Use of Fluorine-Tagged Thia Fatty Acids as Mechanistic Probes for Desaturase-Mediated Sulfoxidation

By

Kim Y.Y. Lao, B.Sc.

A Thesis submitted to the Faculty of Graduate Studies

in partial fulfillment of the requirements for the degree of

Master of Science

to

The Faculty of Graduate Studies

Department of Chemistry

Carleton University

Ottawa, Canada

August 2006

Your file Votre référence
ISBN: 978-0-494-18367-0
Our file Notre référence
ISBN: 978-0-494-18367-0

Abstract

The cryptoregiochemistry of the Δ^9 desaturase (*S. cerevisiae*) was determined using a series of fluorine-tagged thiastearates with the sulfur atom in the 8^{th}, 9^{th}, 10^{th} or 11^{th} position. Percentage conversion of sulfide to sulfoxide was 1.2%, 7 %, 4.5 %, 2.0 % for the 8-, 9-, 10-, and 11-thia analogues respectively and agreed with previous data using non-fluorinated substrates.

Stereochemical analysis of Δ^9 desaturase-mediated sulfoxidation was carried out using methyl 15-fluoro-11-thiapentadecanoate as a test substrate and (*S*)-(+)-MPAA as the chiral shift reagent. Analysis of (*R,S*)-methyl 15-fluoro-11-thiapentadecanoate, S-oxide complexed with MPAA in $CDCl_3$ revealed doubling of the ^{19}F NMR signal ($\Delta\delta$ = 0.01 ppm). Methyl 15-fluoro-11-thiapentadecanoate was oxidized in the yeast whole cell system and the stereochemistry of the predominant enantiomer (*S*, 32 % ee) correlated with the known pro *R* selectivity of the parent dehydrogenation reaction.

Chemoenzymatic synthesis of fluorine-tagged 12-thia and 13-thiaoleates were investigated in preparation for *in vitro* sulfoxidation using an over-expressed membrane-bound Δ^{12} desaturase (desA). *Trans*-olefin formation was observed for a number of 12-thia substrates including 16F-12S (17 % -*trans*), 18F-12S (22% -*trans*) and C18-12S (23 % trans) fatty acid methyl esters. The details of a trial *in vitro* Δ^{12} desaturase experiment are reported.

Acknowledgments

I'd like to take this opportunity to give thanks to Dr. Peter Buist who has given me guidance and encouragement throughout the years working in the lab. A big thank you goes out to Pat Covello, Robert Sasata, Darwin Reed and the rest of the lab group at PBI Saskatoon who helped me in making this thesis possible. Technical assistance from Keith Bourque (Carleton University), Clem Kazakoff (Ottawa University) and Tony O'Neil (Carleton University) was much appreciated throughout my project. I'd also like to give thanks to my family who has supported me throughout the years and who have given me patience in trying times. I'd like to give thanks to my former and current lab mates Laura, Frances, Nigel, Rishi, James and Amy who made my stay enjoyable at Carleton. Finally I'd like to give thanks to Amber who has given me much needed moral support.

Table of Contents

Chapter 3: Experimental

List of Figures **Page**

List of Tables **Page**

Chapter 1: Introduction

1.1 Fatty acids

1.1.1 Major types of Fatty Acid Derivatives found in Biological Systems

Fatty acids are long chain carboxylic acids and are commonly found in biological systems as esters of triglycerides and phospholipids[1]. Chain lengths can vary but the most common fatty acid types encountered range from 14 to 20 carbons in length. Common chain lengths found in plant and animal cell membranes are of the 16 and 18 carbon length. Fatty acids may be substituted to varying degrees with different substituents such as methyl, hydroxyl or keto groups to list a few[1].

The most common form of fatty acid modification (and the focus of this study) is desaturation (dehydrogenation) of the aliphatic component. This biochemical reaction is catalyzed by enzymes known as desaturases. The positioning and level of unsaturation varies greatly but the most common are displayed in Table 1.1.

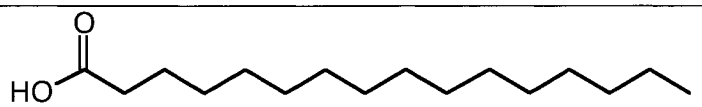
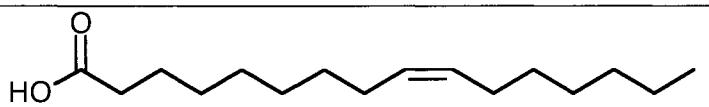
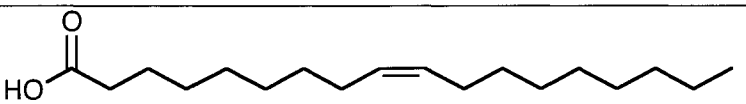
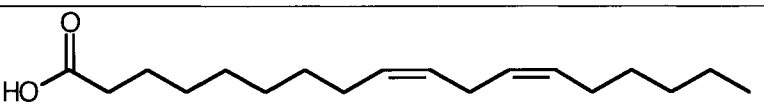
Chain Length	Structure
16:0	
16:1 (Δ9)	
18:1 (Δ9)	
18:2 (Δ9, 12)	

Table 1.1 Structures of common fatty acids.

Common fatty acids found in plants and animals include: oleic acid (Δ^9), linoleic ($\Delta^{9,12}$) acid, α-linolenic acid ($\Delta^{9,12,15}$) and γ-linolenic acid ($\Delta^{6,9,12}$). Linolenic and α-linolenic acids are classified as essential fatty acids, as they are required in human diet in order to maintain good health. These are usually precursors to longer chain fatty acids (arachidonic acid) or converted to other species[6]. γ-Linoleic acid contains *cis*-double bonds at the 6th, 9th and 12th position and is the precursor to longer chains and other poly unsaturated fatty acids (PUFA)[7].

Other major fatty acid types include the "unusual" acetylenic type fatty acids. Crepenynate is an example of a fatty acid containing a triple bond at the 12 position and is derived from the desaturation of linoleic acid[10]. Ring-containing fatty acids also exist such as dihydrosterculic acid (9,10-methylene octadecanoic acid) that have a cyclopropane ring at the 9th and 10th position[8]. This unusual fatty acid is formed by the methylation of oleic acid and can be further desaturated to form the cyclopropene derivative, such as sterculic acid (9,10-methylene octadecenoic acid). It has been shown that sterculic acid inhibits mammalian, membrane bound stearoyl CoA desaturase and this phenomenon is assumed to be a defence mechanism. [11]

1.1.2 Nomenclature

Identification of fatty acids may follow the systematic approach (octadecanoic acid for C18 derivative), however the use of its trivial name is a more common way of communicating. Octadecanoic acid is often referred to as stearic acid and hexadecanoic acid is referred to as palmitic acid[9]. Naming of unsaturated forms of fatty acids follow

similar conventions with their own systematic and trivial names. Table 2.2 displays a small sample of the more common unsaturated forms.

No. of Carbon atoms	Systematic name	Trivial name
8	Octanoic	Caprylic
14	Tetradecanoic	Myristic
16	Hexadecanoic	Palmitic
18	Octadecanoic	Stearic
20	Eicosanoic	Arachidic

Table 1.1 Names of various saturated fatty acids[9]

No. of Carbon atoms	Systematic name	Trivial name
14	*Cis*-9-tetradecenoic	Myristoleic
16	*Cis*-9-hexadecanoic	Palmitoleic
18	*Cis*-9-octadecenoic	Oleic
18	*Trans*-9-octadecenoic	Elaidic

Table 1.2 Names of various unsaturated fatty acid.

As is often the case, shorthand identifiers are employed in order to communicate fatty acid type. The X:Y convention is often used when only the chain length (X) and degree on unsaturation (Y) is needed: eg oleic acid can be referred to as 18:1.

1.1.3 Biological Role of Unsaturated Fatty Acid Derivatives

The biological roles of unsaturated fatty acid derivatives range from 1) adjusting cell membrane fluidity in cold acclimation[2], 2) biosynthesis of defense molecules[3] to 3) biosynthesis of chemical signals between insects[4].

1) It is the level of desaturation that influences the degree of membrane fluidity[12,13] in the phospholipid bilayer. Typically, desaturation of fatty acids lead to the introduction of a *cis*-double bond. This double bond provides a kink in the overall structure of the fatty acid, and in the context of the phospholipid bilayer increases

membrane fluidity. The increase of unsaturation would increase the "fluidity" of the cell membrane as the conformation of the hydrocarbon chain deviates away from linearity. This has been shown to play a role in regulating membrane fluidity under low temperature stress among many organisms.

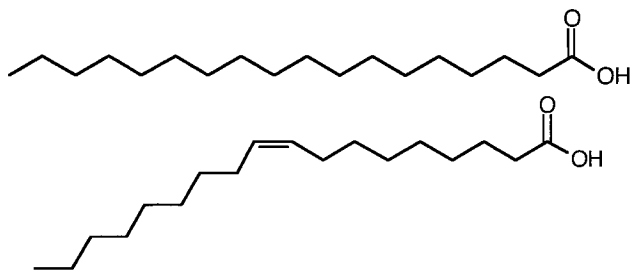


Figure 1.1 Effect of *cis*-double bond on overall shape of fatty acids

2) Various exotic fatty acids are known to be used in defense mechanisms against predators[14]. As mentioned previously, cyclopropenyl type fatty acids have been implicated in defence mechanisms because they inhibit a variety of desaturases in animals leading to various disorders[15]. A family of polyunsaturated fatty acids known as oxylipins[16] was found to delay the growth of bacterial and fungal cultures in liquid medium. These fatty acids are derived from linoleic and α-linoleic acid. Other fatty acids were shown to induce a plant defense response, such as volicitin, a regurgitate of a beet armyworm caterpillar (*Spodoptera exigua*). These oral secretions were found to induce corn seedlings to release volatile compounds attracting natural predators of the beet armyworm caterpillar.

3) Certain fatty acid derivatives have been found to play a role in signaling in insects[17] and mammals[18]. Sphingolipid derivatives containing a *trans*-double bond in the 4th position or a 4-hydroxy moiety have been implicated in cell proliferation and apoptosis in mammals[19]. The Δ^{11} desaturase is well known in its involvement in the

biosynthesis of insect pheromones[20,21]. Females of a cabbage moth (*Mamestra brassicae*) produced a pheromone, *cis*-11-hexadecenyl acetate, which is derived from the desaturation of hexadecanoic acid at the 11th and 10th position.

