2\text{CHFCH}_2(\text{CH}_2)_6\text{CH}_2-$), 0.89 (t, 3H, CH_3CH_2-)

5.8 Racemic methyl *erythro*-9(10)-fluoro,10(9)-hydroxystearate

5.8.1 Racemic methyl *trans*-9,10-epoxystearate

Methyl elaidate (5.12 g, 17.3 mmol) in 50 mL of CH_2Cl_2 was added to a 250 mL, round-bottomed flask equipped with a stirring bar and dropping funnel. The solution was cooled to ca. 0 °C in an ice-bath. *m*-Chloroperoxybenzoic acid (70%, 4.3 g, 25 mmol) in CH_2Cl_2 was added dropwise via dropping funnel with stirring during 1 h. The stirring was continued for a further 0.5 h at room temperature and a white precipitate was formed. After addition of 10% sodium sulfite (15 mL) to destroy excess peracid, the solution was transferred to a separatory funnel, the organic layer was removed and the aqueous layer was extracted with CH_2Cl_2 (2 × 20 mL). The organic extracts were washed with 5% NaHCO_3 (15 mL), saturated NaCl (15 mL), dried over Na_2SO_4 , and concentrated in *vacuo* to yield racemic methyl *trans*-9,10-epoxystearate (5.4 g, 99% est. yield) as a white solid.¹¹⁹

mp 22-25 °C

MS (EI, 70 eV) m/z 294 $[(M - \text{H}_2\text{O})^+]$

^1H NMR (200 MHz) δ 3.69 (s, 3H, $-\text{C}(\text{O})\text{OCH}_3$), 2.66 (m, 2H, $-\text{CH}_2\text{CH}(\text{O})\text{CHCH}_2-$), 2.32 (t, 2H, $-\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OCH}_3$), 1.61 (m, 4H, $-\text{CH}_2\text{CH}_2\text{CH}(\text{O})\text{CHCH}_2\text{CH}_2-$), 1.30 (br s, 22H, $\text{CH}_3(\text{CH}_2)_6\text{CH}_2\text{CH}(\text{O})\text{CHCH}_2(\text{CH}_2)_5\text{CH}_2-$), 0.90 (t, 3H, CH_3CH_2-)

5.8.2 Racemic methyl *erythro*-9(10)-fluoro,10(9)-hydroxystearate

Racemic methyl *trans*-9,10-epoxystearate (194 mg, ca. 0.622 mmol) in 5 mL of CHCl_3 was added to a round-bottomed flask fitted with a stirring bar and a rubber septum. The flask was cooled in an ice- CaCl_2 bath and 0.2 mL of pyridinium polyhydrofluoride (70% $\text{py} \cdot (\text{HF})_x$) was added via a syringe. The mixture was stirred at ca. -5°C for 30 min. The reaction was quenched with ice- H_2O (10 g) and the solution was transferred to a separatory funnel. After removing the organic layer, the aqueous phase was extracted with CHCl_3 (3×15 mL).¹¹⁸ The combined organic layers were washed with saturated NaCl (15 mL), dried over Na_2SO_4 , and concentrated in *vacuo* to yield 151 mg of the crude methyl *erythro*-9(10)-fluoro,10(9)-hydroxystearate. Purification by flash chromatography (15% EtOAc /hexanes) gave 96.8 mg (0.292 mmol, 50% yield) of the pure products as a colourless oil at room temperature. This material contained one or more minor fluorinated contaminant(s) of unknown structure which are detected by ^{19}F NMR (m, -190.05 and -189.15 ppm) but not by GC/MS and ^1H NMR.

R_f 0.52 (20% EtOAc /hexanes)

MS (EI, 70 eV) m/z 312 $[(M - \text{HF})^+]$, 294 $[(M - \text{HF}) - \text{H}_2\text{O}]^+$

^{19}F NMR (376.5 MHz) δ -191.48, -191.56

^1H NMR (400 MHz) δ 4.37 (d of m, 1H, $J_{\text{HF}} = 49$ Hz, $-\text{CH}_2\text{CHFCHOH}-$), 3.71 (m, 1H, $-\text{CH}_2\text{CHOHCHF}-$), 3.67 (s, 3H, $-\text{C}(\text{O})\text{OCH}_3$), 2.30 (t, 2H, $-\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OCH}_3$), 1.62 (m, 2H, $-\text{CH}_2\text{CH}_2\text{CHFCHOH}-$), 1.51 (m, 2H, $-\text{CH}_2\text{CH}_2\text{CHOHCHF}-$), 1.33 (br s, 22H, $\text{CH}_3(\text{CH}_2)_6\text{CH}_2\text{CHF}(\text{OH})\text{CHOH}(\text{F})\text{CH}_2(\text{CH}_2)_5\text{CH}_2-$), 0.88 (t, 3H, CH_3CH_2-)

5.9 Racemic methyl *threo*-9(10)-fluoro,10(9)-hydroxystearate

5.9.1 Racemic methyl *cis*-9,10-epoxystearate

The epoxidation was performed as previously described (5.8.1). Racemic methyl *cis*-9,10-epoxystearate (2.9 g, 92% est. yield) was prepared via the reaction of methyl oleate (2.99 g, 10.1 mmol) with *m*-chloroperoxybenzoic acid (2.4 g, 9.3 mmol).¹¹⁹

mp 18-20 °C

MS (EI, 70 eV) m/z 294 $[(\text{M} - \text{H}_2\text{O})^+]$

^1H NMR (200 MHz) δ 3.69 (s, 3H, $-\text{C}(\text{O})\text{OCH}_3$), 2.93 (m, 2H, $-\text{CH}_2\text{CH}(\text{O})\text{CHCH}_2-$), 2.33 (t, 2H, $-\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OCH}_3$), 1.53 (m, 4H, $-\text{CH}_2\text{CH}_2\text{CH}(\text{O})\text{CHCH}_2\text{CH}_2-$), 1.36 (br s, 22H, $\text{CH}_3(\text{CH}_2)_6\text{CH}_2\text{CH}(\text{O})\text{CHCH}_2(\text{CH}_2)_5\text{CH}_2-$), 0.90 (t, 3H, CH_3CH_2-)

5.9.2 Racemic methyl *threo*-9(10)-fluoro,10(9)-hydroxystearate

Racemic methyl *cis*-9,10-epoxystearate (221 mg, ca. 0.711 mmol) was treated with 0.2 mL of pyridinium polyhydrofluoride (70% $\text{py} \cdot (\text{HF})_x$) at -5°C for 15 min. A work-up similar to that used to prepare *erythro*-derivative was employed (5.8.2). After purification by flash chromatography (15% EtOAc/hexanes), racemic methyl *threo*-9(10)-fluoro,10(9)-hydroxystearate (199 mg, 0.599 mmol, 85% yield) was obtained as a low-melting waxy solid.¹¹⁸ This material also contained one or more minor fluorinated contaminant(s) of unknown structure which are detected by ^{19}F NMR (m, -192.85 and -189.15 ppm) but not by GC/MS and ^1H NMR.

R_f 0.52 (20% EtOAc/hexanes)

MS (EI, 70 eV) m/z 312 $[(M - \text{HF})^+]$, 294 $[(M - \text{HF}) - \text{H}_2\text{O}]^+$

^{19}F NMR (376.5 MHz) δ -195.53 , -195.56

^1H NMR (400 MHz) δ 4.31 (d of m, 2H, $J_{\text{HF}} = 48$ Hz, $-\text{CH}_2\text{CHFCHF}-$), 3.66 (s, 3H, $-\text{C}(\text{O})\text{OCH}_3$), 3.51 (m, 1H, $-\text{CH}_2\text{CHOHCHF}-$), 2.30 (t, 2H, $-\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OCH}_3$), 1.62 (m, 2H, $-\text{CH}_2\text{CH}_2\text{CHFCHOH}-$), 1.48 (m, 2H, $-\text{CH}_2\text{CH}_2\text{CHOHCHF}-$), 1.35 (br s, 22H, $\text{CH}_3(\text{CH}_2)_6\text{CH}_2\text{CHF}(\text{OH})\text{CHOH}(\text{F})\text{CH}_2(\text{CH}_2)_5\text{CH}_2-$), 0.88 (t, 3H, CH_3CH_2-)