1.2 Desaturation of Fatty Acids

1.2.1 Desaturase

Desaturases are a class of enzymes that introduce a double bond into a fatty acid by dehydrogenation (desaturation)[22]. Their naming convention follows a Δ^x standard, where x is the location of the double bond starting in the position closest to the carbonyl end group. As an example, a desaturase that forms a double bond between the 9th and 10th position is a Δ^9 desaturase; while one that adds a double bond between the 4th and 5th positions is a Δ^4 desaturase, and so on. Desaturases can be divided into two categories, those that are membrane-bound[23] or those that are soluble[24]. Membrane-bound desaturase utilize fatty acyl CoA or glycerolipid derivatives as substrate while the soluble counterpart exclusively uses ACP thioesters. Attempts to determine the structure of fatty acid desaturase has been met with mixed results.

The membrane bound family of desaturase have been proven difficult to purify and isolate outside of its native environment. Attempts to obtain X-ray crystal structures are ongoing in at least two laboratories. Fractionation of rat liver microsomes[25] led to the first documented isolation and purification of an integral membrane-bound desaturase that allowed elucidation of specific structural components. The Δ^9 desaturase was found to consist of 3 major components; (1) NADH-cytochrome b_5 reducase; (2) cytochrome b_5 components; (3) and the integral membrane bound desaturase[51]. Detailed analysis

coupled with site directed mutagenesis revealed the presence of eight histidines believed to coordinate a di-iron center[25-27].

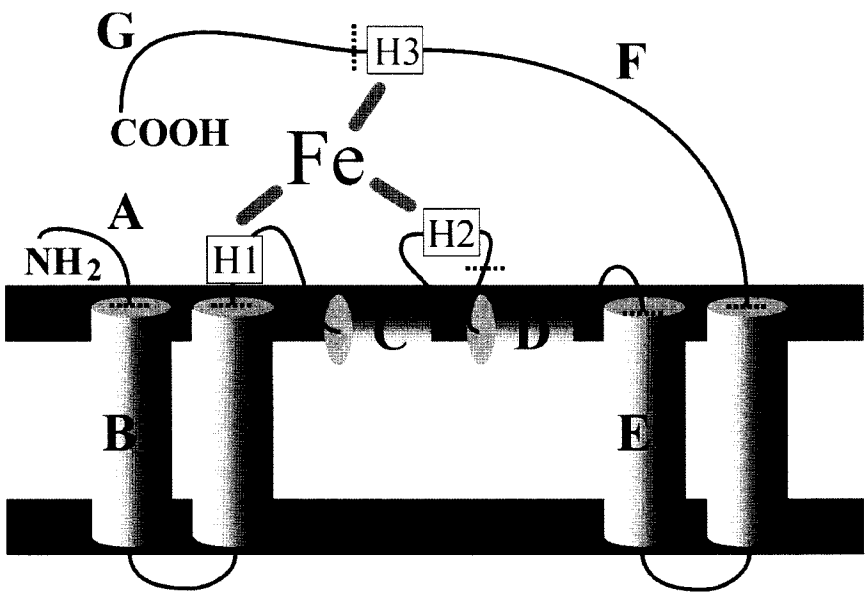


Figure 1.2 Proposed topology of a membrane bound desaturase. (adapted from Sasata et al.[50])

In contrast, progress in the structural study of soluble desaturases has been substantial. Stearoyl-ACP Δ^9 desaturase was purified from castor[28] and safflower[29] embryos and overexpressed in *E. coli.* Sufficient amounts of active enzyme could be isolated and allowed for a crystal structure determination of the soluble Δ^9 desaturase by Lindqvist and Shanklin[30]. The structure revealed the presence of a non-heme carboxylate-bridged di-iron at the putative active site. A hydrophobic region within the soluble enzyme was also discovered that is presumed to accommodate the substrate and is designed to force it into a gauche conformation at the C9-C10 position[30].

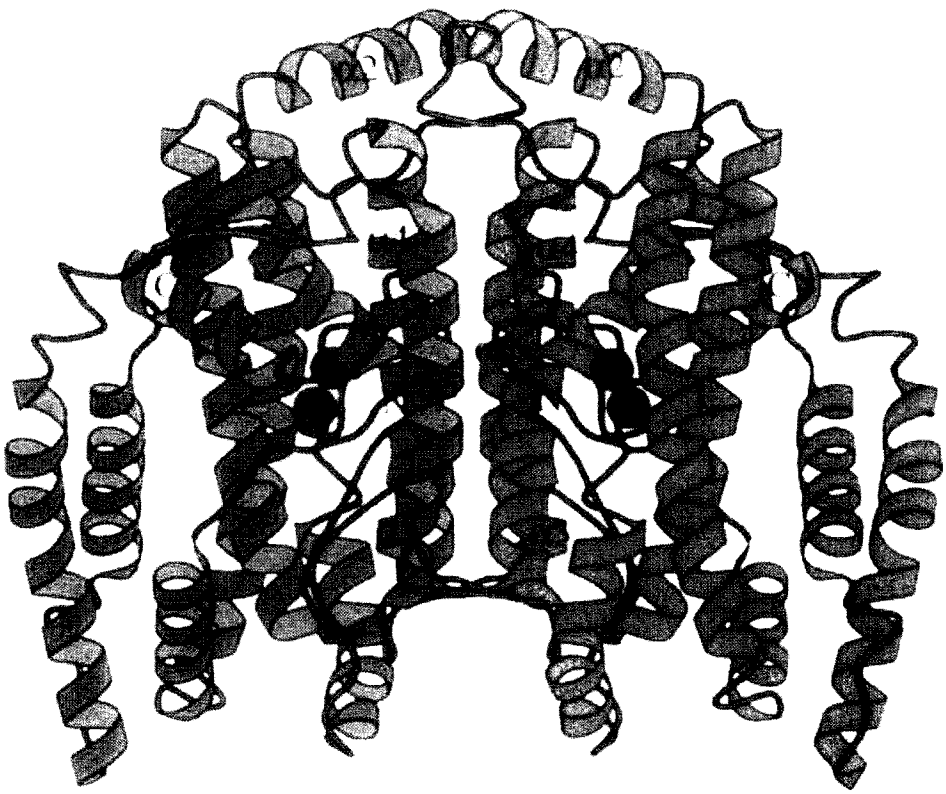


Figure 1.3 3D structure of soluble Δ^9 desaturase dimer; Image adapted from Lindqvist et al[30].

1.2.2 Proposed General Mechanism

Current knowledge of the desaturation process has been established using *in vivo* and *in vitro* Δ^9 desaturase experiments using labeled substrates. What was found was that the removal of hydrogens occurred in a stepwise, pro *R* selective, *syn*-dehydrogenation process[31,32]. It is in agreement that the current mechanistic model is valid for both soluble and membrane bound desaturases since both enzymes are thought to utilize a non-heme diiron catalytic centre and are NADH and O_2-dependent.

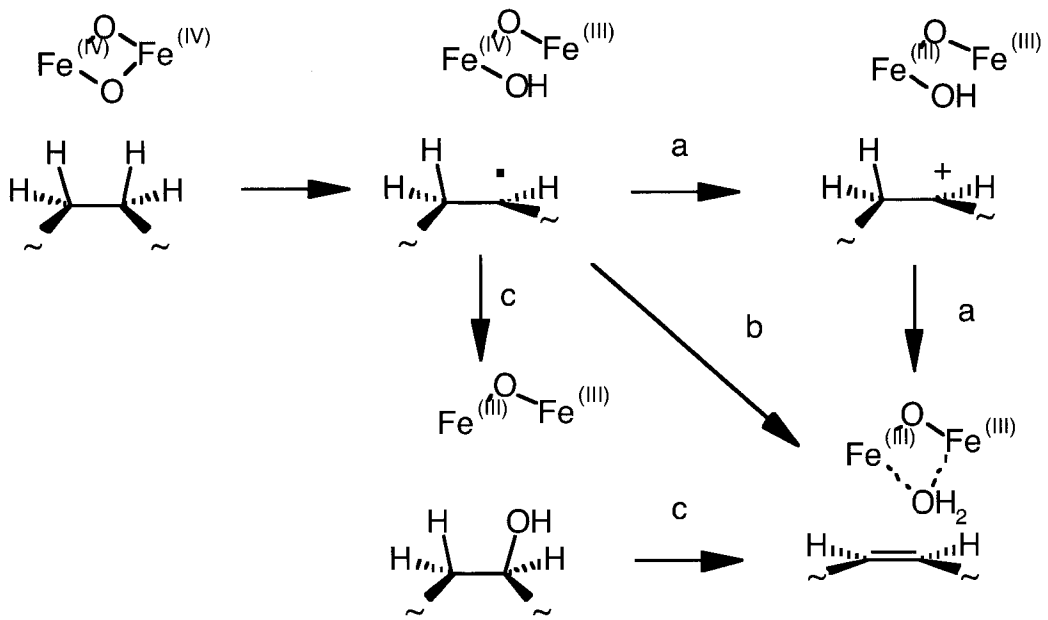


Figure 1.4 Generic mechanism of a *syn*-dehydrogenation process resulting in the formation of a double bond. Initial hydrogen abstraction considered to be a kinetically slow process. Route (b) is the accepted pathway. Refer to review[68] for expanded scheme of desaturase regeneration of active site.

Figure 1.3 outlines the current proposed mechanistic model for fatty acid desaturation. Pro *R*- hydrogen abstraction leading to a carbon-centred radical begins[33] the process and is kinetically the slowest step in this mechanism. What follows is assumed to be a quick collapse of the intermediate to produce the expected olefin group with iron-bound water as the by-product (Pathway B). Pathway A comes from early suggestions that stem from reports on Cytochrome P450[35] that rapid oxidation of the radical center could lead to a carbocationic intermediate followed by a rapid collapse to the double bond[36] by loss of a proton instead of a hydrogen atom. Path C shows that some membrane bound desaturase may permit a competing pathway that results in a "hydroxyl rebound " and dehydration of the fatty acid would lead to the double bond formation. Indeed, small amounts (ca 1 %) of the secondary alcohol are observed as byproducts in many membrane-bound desaturase systems[34]. For example, recent studies

using GC-MC analysis of enzymatic products obtained from an *in vivo* yeast Δ^9 desaturase yeast have shown that the ratio of double formation to a 9-hydroxylated "error" product was 99:1. Interestingly the FAD2 Δ^{12} desaturase was reported to increase the formation of 12-hydroxy byproduct when certain amino acids in the enzyme are changed. This re-enforces the notion that desaturase type enzymes are closely related to non-heme hydroxylating enzymes such as methane monooxygenase and that they may follow a similar mechanistic pathway.