5.10 Methyl [12,12-²H₂]-7-thiastreate

5.10.1 [1,1-²H₂]-Heptan-1-ol

Heptanoic acid (7.51 g, 57.7 mmol) was reduced to [1,1-²H₂]-nonan-1-ol (6.7 g, 99% est. yield) using LiAlD₄ (2.75 g, 65.5 mmol). A procedure similar to that described in 5.2.1 was employed.¹⁰³

MS (EI, 70 eV) *m/z* 100 [(M - H₂O)⁺]

¹H NMR (200 MHz) δ 1.56 (br t, 2H, -CH₂CH₂CD₂OH), 1.29 (br s, 8H, CH₃(CH₂)₄CH₂-), 0.88 (t, 3H, CH₃CH₂-)

5.10.2 [1,1-²H₂]-1-Bromoheptane

8.8 g of 48% HBr was added to a 250 mL, round-bottomed flask equipped with a reflux condenser. 1.4 mL of concentrated H₂SO₄ was added in portions with stirring;

some HBr was evolved. [1,1-²H₂]-Heptan-1-ol (5.00 g, ca. 42.3 mmol) followed by 1.1 mL of concentrated H₂SO₄ was added to the flask in several portions with stirring and the mixture was refluxed gently for 1.5 h.¹³⁷ The contents of the flask was cooled to the room temperature. After addition of dH₂O (100 mL), the solution was transferred to a separatory funnel, and the aqueous layer was extracted with hexanes (3 × 50 mL). The combined organic phases were washed with NaHCO₃ (5%, 50 mL), dH₂O (2 × 50 mL), dried over Na₂SO₄ and concentrated in *vacuo* to give 5.8 g (75% est. yield) of a yellowish oily product.

MS (EI, 70 eV) *m/z* 100 [(M - HBr)⁺]

¹H NMR (200 MHz) δ 1.85 (br t, 2H, -CH₂CH₂CD₂Br), 1.31 (br s, 8H, CH₃(CH₂)₄-CH₂-), 0.89 (t, 3H, CH₃CH₂-)

5.10.3 [6,6-²H₂]-1-Dodecene

4-Penten-1-yl magnesium bromide (Grignard reagent) was prepared using dry magnesium turnings (1.11 g, 45.6 mmol), 5-bromo-1-pentene (6.80 g, 45.6 mmol) and 45.6 mL of anhydrous THF, following the procedure given in 5.7.1. [1,1-²H₂]-1-Bromoheptane (5.34 g, ca. 29.5 mmol) followed by 1.5 mL of a solution of dilithium tetrachlorocuprate (LiCl (20 mmol) and CuCl₂ (10 mmol) in 100 mL of anhydrous THF) was added via a syringe to the cooled flask (ca. -10 °C) in an ice-CaCl₂ bath. The

reaction mixture was then stirred for 3 h at ca. 0 °C.¹³⁸ The solution was quenched with crushed ice (30 g) and extracted with Et₂O (3 × 40 mL). The combined ethereal layers were washed with NH₄OH (7 M, 4 × 40 mL), dH₂O (2 × 40 mL), dried over Na₂SO₄, and concentrated in *vacuo* to give 4.99 g of the crude product. The purity of the product was estimated to be ca. 80% by GC/MS and ¹H NMR for unlabelled material. The major impurity was [1,1-²H₂]-1-bromoheptane (starting material). This intermediate was used in the next step without further purification.

MS (EI, 70 eV) *m/z* 170 (M)⁺

¹H NMR (200 MHz) δ 5.85 (m, 1H, -CH₂CH=CH₂), 4.96 (m, 2H, -CH=CH₂), 2.07 (m, 2H, -CH₂CH₂CH=CH₂), 1.85 (br t, 2H, -CH₂CH₂CD₂Br, starting material), 1.32 (br s, 14H, CH₃(CH₂)₅CD₂(CH₂)₂CH₂-), 0.89 (t, 3H, CH₃CH₂-)

5.10.4 [5,5-²H₂]-Undecanoic acid

[6,6-²H₂]-1-Dodecene (5 g, ca. 80% pure) was oxidized to [5,5-²H₂]-undecanoic acid (3.4 g, ca. 85% pure) using a 2 M KMnO₄ solution.¹⁰⁶ A procedure similar to that used to synthesize [2,2-²H₂]-undecanoic acid was employed (5.2.4). The major impurity was [1,1-²H₂]-1-bromoheptane as judged by ¹H NMR (ca. 15%). This intermediate was used in the next step without further purification.