1.2.3 Stereochemistry of Fatty Acid Desaturation

Stereochemical issues that arise in the study of fatty acid desaturation have two main features: the enantioselective removal of prochiral hydrogens and the relative stereochemistry of C-H bond cleavage at the adjacent carbons to give *cis*- or *trans*-double bonds (*syn* vs. *anti*).

The enantioselectivity of hydrogen removal for Δ^9 desaturation was initially determined through the use of tritium-labeled substrates derived from naturally occurring alcohols of known absolute configuration.[37] The results showed that when the tritium label occupied the pro-*R* positions of C9 and C10, these labels were removed upon reaction of active Δ^9 desaturase. On the other hand, tritium labels at the pro-*S* configuration were not removed.

The relative stereochemistry of hydrogen removal (*syn* vs. *anti*) of the two methylene groups can be easily verified using *erythro*- and *threo*- dideuterated substrates. A schematic of the experimental approach could be seen in Figure 1.4. Racemic *erythro*- and *threo*-9,10-dideuterated stearate were prepared and incubated with an active Δ9 desaturase enzyme.

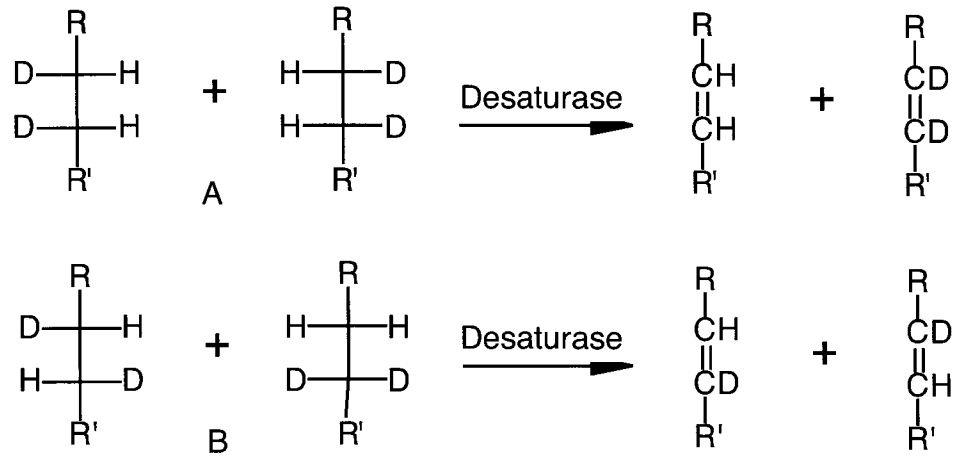


Figure 1.5 Racemic *Erythro* (A) and *Threo* (B) compounds in an incubation experiment that confirms *syn*-removal of methylene protons.

Analysis of the resultant oleate extracts was performed by mass spectrometry and showed the formation of dideuterated products with the racemic *erythro* substrates; this indirectly confirms the results obtained from the tritium-labelling experiments. Incubation of the *threo*-substrates produced mainly monodeuterated products. Both results imply that the removal of hydrogen atom in the desaturation process proceeds exclusively in *syn*-fashion.

New approaches are now being used to study stereochemistry of desaturation by employing thia-fatty acid substrates as stereoselective oxo-traps in conjunction with the use of suitable chiral shift reagents[38] to determine the absolute stereochemistry of the resultant sulfoxide product (See Section 1.3.2).

1.2.4 Cryptoregiochemistry

The cryptoregiochemistry[39] of desaturation refers to the "site of initial oxidative attack" involved in double bond formation. It is a fundamental mechanistic feature that

answers the question; "which hydrogen is removed first" and is an important factor concerning the reaction scheme in Figure 1.2. Remarkably all membrane-bound desaturases studied to date initiate double bond formation at the position closest to the carboxyl group[43]. As an example, the Δ^{12} (FAD2) desaturase initially cleaves the C-H group at the 12^{th} position and leads to olefin formation at C12 and C13.

1.3 Mechanistic Probes for Cryptoregiochemistry

1.3.1 Deuterium Labeled Substrates: KIE Studies

Evalutation of the kinetic isotope effects (KIE) involved in the C-H cleavage steps is the most effect means of determining the cryptoregiochemistry of a desaturase enzyme. This is based on the premise that C-D bond cleavage should be energetically more difficult than that of C-H. The prototypical KIE experiment involves the incubation of a 1:1 mixture of a regiospecifically dideuterated substrate and its non-deuterated analogue. By means of mass spectrometry[42], the ratios of d_1/d_0-product are compared to the d_2/d_0 ratio of the olefinic product after incubation. (See Figure 1.6). Indeed, KIE results for a membrane-bound yeast Δ^9 desaturase show that k_H/k_D was 6.6 ± 0.3 when deuterium was at the 9^{th} position of the substrate but when the labels were in the 10^{th} position, the ratio k_H/k_D was essentially 1. This reveals that hydrogen removal is initiated at the 9^{th} position assuming that the first hydrogen abstraction is energetically more difficult than the second. This methodology was used for a whole host of desaturase systems and most of those findings can be found in a recent review.[43]

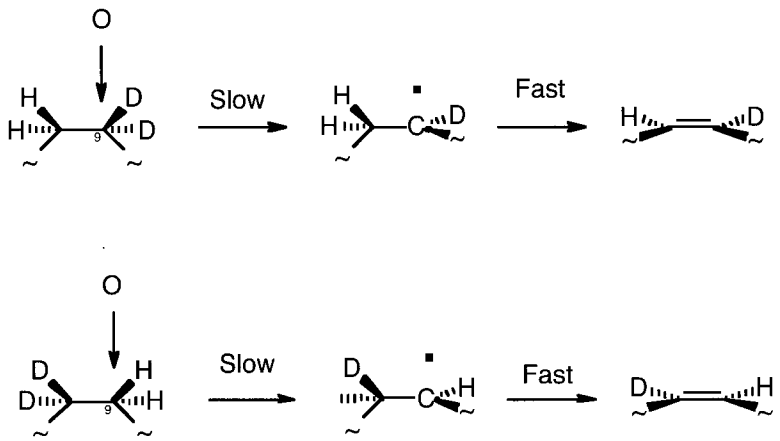


Figure 1.6 Determination of cryptoregiochemistry of a Δ^9 desaturase using dideuterated substrates in the 9th and 10th position.

1.3.2 Thia Probes for Stereochemistry and Cryptoregiochemistry of Fatty Acid Desaturation.

Recently, the use of thia fatty acid substrates have been used to study the cryptoregiochemistry of desaturase enzymes[44]. Systematic replacement of the methylene groups of substrate with a sulfur atom leads to a series of analogues whose ability to undergo oxo transfer in a desaturase active site is then measured. As shown below, more efficient sulfoxidation of a 9-thia analogue by a yeast Δ^9 desaturase as compared to the 10-thia regioisomer indicates that the diiron oxidant is asymmetrically located between the C-9 and C-10 bond. This result corroborates the KIE result (C-9 H removed before C-10H).

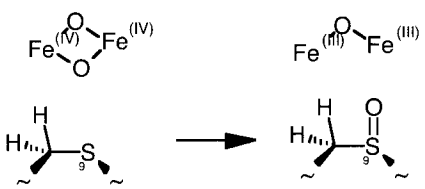


Figure 1.7 Schematic diagram of desaturase-mediated sulfoxide formation.

Along with the cryptoregiochemistry, the stereochemistry of desaturase- mediated sulfoxidation can also be obtained. In order to proceed with analysis, use of a chiral shift reagent is needed in order to distinguish between enantiomers. The most common chiral shift reagents used for analysis are Pirkle-type[46] reagents such as the commercially available (*S*)-(+)-MPAA (α-methoxyphenylacetic acid).[53] These reagents share common features. The first is a functional group that hydrogen bonds with the sulfoxy oxygen, such as a hydroxyl or carboxyl group[47]. Secondly an aromatic group is required to induce non-equivalence by shielding nuclei that are spatially within its cone of shielding. An example model of MPAA interacting with a sulfoxy group is displayed in Figure 1.8.

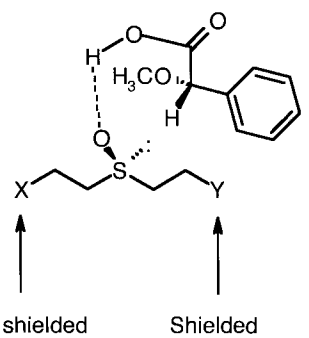


Figure 1.8 Pirkle-type binding model of (S)-MPAA with a sulfoxide.

It is not clear exactly how the shift reagent interacts with the sulfoxy moiety; however it is proposed that a two-point interaction is required. It has been found that a methine proton on the shift reagent is needed for this binding model to work. The model has been validated as a means of evaluating % ee and absolute configuration through the use of a dozen or more sulfoxide reference standards.[47]

The chief advantage of using MPAA is its commercial availability. The accessibility of this reagent makes it an ideal candidate for stereochemical analysis and

prevents the need to perform complex synthesis of more exotic reagents[48]. The second is the elimination of the need to form a covalent derivative of the sulfoxide[52]. This obviates the need to perform an extra reaction and allows for easy recovery of the oxidized substrate.

Previous work[53] on the yeast desaturase-mediated biosulfoxidation of a 9-thiastearate has shown that (*R*)-configured enantiomer is formed as expected. Use of MPAA revealed that enantiomeric excess was greater than 96%. ^{1}H and ^{13}C sulfoxide resonances were used to analyze and determine enantiomer enrichment. Incubation of the 10-thia analogues also resulted in (*R*)-configured (>91% ee) sulfoxides, indicating that enantioselectivity of oxo transfer is retained even if the sulfur oxo-trap is in a less than ideal position. The yield of 10-sulfoxide was approximately 50% less than the yield of the corresponding 9-regioisomer.

1.3.3 Fluorine-tagged Thia Fatty Acids: A new Probe

Contemporary approaches to the study of desaturase mechanism are beginning to take advantage of fluorine-tagged substrates and ^{1}H-decoupled ^{19}F NMR[49]. Used in conjunction with the traditional thia probes, this method of analysis has a number of advantages over other traditional analytical tools such as GC-MS, ^{1}H and ^{13}C NMR. Firstly, is the relative lack of interfering signals in the ^{19}F spectra. This is mainly due to the relative lack of abundance of fluorine atoms in biological matrices. This diminishes the need to purify the final sample for analysis. The second advantage is the relative sensitivity of ^{19}F chemical shifts to changes in oxidation state of atoms located several bonds away from the fluorine tag.[45] Finally, fluorine-substituted substrate analogues

frequently exhibit good binding to enzymes because of the small size of a monofluorine substituent compared to the corresponding hydrogen atom it replaces.