MS (EI, 70 eV) *m/z* 188 (M)⁺

^1H NMR (200 MHz) δ 2.33 (t, 2H, $-\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.59 (m, 2H, $-\text{CH}_2\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.85 (br t, 2H, $-\text{CH}_2\text{CH}_2\text{CD}_2\text{Br}$, starting material), 1.23 (br s, 12H, $\text{CH}_3(\text{CH}_2)_5\text{CD}_2\text{CH}_2\text{CH}_2-$), 0.89 (t, 3H, CH_3CH_2-)

5.10.5 $[5,5\text{-}^2\text{H}_2]$ -Undecan-1-ol

Following the procedure described in 5.4.1, 2 g of the crude $[5,5\text{-}^2\text{H}_2]$ -undecanoic acid was reduced to $[5,5\text{-}^2\text{H}_2]$ -undecan-1-ol (1.3 g, 25% est. yield, over three steps) using LiAlH_4 (0.62 g, 16 mmol).¹⁰³ $[1,1\text{-}^2\text{H}_2]$ -1-Bromoheptane was no longer present as judged by ^1H NMR and GC/MS.

MS (EI, 70 eV) m/z 156 $[(\text{M} - \text{H}_2\text{O})^+]$

^1H NMR (200 MHz) δ 3.65 (t, 2H, $-\text{CH}_2\text{CH}_2\text{OH}$), 1.58 (m, 2H, $-\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 1.24 (br s, 12H, $\text{CH}_3(\text{CH}_2)_5\text{CD}_2(\text{CH}_2)_2\text{CH}_2-$), 0.88 (t, 3H, CH_3CH_2-)

5.10.6 $[5,5\text{-}^2\text{H}_2]$ -1-*p*-Toluenesulfonyloxyundecane

[5,5-²H₂]-Undecan-1-ol (1.1 g, ca. 7.3 mmol) was converted to the corresponding tosylate (1.6 g, 67% est. yield) using *p*-toluenesulfonyl chloride (2.1 g, 11 mmol).¹⁰⁴ A procedure similar to that reported in 5.2.2 was employed.

MS (EI, 70 eV) *m/z* 173 [(M - Ts)⁺]

¹H NMR (200 MHz) δ 7.80 (d, 2H, Ar-H), 7.34 (d, 2H, Ar-H), 4.02 (t, 2H, -CH₂CH₂OTs-), 2.44 (s, 3H, Ar-CH₃), 1.59 (br t, 2H, -CH₂CH₂CH₂OTs-), 1.21 (br s, 12H, CH₃(CH₂)₅CD₂(CH₂)₂CH₂-), 0.87 (t, 3H, CH₃CH₂-)

5.10.7 [12,12-²H₂]-7-Thiastearic acid

S-alkylation of the dilithio salt of 7-mercaptohexanoic acid (730 mg, ca. 4.93 mmol) using [5,5-²H₂]-1-*p*-toluenesulfonyloxyundecane (1.6, ca. 5.0 mmol) gave 0.74 g of the crude [12,12-²H₂]-7-thiastearic acid.¹⁰⁰ A procedure similar to that used to synthesize [9,9-²H₂]-7-thiastearic acid was employed (5.2.7). The chromatographic and spectral properties were similar to those of unlabelled material except for the following:

MS (EI, 70 eV) *m/z* 304 (M⁺)

5.10.8 Methyl [12,12-²H₂]-7-thiastearate

Methylation of the crude [12,12-²H₂]-7-thiastearic acid (0.74 g) using BF₃/MeOH¹⁰⁰ followed by flash chromatography (4% EtOAc/hexanes) yielded 420 mg (1.32 mmol, 26% over two steps and 5.5% est. overall yield) of the colourless oily product at room temperature. A procedure similar to that described in 5.1.3 was employed. The physical, chromatographic and spectral properties were similar to those of unlabelled material except for the following:

MS (EI, 70 eV) *m/z* 318 (*M*⁺), 287 [*M* - OCH₃]⁺, 189 [(CH₃(CH₂)₅CD₂(CH₂)₄S)⁺], 129 [*M* - (CH₃(CH₂)₅CD₂(CH₂)₄S)⁺]

¹³C NMR (100 MHz) δ 29.43 (C-13, upfield β-isotope shift (0.21 ppm)), 29.58 (C-14, upfield γ-isotope shift (0.06)), 29.09 (C-11 upfield β-isotope shift (0.21 ppm)), 28.93 (C-10, upfield γ-isotope shift (0.06 ppm))

5.11 Methyl [13,13-²H₂]-7-thiastearate

The title compound (403 mg, 1.27 mmol, 4% est. overall yield) was prepared using experimental procedures analogous to those described in 5.10. The sequence of steps is as follows: [1,1-²H₂]-1-bromohexane (23 mmol, 73% est. yield), obtained from the bromination¹³⁷ of the corresponding alcohol,¹⁰³ was coupled with 6-bromo-1-hexene

to give crude [7,7- $^2\text{H}_2$]-1-dodecene.¹³⁸ This material was cleaved¹⁰⁶ to yield crude [6,6- $^2\text{H}_2$]-undecanoic acid which in turn was reduced¹⁰³ to provide [6,6- $^2\text{H}_2$]-undecan-1-ol (57.6% over three steps). The corresponding tosylate, [6,6- $^2\text{H}_2$]-1-*p*-toluenesulfonyloxyundecane (67% est. yield),¹⁰⁴ was used to S-alkylate¹⁰⁰ the dilithio salt of 6-mercaptohexanoic acid. Methylation¹⁰¹ of the carboxyl function via BF_3/MeOH yielded the title compound. The physical, chromatographic and spectral properties of the title compound were similar to those of unlabelled material except for the following:

MS (EI, 70 eV) m/z 318 (M^+), 287 [$(\text{M} - \text{OCH}_3)^+$], 189 [$(\text{CH}_3(\text{CH}_2)_4\text{CD}_2(\text{CH}_2)_5\text{S})^+$], 129 [$(\text{M} - (\text{CH}_3(\text{CH}_2)_4\text{CD}_2(\text{CH}_2)_5\text{S}))^+$]

^{13}C NMR (100 MHz) δ 29.64 (C-13, absent), 29.43 (C-14, upfield β -isotope shift (0.21 ppm)), 29.38 (C-12, upfield β -isotope shift (0.19 ppm)), 29.30 (C-15, upfield γ -isotope shift (0.07 ppm)), 29.24 (C-11, upfield γ -isotope shift (0.06 ppm))

Part 3: Biological Experiments

5.12 General

All components of the biological media were purchased from Difco with the exception of D-glucose which was obtained from BDH. The “dropout powder” (5.13.2) was donated by Dr. Pat Covello (PBI, Saskatoon). Distilled deionized water (ddH_2O) was used in the preparation of all media and was obtained from Millipore Milli Q water system CD0F01205 equipped with a Super C Carbon cartridge, two ion Ex cartridges, an Organex Q cartridge and a Milli Pak Filter. All glassware and media were sterilized in an Amsco Eagle series (model 2021) gravity autoclave at 120 °C for 30 min. All biological transfers were performed in a sterile laminar-flow fumehood under a Bunsen burner flame. All cultures were grown in a New Brunswick Scientific incubator shaker, either model G25 or Gyrotory water bath model G75, at 150 rpm. The cells were separated

from the medium by centrifugation for 10 min (5500 rpm) at ca. 4 °C using a Sorvall model RC-5B refrigerated centrifuge (Biology Department, Carleton University).

The yeast, *S. cerevisiae*, strain ATCC #12341 was obtained from the American Type Culture Collection, Rockville, Maryland. A pDR₁ transformed strain of *S. cerevisiae* containing a functional oleate Δ^{12} desaturase from *A. thaliana*, as well as detailed instructions on its growth and maintenance, were generously provided by Dr. Pat Covello (PBI, Saskatoon).