Cryptoregiochemical studies were performed on the castor stearoyl-ACP soluble Δ^9 desaturase[45] using ω-fluorine tagged thia-fatty acid esters. Incubation of 9- and 10-thia fatty acids were performed *in vitro* and produced sulfoxy product as detected by ^{19}F NMR. Surprisingly sulfoxidation of the 10-thia substrate was found to be more efficient than oxidation of the 9-thia isomer. This data confirmed the results of earlier work using non-fluorine-tagged thia substrates and demonstrated that the site of initial oxidation for soluble versus membrane-bound Δ^9 desaturase is different. Also it became clear that ^{19}F based methodology was suitable for *in vitro* mechanistic work.

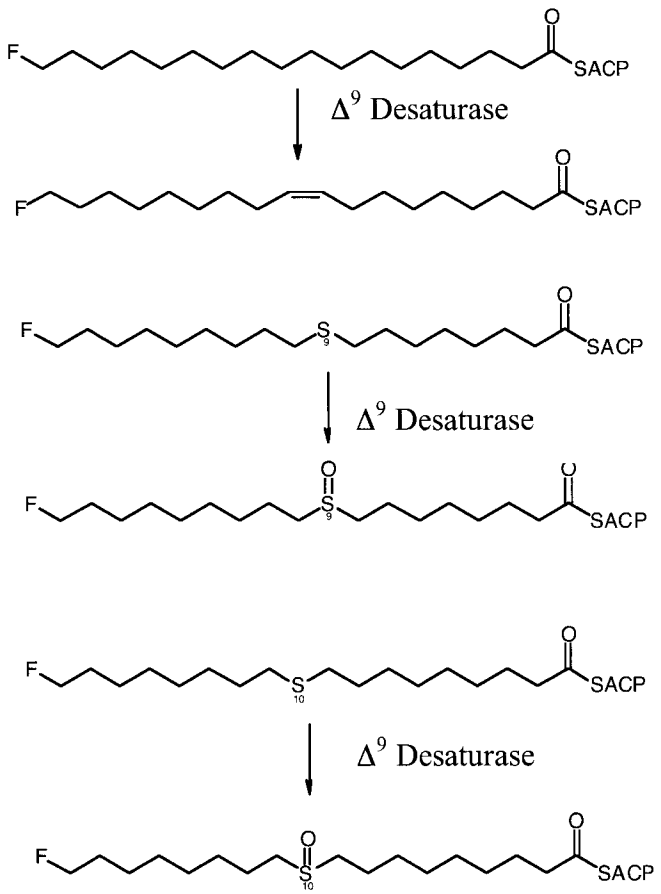


Figure 1.9 In vitro sulfoxidation of fluorine-tagged thia substrates.

It was the purpose of this thesis project to apply ^{19}F NMR methodology in combination with the use of fluorine- tagged thia fatty acid substrates to the study of important membrane-bound desaturases such as stearoyl CoA Δ^9 desaturase and oleate Δ^{12} desaturase. The ultimate goal of this line of investigation is to facilitate the *in vitro* mechanistic study of membranous desaturases.

Chapter 2: Results & Discussion

2.1 Experimental Approach

The approach used in this thesis involved three parts: 1) To test whether the use of fluorine-tagged thia fatty acid methyl esters as probes for desaturase cryptoregiochemistry was valid, a series of ω-fluorooctadecenoates bearing a sulfur atom at the 8^{th}, 9^{th}, 10^{th} or 11^{th} position were synthesized. These compounds were incubated with a whole cell Δ^9 desaturase system and the relative % desaturase-mediated sulfoxidation as a function of sulfur position was compared to data obtained previously for non-fluorinated analogues.

2) The use of ^{19}F NMR as a micromethod to assess the stereochemistry of desaturase-mediated sulfoxidation was probed using methyl 15-fluoro-11-thiapentadecanoate substrate. This material was synthesized and incubated with a whole cell Δ^9 desaturase system similar to previous experiments. The stereochemistry of the resultant sulfoxide was analyzed by ^{19}F NMR with the help of a chiral shift reagent: (*S*)-MPAA.

3) The final portion of the thesis covers the chemoenzymatic synthesis of fluorine-tagged 12- and 13-thiaoleates and related analogues. These compounds were potentially useful as probes to study the active site topology of a well-characterized cyanobacterial Δ^{12} desaturase (desA). The latter enzyme is currently the subject of ongoing structural studies at the Canada Light Source (Saskatoon). An initial trial incubation using desA was carried out.

2.2 Proof of concept: Fluorine tagged fatty acids used to probe cryptoregiochemistry of Δ^9 Desaturase

2.2.1 Synthesis of Fluorine-Tagged Thia Fatty Acids

^{1}H-decoupled ^{19}F NMR can potentially be used to monitor the regioselectivity of Δ^9 desaturase-mediated sulfoxidation by monitoring the fate of a series of fluorine-tagged thia fatty acyl substrates. The methyl ω-fluoro-thiaoctadecanoates required for such a study were synthesized via the scheme outlined in Figure 2.1.

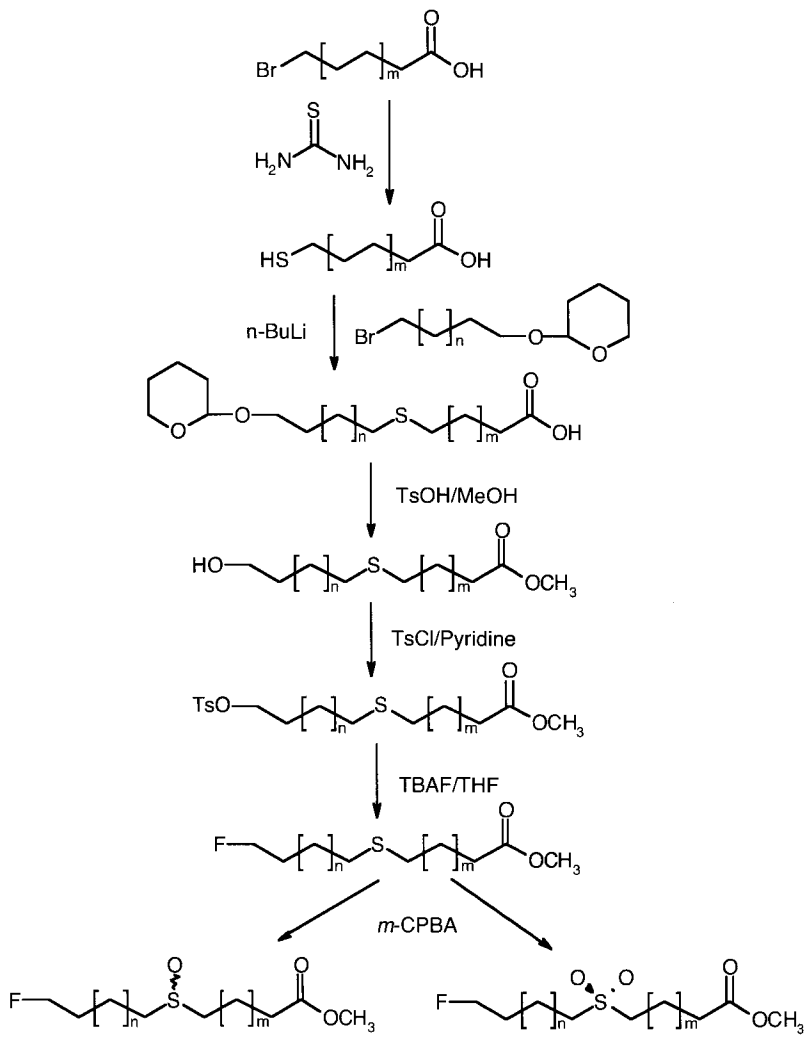


Figure 2.1 Generalized scheme for the synthesis of fluorine-tagged thiastearates and corresponding sulfoxide and sulfones.

Alkylation of the appropriate ω-thioacid with the THP-protected ω-hydroxyl alkyl bromides of the correct chain length furnished the methyl 18-hydroxy-thiaoctadecanoates after deprotection. These intermediates were fluorinated via a conventional tosylation/TBAF sequence. Purity of the target compounds was shown to be high (95-98 % based on GC-MS) after flash chromatography and thus suitable for biological experiments. Typical overall yields ranged from 3 - 5% based on starting bromoacid. Oxidation of the fluorine-tagged thia fatty acids using one or two equivalents of *meta*-chloroperbenzoic acid (*m*-CPBA) respectively yielded the corresponding sulfoxides and sulfones. These compounds served as synthetic reference standards for the incubation experiments.

Characterization of fluorine-tagged compounds relied on the use of standard spectroscopic techniques. IR spectra of the sulfide analogue featured a strong carbonyl stretch (1741 cm^{-1}) typical of ester functionality. In addition to the carbonyl IR band, sulfoxides exhibited a characteristic absorption at 1088 cm^{-1} (SO stretch). In contrast, sulfones absorbed at 1136 cm^{-1} as expected[66] Typical mass spectra of substrates resemble that displayed in Figure 2.2. The parent ion of sulfide appears at *m/z* 334 while the fragment ion at 303 is due to the typical loss of the methoxy group. Fragment ions at *m/z* 185 and 149 correspond to the cleavage between the carbon closest to the carbonyl group and the sulfur atom and confirms the position of the sulfur in the hydrocarbon chain.

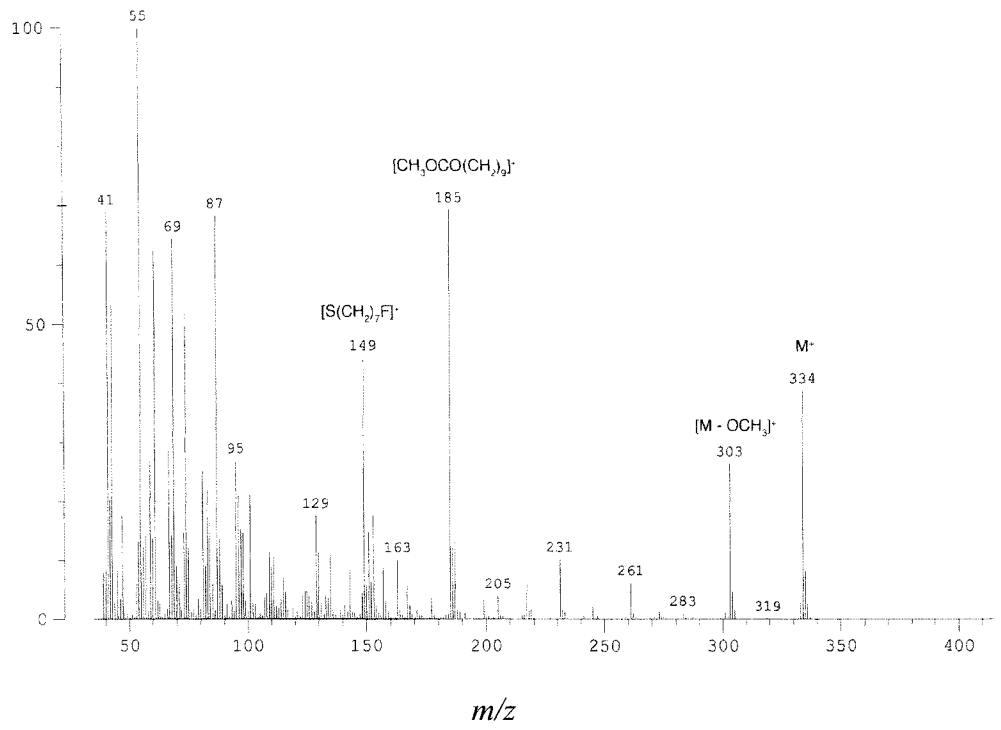


Figure 2.2 Mass spectrum of Methyl 18-fluoro-11-thiaoctadecanoate.