5.13 Incubation Experiments

5.13.1 Yeast Δ^9 Desaturase (*S. cerevisiae*)

S. cerevisiae, strain ATCC #12341 was maintained on slant cultures containing YPD (1% Yeast extract, 2% bacto-Peptone, and 2% D-glucose) medium.⁸⁹ The slant medium was prepared as follows: A solution of yeast extract (5 g), bacto-peptone (10 g), and bacto-agar (15 g) in 400 mL of ddH₂O was prepared in a 1 L Erlenmeyer flask. A solution of glucose (10 g) in 100 mL of ddH₂O was prepared in a 500 mL Erlenmeyer flask. The two flasks were then plugged with cotton wool, capped with a double layer of tinfoil, and autoclaved along with culture tubes and a cotton-plugged, 5 mL, graduated, wide-mouthed pipette (foil-wrapped). The two hot solutions were immediately combined and then ca. 5 mL aliquots of this solution were added to each of the culture tubes using the sterile 5 mL graduated pipette. All transfers were carried out under sterile conditions. The agar was allowed to solidify with the culture tubes lying on a “slant”. The resultant “slants” were then stored at 4 °C until needed. To obtain a batch of freshly grown cells for use in a feeding experiment, the required number of slants were streaked with *S. cerevisiae* cells (stored at 4 °C) and incubated at 30 °C for 24 h. Every three months a new slant was inoculated from a stock culture of *S. cerevisiae*.

The YPD culture medium for incubation of ca. 25 mg of substrate was prepared as follows: A solution of D-glucose (6.5 g) in 150 mL of ddH₂O was prepared in a 1 L

Erlenmeyer flask; a solution of bacto-peptone (6.5 g), and yeast extract (3.25 g) in 200 mL ddH₂O was prepared in a 5 L beaker. 9.5 mL of the D-glucose solution and 15.5 mL of the bacto-peptone and yeast extract solution were added to a 250 mL Erlenmeyer flask and served as the medium for the “starter culture”.⁸⁹ The remaining bacto-peptone and yeast extract solution was then equally divided between two 500 mL Erlenmeyer flasks. The four Erlenmeyer flasks were plugged with cotton wool, capped with a double layer of tinfoil and sterilized along with a 10 mL graduated cylinder, three cotton-plugged glass pipettes (foil-wrapped) and a 140 mL beaker for 30 min. The D-glucose solution was equally divided among each 500 mL Erlenmeyer flask using the sterile 140 mL beaker.

The “starter culture” flask was inoculated with a small portion of yeast from a slant culture using a flamed wire loop and incubated at 30 °C for ca. 24 h. Under sterile conditions, 5-7 mL of the “starter culture” was then distributed among two 500 mL Erlenmeyer flasks using the sterile 10 mL graduated cylinder. Ca. 25 mg of the substrate in 1 mL of absolute EtOH was added to one of the 500 mL Erlenmeyer flasks and 1 mL of absolute EtOH was transferred to the other flask (control) using the autoclaved pipettes. These two cultures were then incubated with shaking (150 rpm) at 30 °C for 18-24 h. The turbid samples were poured into four pre-chilled polyethylene centrifuge bottles and centrifuged at 5500 rpm for 10 min at ca. 4 °C. The supernatants were discarded and the cell pellets were re-centrifuged after addition of 25 mL of ddH₂O to each pellet.⁸⁹

5.13.2 Oleate Δ^{12} Desaturase (*A. thaliana*)

The pDR₁ strain of *S. cerevisiae* (PBI, Saskatoon) slants containing a functional oleate Δ^{12} desaturase from *A. thaliana* was grown on SGal_{ura} medium.¹³⁹ 2% D-galactose, 0.7% yeast nitrogen base without amino acids, 0.2% “dropout powder” (the essential base and amino acid mixture for this yeast strain to grow (Appendix D)), 2% bacto-agar. The SGal_{ura} medium for incubation of ca. 25 mg of substrate contained: D-galactose (6.6 g) in 150 mL of ddH₂O and a solution of yeast nitrogen base without amino acids (2.2 g) and “dropout powder” (0.5 g) in 200 mL of ddH₂O. The media were

prepared as described in 5.13.1. 8 mL of starter culture grown at 30 °C for 48 h was used to inoculate 150 mL of sterile medium contained in a 500 mL Erlenmeyer flask.

A ca. 25 mg portion of thia fatty acid methyl ester was added as a solution (5% w/v) in absolute EtOH to each flask and the inoculated cultures were then incubated in a portable shaker (150 rpm) inside a refrigerated plant growth chamber set at 15 °C for 1 wk (Environmental Laboratories, Carleton University). Cells were harvested as previously described (5.13.1). Sterile, and transfer procedures as well as other experimental details were similar to those used for Δ^9 desaturase (5.13.1).

5.14 Isolation of Fatty Acids

5.14.1 Extraction of Cellular Fatty Acids

The harvested yeast cells were transferred to a 100 mL, round-bottomed flask, equipped with a stirring bar, and a condenser using 50 mL of 1 M KOH in 50% EtOH. The mixture was hydrolyzed for 3 h at 80 °C under N₂. The resultant mixture was then filtered using a Buchner funnel equipped with 5.5 cm filter paper. The clear yellow solution was added to a 1 L round-bottomed flask and the EtOH was removed in *vacuo*. Bumping of the solution was minimized by using a high rpm setting and a lukewarm water bath (30-35 °C). The resulting brown solution was diluted with 100 mL dH₂O, transferred to a separatory funnel, acidified with 5 mL of H₂SO₄ to ca. pH 2 (Universal litmus paper), and extracted with CH₂Cl₂ (4 × 40 mL). The combined organic extract was washed with H₂O (20 mL), saturated NaCl (20 mL), dried over Na₂SO₄, and concentrated in *vacuo* to yield a fatty acid mixture as a slightly yellow solid.⁸⁹

5.14.2 Extraction of Fatty Acids in Culture Medium

The culture medium was acidified with 5 mL of the 50% H_2SO_4 to ca. pH 2 (Universal litmus paper) and extracted with CH_2Cl_2 (2×40 mL), and CHCl_3 (2×40 mL). The organic extracts were combined, washed with dH_2O (40 mL), saturated NaCl (40 mL), dried over Na_2SO_4 and concentrated in *vacuo* to give a slightly yellow residue.⁸⁹

5.15 Derivatization of Fatty Acids Using Diazomethane

An ethereal solution of diazomethane was prepared by the following method: 3 mL of a 50% (w/v) aqueous solution of KOH and 15 mL of Et_2O were added to a 25 mL test tube. The test tube was placed on ice and 250 mg of nitrosomethylurea was added portionwise. Diazomethane gas bubbled into the ether layer and turned it yellow in colour. The ethereal layer was transferred to another test tube and Na_2SO_4 was added to remove any remaining H_2O .

The fatty acid extracts were dissolved in 1-2 mL Et_2O , cooled in a ice-bath, and then the ethereal diazomethane solution was added until no bubbling (occurring from the release of N_2 gas) was observed and a yellow colour persisted. The Et_2O was removed in *vacuo* and the resulting product was stored in Reactivials under N_2 at 0 °C. CH_2Cl_2 was added to the residue in order to perform the GC/MS analyses.

Appendix A

Primary Deuterium Kinetic Isotope Effects (KIEs)

Kinetic isotope effects (KIEs) originate in isotopically sensitive zero-point energy (the vibrational energy level at absolute zero ($n = 0$)) differences between initial states and transition states. In the case of primary isotope effects, it seems natural to identify this difference with the loss of zero-point energy ($E_0 = 1/2 h\nu$) which accompanies the conversion of vibrational into translational motion as the reacting bond breaks (Figure A).⁹⁶ This picture of primary isotope effects leads to the well known estimate of k_H/k_D for the primary KIE on C–H bond breaking: the zero-point energy of a typical C–H stretching vibration with $\nu = 3,000\text{ cm}^{-1}$ is 4.3 kcal/mole; deuterium substitution reduces the frequency to $2,200\text{ cm}^{-1}$ and the zero-point energy to 3.2 kcal/mole; and the difference, $\Delta E_0 = 1.15\text{ kcal/mole}$, using $k_H/k_D = \exp(\Delta E_0/RT)$ at 300 K gives $k_H/k_D = 6.9$.

Figure A. Energy profile for C–H and C–D bonds.