Mass spectra of the sulfoxide and sulfone analogues were more difficult to interpret since the parent molecular ion of the sulfoxide and sulfone product were not prominent. However, a fragment ion of *m/z* 333 (loss of OH) and 335 (loss of OCH_3) were present as the heaviest ion for the sulfoxide and sulfone spectra respectively. Characteristic fragment ions derived from carbon-sulfur cleavage specific to the compounds were also found.

^{1}H and ^{13}C data of all thiastearates and their corresponding sulfoxide and sulfone derivates were consistent with the expected structures. A ^{1}H NMR spectrum of a representative ω-fluorothiastearate and the corresponding sulfoxide is displayed in Figure 2.3.

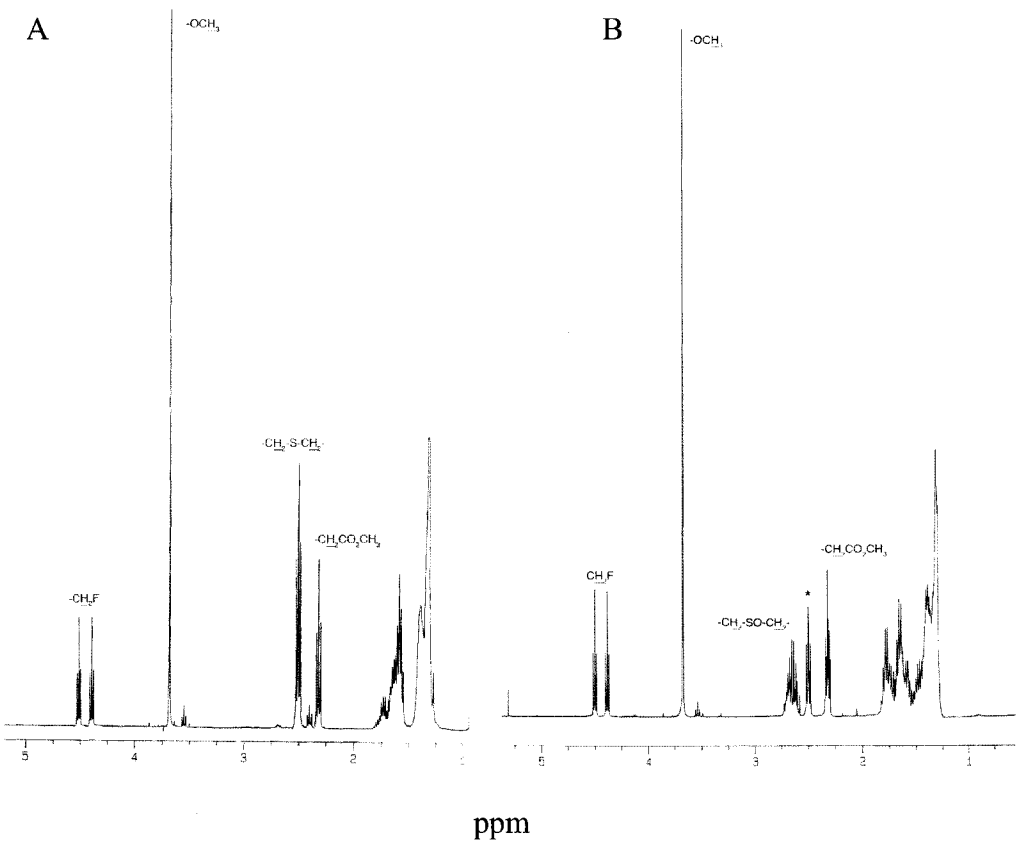


Figure 2.3 A) ^{1}H NMR spectrum of Methyl 18-fluoro-9-thiaoctadecanoate and B) Methyl (*R,S*)-18-fluoro-9-thiaoctadecanoate S-oxide. *Minor impurity due to sulfide.

^{1}H spectra of ω-fluorothiastearates featured a characteristic doublet of triplets centered on δ 4.4 ppm with a large $^{2}J_{HF}$ coupling constant of ca. 47 Hz. A unique set of resonances is present around 2.4 ppm and is characteristic of the methylene groups adjacent to either side of the sulfur atom. The profile of this resonance strongly depends on the distance between the fluorine atom and the sulfur atom; if the distance is great enough, a simple triplet pattern would be observed. However two partially resolved triplets are observed with decreasing sulfur -fluorine atom distances. A characteristic singlet of the methyl ester (δ 3.67 ppm) and a triplet of the methylene protons adjacent to the carbonyl group (δ 2.31 ppm) were present as well. ^{1}H spectrum obtained for the sulfoxide product was very similar. Most resonances remained unchanged except in one

key area. The protons of each methylene group in the sulfoxide are diastereotopic due to the presence of the stereogenic sulfonyl group. This fact results in an exceedingly complex ABXY pattern of signals for the ^{1}H resonances due to the α-sulfinyl hydrogens centered around 2.66 ppm.

^{13}C data for all compounds showed characteristic carbonyl (δ 174 ppm) and methyl ester (δ 51 ppm) resonances (Figure 2.4). Characteristic splitting of ^{13}C resonances due to coupling with fluorine was observed at C-18: δ 84.2 ppm ($^{1}J_{CF}$ = 164.0 Hz), C17: δ 30 ppm, ($^{2}J_{CF}$ = 19.3 Hz) and at C16: δ 25.1 ppm ($^{3}J_{CF}$ = 5.5 Hz) Carbon atoms α to the sulfide group lie near the δ 32 ppm range. ^{13}C spectra of the sulfoxides and sulfones were similar to that of the sulfide analogue with the exception of the further downfield shift of the carbon resonances neighbouring the sulfoxide and sulfone group.

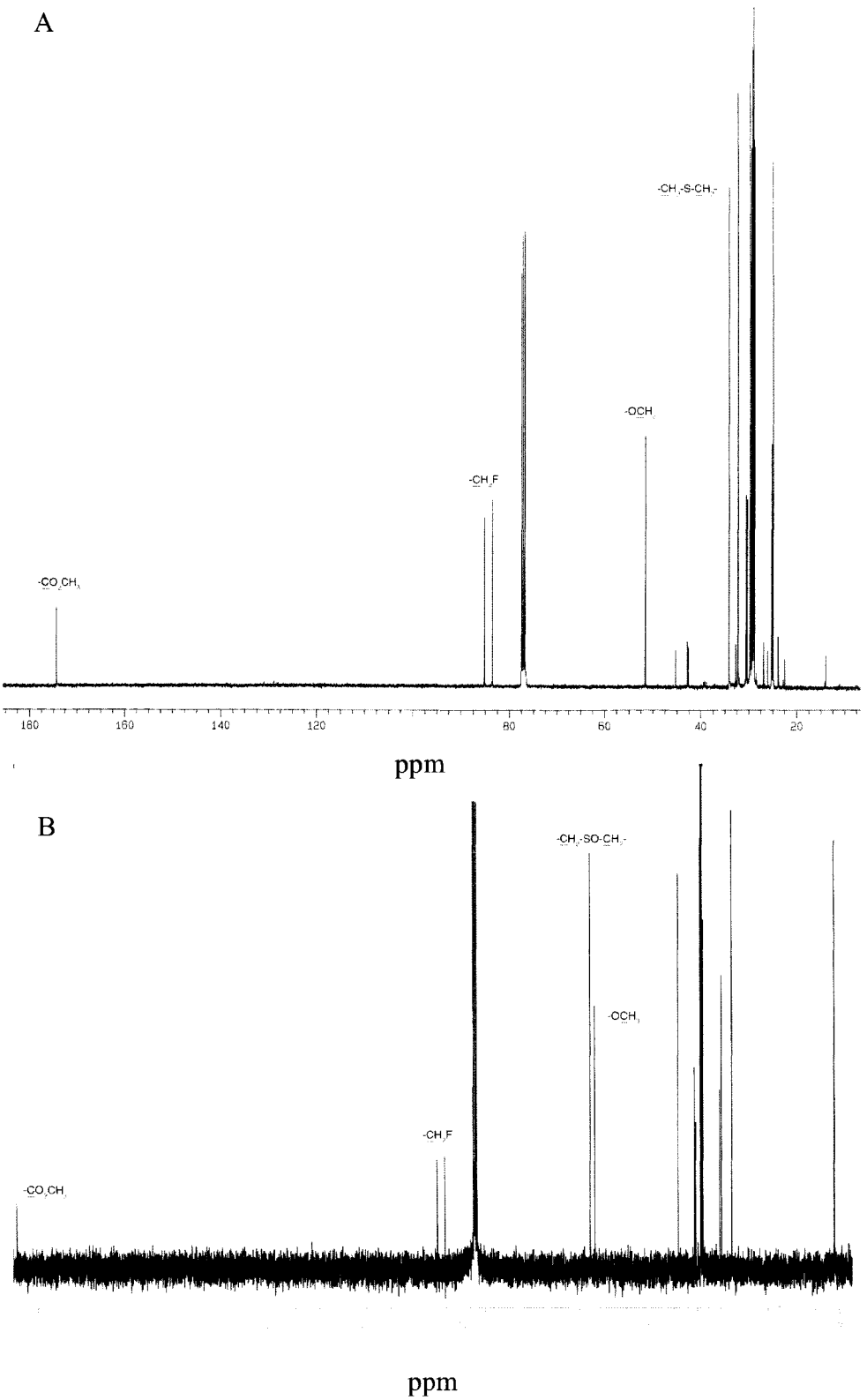


Figure 2.4 ^{13}C spectra of A) 18-fluoro-9-thiaoctadecanoate and B) (*R*,*S*) 18-fluoro-9-thiaoctadecanoate, S-oxide in $CDCl_3$.

2.2.2 ^{19}F NMR Analysis

The ^{1}H-decoupled ^{19}F NMR spectra of all compounds were recorded in $CDCl_3$. Importantly, ^{19}F resonances of each series of ω-fluorothiastearate and the corresponding oxidized derivatives were resolved to baseline in all cases. This phenomenon allowed facile detection of sulfoxidation reactions in complex biological matrices.

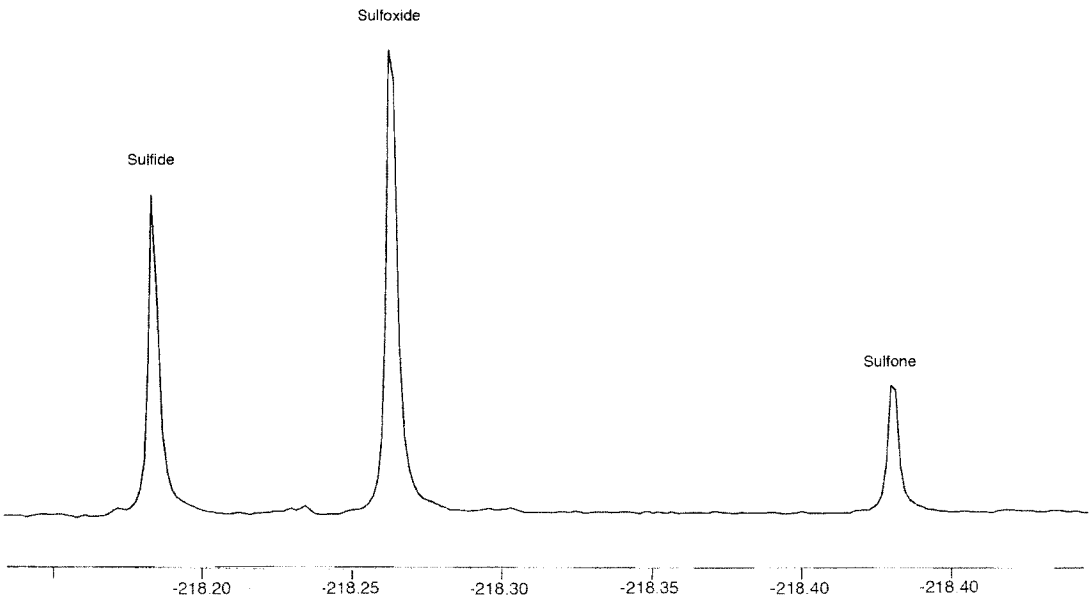
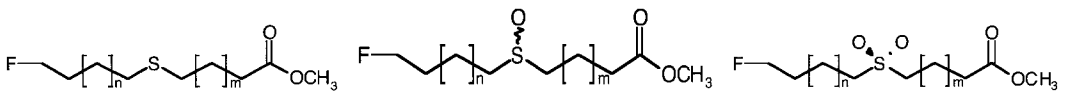


Figure 2.5 ^{19}F NMR spectrum of methyl 18-fluoro-8-thiaoctadecanoate and its corresponding S-oxides.



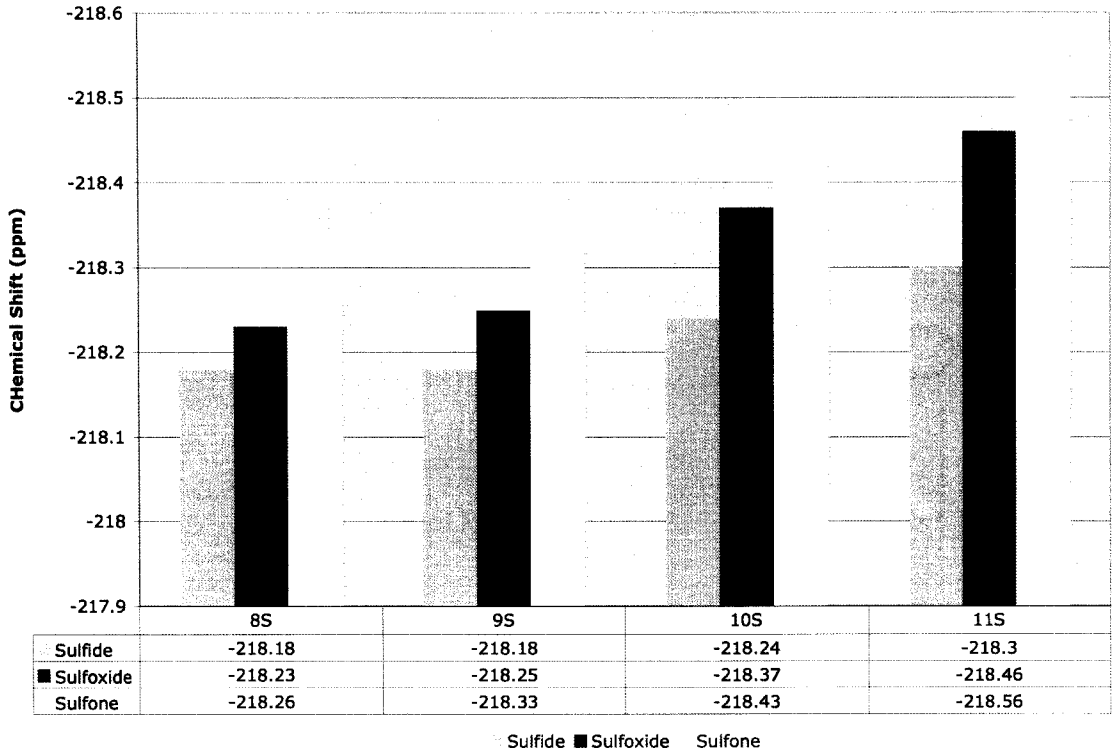


Figure 2.6 ^{19}F NMR chemical shift data of synthetic standards and their representative structure. 8S: m = 4, n = 8; 9S: m = 5, n = 7; 10S: m = 6, n = 6; 11S: m = 7, n = 5.

As is readily apparent by inspection of the data displayed in Figure 2.6, the ^{19}F NMR chemical shift difference between oxidized deriviatives and the sulfide parent increases as the distance between the fluorine and sulfur atom decreases (7-10 carbon atoms away). This dependence of chemical shifts on distance of the reporter nucleus from the substituent is a common phenomenon in NMR[45].

2.2.3 Incubation of FluoroSubstrates with Yeast Cultures

To evaluate the suitability of the fluorothiastearates as probes for desaturase cryptoregiochemistry, the well-characterized yeast Δ^9 desaturase model was utilized. Each ω-fluorothiastearate (25 mg) was incubated separately with actively growing cultures (200 mL) of *Saccharomyces cerevisiae S522C* to maximize production of the corresponding sulfoxide. Our whole cell system allows us to use methyl ester substrates that are presumably converted, intracellularly, to the corresponding CoA thioesters prior to desaturase-mediated sulfoxidation. The product sulfoxide is then hydrolyzed and excreted into the medium.

^{19}F NMR analysis of the CH_2Cl_2 extracts of the acidified supernatant derived from each ω-fluorothiastearate incubation revealed the presence of sulfide and the corresponding sulfoxide as the major analytes in each case. A typical ^{1}H-decoupled ^{19}F NMR spectrum is displayed in Figure 2.7. The sulfoxy products were identified on the basis of ^{19}F NMR chemical shift data (agreement within 0.01 ppm) as well as TLC analysis of each extract using authentic standards. In the case of 18-fluoro-11-thiastearate oxidation, three additional minor, unidentified products (12 % of total) - presumably derived from chain cleavage were observed.

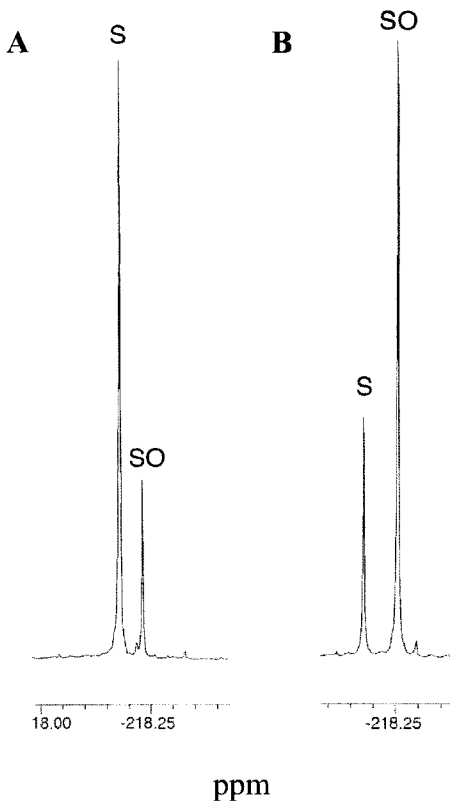


Figure 2.7 Representative ^{19}F NMR spectra of yeast medium extract showing the increase in sulfoxide production from A) 8-thia- as compared to B) 9-thia substrate.

The relative amounts of sulfoxidized product were quantitated by the use of an external standard (flunisolide) and the % sulfoxidation of fatty acid series were calculated to be 1.2 %, 7 %, 4.5 %, 2.0 % respectively. Details of the calculation can be found in appendix A.1. The results were compared to data obtained previously[39,54] for non-fluorinated thiastearoyl substrates and the % sulfoxidation as a function of sulfur position showed a similar reactivity profile.

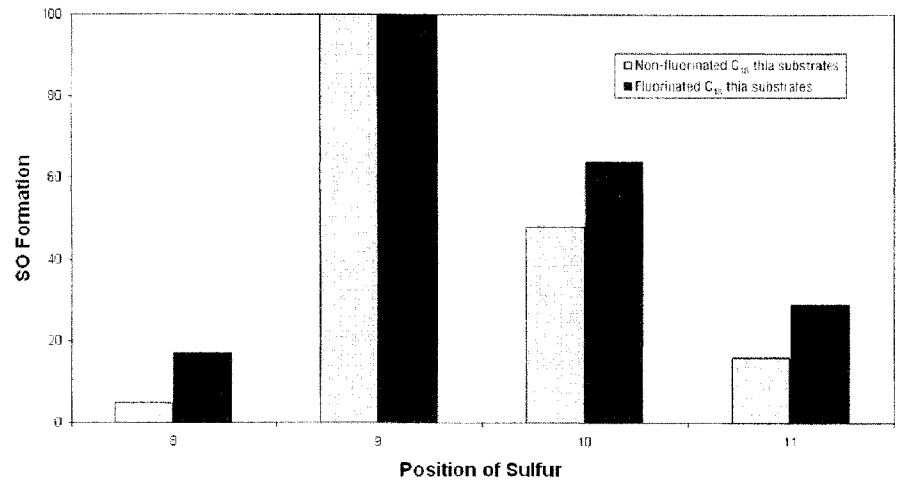


Figure 2.8 Regioselectivity profile for yeast Δ^9 desaturase-mediated sulfoxidation of fluorine-tagged sulfur-containing fatty acids from yeast extracts.