Appendix B

¹³C Chemical Shifts of Labelled and Unlabelled Methyl 7-Thiastearate Fatty Acids

C #	Methyl 7-thiastearate	Methyl 7-thiastearate-9d ₂	Methyl 7-thiastearate-10d ₂	Methyl 7-thiastearate-12d ₂	Methyl 7-thiastearate-13d ₂
1	174.09	174.01	174.12	174.04	174.07
2	33.98	33.98	33.96	33.97	33.97
3	24.60	24.60	24.58	24.60	24.60
4 or 5	28.43	28.43	28.41	28.43	28.43
4 or 5	29.37	29.37	29.36	29.38	29.36
6 or 16	31.98	31.94	31.92	31.99	31.99
8	32.26	32.07	32.17	32.27	32.27
9	29.77	Absent	29.51	29.78	29.77
10	28.99	28.80	Absent	28.93	28.99
11	29.30	29.24	29.08	29.09	29.24
12	29.57	29.57	29.51	Absent	29.38
13	29.64	29.64	29.63	29.43	Absent
14	29.64	29.64	29.63	29.58	29.43
15	29.37	29.37	29.36	29.38	29.30
6 or 16	31.94	31.98	31.92	31.93	31.95
17	22.71	22.70	22.70	22.67	22.70
18	14.12	14.12	14.13	14.11	14.11
O-Me	51.49	51.49	51.51	51.45	51.47

Appendix C

Evaluation of k_H/k_D in an Intermolecular Competition⁹⁹

Assuming that two isotopic molecules, S_H and S_D , undergo analogous irreversible reactions, according to

it is possible to write the general rate expression:

$$v_H = -\frac{dS_H}{dt} = k_H S_H \quad \text{and} \quad v_D = -\frac{dS_D}{dt} = k_D S_D$$

Since dt is the same for both equations, it must consequently hold that

$$\frac{1}{k_H} \times \frac{dS_H}{S_H} = \frac{1}{k_D} \times \frac{dS_D}{S_D}$$

With the initial conditions $S_H = S_H^0$ and $S_D = S_D^0$, integration and rearrangement gives

$$\frac{k_H}{k_D} = \frac{\ln(S_H / S_H^0)}{\ln(S_D / S_D^0)}$$

Introducing the fractional amount of conversion, or fraction of reaction, F , yields:

$$\frac{k_H}{k_D} = \frac{\ln(1 - F_H)}{\ln(1 - F_D)}$$

Since the limit of $\ln(1 - x)$ as $x \rightarrow 0$ is $-x$, at low level of conversion (10 - 20%) this eq. reduces to:

$$\frac{k_H}{k_D} = \frac{F_H}{F_D}$$

F_H as a function of F_D is shown in Figure B. It is evident that for small conversion of either isotopic species the curves are almost straight lines (lower left corner). The simple meaning of all this is that the fraction of reaction of each species is proportional to the specific reaction rate of that species as long as the supply of the reactants is not appreciably decreased by consumption.

Since the fractional conversion can be measured via:

$$F_H = 1 - \frac{S_H}{S_H^0} = \frac{P_H}{S_H^0}$$

Thus, k_H/k_D can be calculated using the following equation:

$$\frac{k_H}{k_D} = \frac{P_H / S_H^0}{P_D / S_D^0}$$

or

$$\frac{k_H}{k_D} = \frac{P_H / P_D}{S_H^0 / S_D^0}$$

The ratio of the two isotopic species in the product (P_H / P_D), and starting mixture (S_H^0 / S_D^0) can experimentally be obtained using a mass spectrometer.

Figure B. The relation between the fractions of reaction of two competing isotopic reactants. The rate-constant ratio k_H/k_D indicated at each curve

Appendix D

Composition of “Dropout Powder”

Supplement	Final Concentration	
	(mg / L)	g / 100 L
Adenine sulphate	39.48	4.0
L-Arginine-HCl	19.92	2.0
L-Aspartic acid	99.60	10.0
L-Glutamic acid	99.60	10.0
L-Histidine-HCl	19.92	2.0
L-Isoleucine	29.88	3.0
L-Leucine	56.76	6.0
L-Lysine-HCl	29.88	3.0
L-Methionine	19.92	2.0
L-Phenylalanine	49.80	5.0
L-Serine	373.50	37.4
L-Threonine	199.20	20.0
L-Tryptophan	39.84	4.0
L-Tyrosine	30.00	3.0
L-Valine	149.40	14.9

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