Consistent with a mass spectral study using non-fluorinated substrates[4], oxo transfer was maximal when the sulfur was at the "C-9" of substrate. Less sulfoxide formation was found for substrates bearing sulfur at the 8,10th and 11th positions, probably due to increasing distance from the putative diiron oxidant that was thought to be asymmetrically located between C-9 and C-10.

The primary advantage of the ^{19}F NMR approach to analyzing desaturase cryptoregiochemistry as described above relative to traditional methods used in biological experiments of similar design can be traced to the lack of background interferences and the inherent sensitivity of this technique. The use of ^{19}F NMR as an analytical tool eliminates the need to obtain milligram quantities for analysis. Previously, mass spectral analysis of regioisomeric mixtures derived by direct competition experiments was used to evaluate desaturase-mediated regioselectivity; however the need to purify yeast extracts by flash chromatography (or other preparative chromatography) extends the time required to prepare the sample for analysis. Furthermore, although mass spectral analysis is a

sensitive tool in detecting small quantities of analyte, background interferences would be a major issue. GC-MS could not be employed to analyze the sulfoxy product due to the fact that sulfoxides are thermally unstable under GC conditions. Use of HPLC is problematic because saturated fatty acid methyl esters are difficult to detect at low concentrations due to the lack of a suitable chromophore. In contrast, the use of the ^{19}F NMR eliminates the need to purify the product for analysis and the method is sensitive enough to obtain a good signal to noise ratio at the μMolar concentrations of the analyte. This obviates the need to "scale up" in order to acquire adequate amounts of product for easily handling and detection.

In summary, the success of a ^{19}F NMR-based approach to studying desaturase-mediated sulfoxidation depends on three considerations: 1) synthetic accessibility of the fluorinated substrates 2) ability to detect product formation at trace analytical levels and 3) unobtrusive nature of the remote fluorine probe.

The synthetic scheme used to obtain the appropriate fluorine-tagged thia-fatty acid methyl ester was adequate and could be easily adapted to produce fatty acids of varying chain length or with different sulfur positions. Difficulties encountered during the synthesis were mainly due to low yields in the coupling reaction. However, this is not a serious problem due to the availability of the required starting materials allowing for the scale of the reaction to be increased in order to compensate for low yields.

The second factor requires that ^{19}F NMR resonances be sensitive to the oxidation state of the sulfur atom. This is important to ensure clear identification of the expected sulfoxy product derived from the biological oxidation of the substrates. The data displayed in Figure 2.5 and 2.6 illustrates that ^{19}F chemical shift of the sulfoxy

resonances are sufficiently different from sulfide so that identification could be made with relative ease.

Lastly, use of the fluorine tag should not cause a severe perturbation in substrate binding such that desaturation activity decreases in an unpredictable manner. The results from these experiments have shown that any perturbation caused by the fluorine atom was minimal and good agreement with the crytoregiochemical data obtained with non-fluorinated substrates were achieved.

2.3 Probing Stereochemistry of Desaturase-mediated sulfoxidation using an ^{19}F NMR approach

In this section, the use of fluorine-tagged thia-fatty acids to study the stereochemistry of desaturase-mediated sulfoxidation is described. The methodology features a ^{19}F NMR based approach in combination with the use of a well-studied chiral NMR shift reagent- (*S*)-(+)-α-methoxyphenylacetic acid (MPAA) (as explained in the Introduction).

2.3.1 Synthesis of Methyl 15-fluoro-11-thiapentadecanoate

Methyl 15-fluoro-11-thiapentadecanoate S-oxide (Figure 2.9) was selected as a test compound for three reasons. 1) The fluorine atom is situated close enough to the sulfoxy centre to facilitate use of the commercially available chiral NMR shift reagent (MPAA) used to assign the absolute configuration of the sulfoxy chiral center[47,55]. 2) Enantiomerically enriched methyl (*S*)-15-fluoro-11-thiapentadecanoate S-oxide was potentially available via whole cell yeast Δ^9 desaturating system. Previous studies[55] have shown that C-15 substrates bind well to membrane-bound Δ^9 desaturases and that the "C-

11" position is accessible to the oxidant.[54] The fluorinated sulfide was readily synthesized using commercially available synthons.

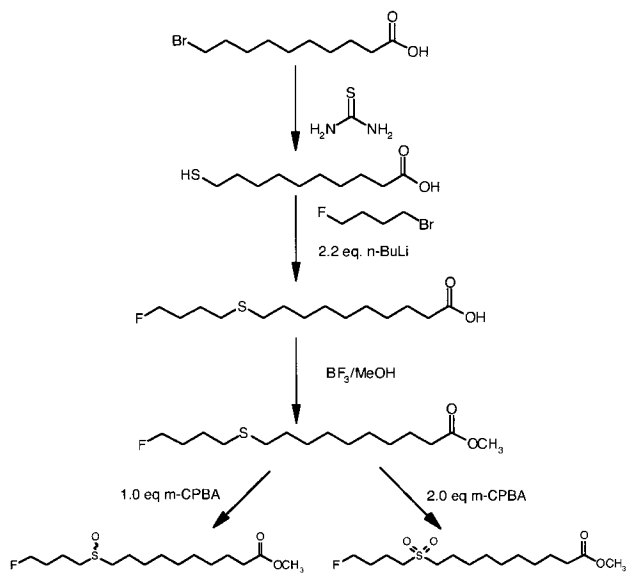


Figure 2.9 Synthesis of Methyl 15-fluoro-11-thiapentadecanoate and the corresponding sulfoxide and sulfone.

The target fatty acid methyl ester was synthesized by thio-alkylation of 10-mercaptodecanoic acid with commercially available 1-bromo-4-fluorobutane followed by methylation ($MeOH/BF_3$) using previously published procedures[56]. The crude product was purified by flash chromatography (SiO_2, 5% AcOEt/hexane) to give the fluorine-tagged thia-fatty acid methyl ester in an overall yield of 8 % based on 10-bromodecanoic acid. Purity of fluorinated thia fatty acid was approximately > 95% based on gas chromatogram. The corresponding racemic sulfoxide and sulfone analogues were prepared by oxidation of the pure sulfide with one and two equivalents of *meta*-chloroperbenzoic acid (*m*-CPBA) respectively. The sulfoxide analogue was purified by flash chromatography (100% AcOEt).

IR, MS, ^{1}H, ^{13}C and ^{19}F NMR spectra of methyl 15-fluoro and oxidized derivatives were consistent with the proposed structures; the spectral data was similar to

that previously discussed for the fluorine-tagged thiaoctacanoates (See Section 2.2). For example, in the ^{1}H NMR spectrum, the typical doublet of triplet ($^{2}J_{HF}$ 47 Hz, centered at 4.44 ppm) could be found on both spectra of the sulfide and sulfoxide analogue. Characteristic methyl ester (singlet, 3.67 ppm) and H-2 resonances (triplet, 2.30 ppm) were found for both spectra. Due to proximity of the fluorine atom to the methylene groups adjacent to the sulfide, a pair of triplets could be resolved as the methylene group closest to the fluorine atom was deshielded due to the strong electronegative effects of the fluorine atom. Resonances of the sulfoxide product displayed the complex pattern (multiplet, 2.67 ppm) normally found in sulfoxy analogues. Mass spectrum of the 11-thia fatty acid displayed a molecular ion at *m/z* 185 that indicates cleavage of C10 and S11 bond.

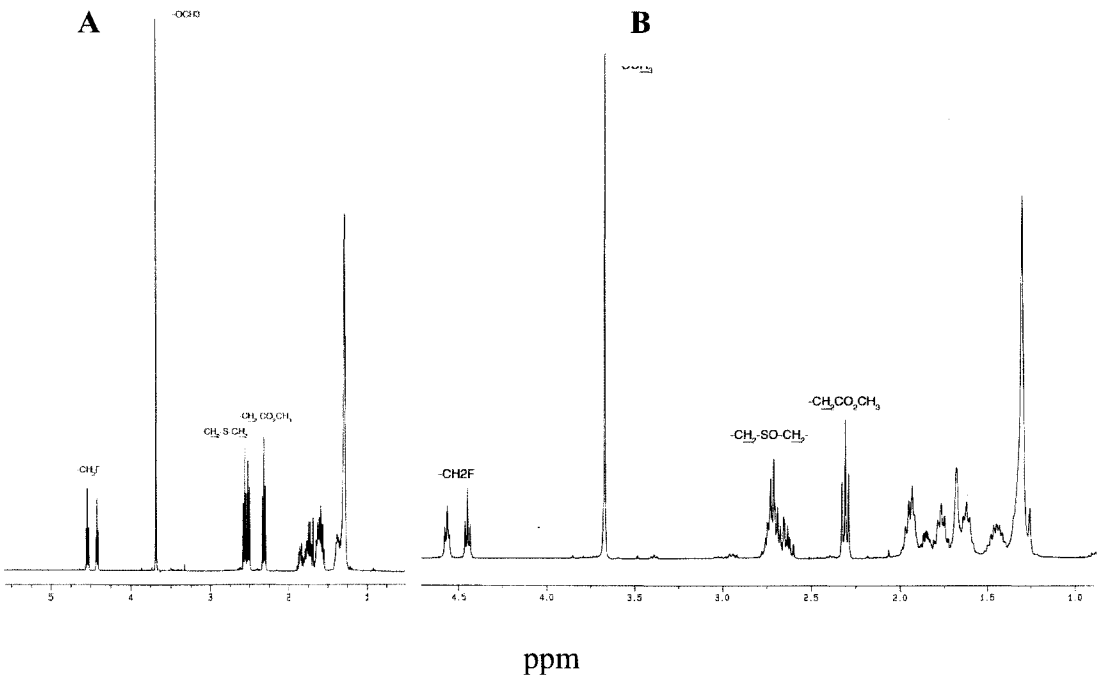


Figure 2.10 ^{1}H NMR spectrum of A) Methyl 15-fluoro-11-thiapentadecanoate and its B) S-oxide derivative

The ^{1}H-decoupled ^{19}F NMR spectrum of each individual compound was recorded in $CDCl_3$ and values for the chemical shifts were found to be as follows: sulfide (δ - 218.82 ppm) and sulfoxide (δ -219.24 ppm). For the purposes of comparison, the ^{19}F resonances of the sulfide, sulfoxide and sulfone (ca. 1:1:1 mixture) are displayed in Figure 2.11A. The effect of sulfur oxidation on the reporter ^{19}F NMR signal of ω-fluorothia-analogues was consistent with previous observations both with respect to direction (upfield) and absolute magnitude of the substituent effect ($\Delta\delta_{SO-S}$ = 0.42 ppm < $\Delta\delta_{SO2-S}$ = 0.91 ppm).

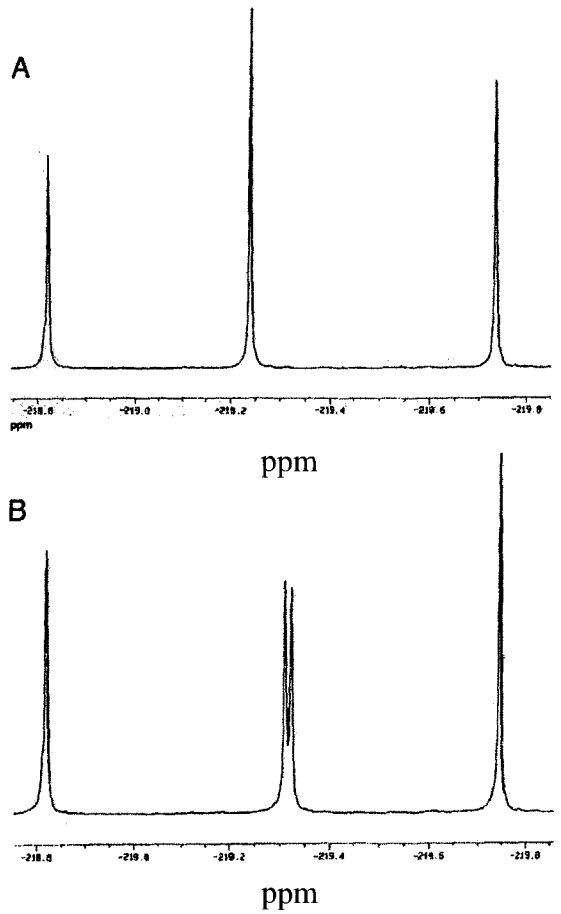


Figure 2.11 ^{19}F spectra of sulfide, sulfoxide and sulfone mixture (1:1:1). A) Mixture of fatty acid analogues before and B) after addition of (S)-(+)-MPAA (3 equiv).

2.3.2 Racemic Sulfoxides and Shift Reagent Experiments

The ^{1}H-decoupled ^{19}F NMR spectrum of the sulfide, sulfoxide and sulfone mixture (ca 2.5 μMoles each) to which (*S*)-(+)-MPAA (70 μMoles) was added as is shown in Figure 2.11B. Only the resonance due to racemic sulfoxide is shifted upfield (0.05 ppm) and split ($\Delta\delta$ = 0.01 ppm) due to Pirkle-type interaction of racemic sulfoxide with this shift reagent. In this experiment, interaction of the NMR shift reagent and the sulfoxide was facilitated by the hydrogen bonding of the carboxylic acid to the sulfoxy oxygen atom (Recall Introduction, p. 13). It is also assumed that a secondary interaction between the methine proton on the shift reagent and the sulfur lone pair contributes to the interaction of the analytes. These specific interactions allow the phenyl ring-induced non equivalence to be expressed for sulfoxides and thus permit designation of absolute configuration.

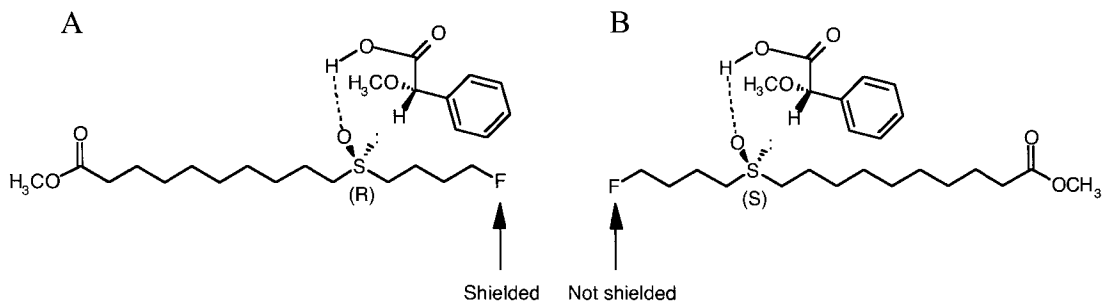


Figure 2.12 Pirkle binding model of NMR shift reagent ((S)-(+)-MPAA) and ω-fluoro thia fatty acid methyl ester S-oxide.

Similar doubling ($\Delta\delta$ = 0.01 ppm) of the corresponding terminal methylene resonances in the ^{1}H NMR spectrum of a sulfoxide / (*S*)-(+)-MPAA mixture was also observed.

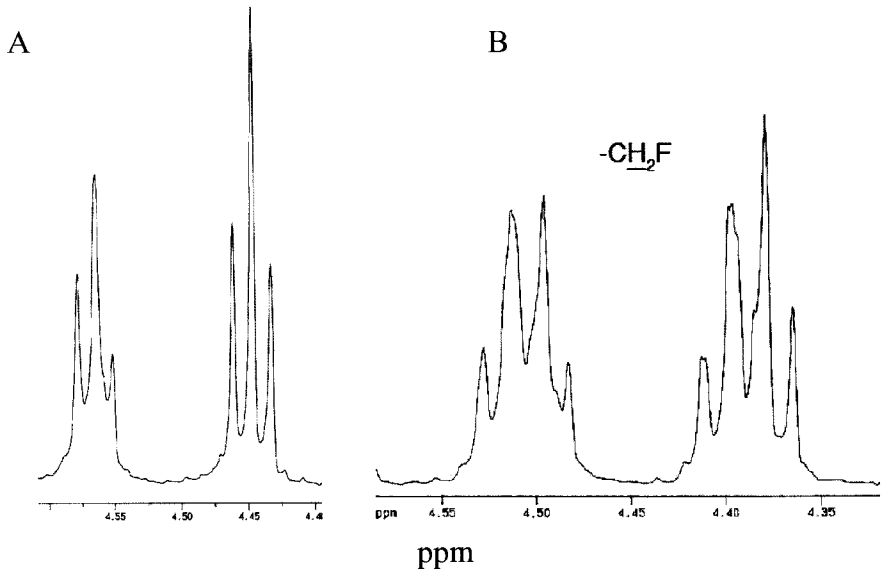


Figure 2.13 Induced non-equivalence of ^{1}H resonances of the terminal methylene group of methyl (*S*)-15-fluoro-11-thiapentadecanoate before (A) and after (B) addition of (S)-(+)-MPAA.

The ^{13}C resonances (d, $^{1}J_{CF}$ = 166 Hz) of the terminal carbon group of racemic sulfoxide analogue were broadened but not differentiated under these conditions. However, weak splitting was observed for one of the α-sulfinyl resonances (Figure 2.14B)

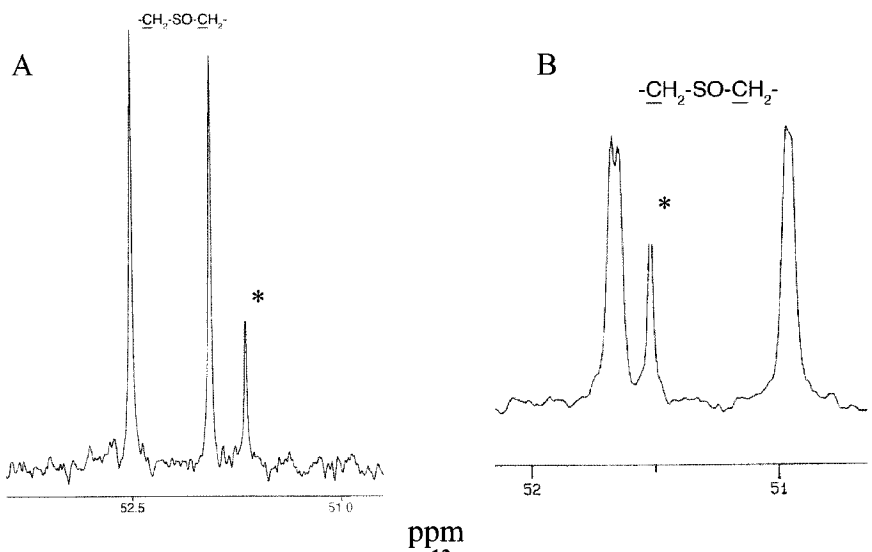


Figure 2.14 Induced non-equivalence of ^{13}C resonances splitting of C12 of methyl (*S*)-15-fluoro-11-thiapentadecanoate before (A) and after (B) addition of (*S*)-(+)-MPAA. *Methyl ester resonance.

Concentration experiments were performed on the racemic sulfoxides to test the limits of the observed splitting. It was found that splitting of the observed ^{19}F resonance was still maintained at concentrations as low as 3.9 mM. However, splitting was less pronounced at this concentration and a large excess of shift reagent was required in order to restore splitting to previous levels.

To evaluate the applicability of this methodology to the stereochemical analysis of enantiomerically enriched sulfoxides isolated from a biological system, we incubated the methyl 15-fluoro-11-thiapentadecanoate (25 mg) with actively growing cultures (200 mL) of *Saccharomyces cerevisiae* as described in the Experimental section. The sulfoxy product was isolated by a series of steps including centrifuging the culture medium, acidifying the supernatant that was then extracted with CH_2Cl_2.

^{19}F NMR analysis of the methylated CH_2Cl_2 extract revealed the presence of strong signals corresponding to residual sulfide and the corresponding sulfoxide. No overoxidation of sulfoxide product to ω-fluoro-sulfone could be detected in this mixture (< 1% of total fluorinated analytes). To expedite the stereochemical analysis of sulfoxide, the extract was purified on a short flash chromatography column (SiO_2, EtOAc) to remove the majority of unidentified byproducts. The ^{1}H-decoupled ^{19}F NMR spectrum of purified sulfoxide dissolved in dry $CDCl_3$ is presented in Figure 2.15A; the amount of sulfoxide (40 nmoles, 12 μg) was quantified through the use of a calibrated, external reference standard (fluperolone acetate, a fluorinated steroid derivative).

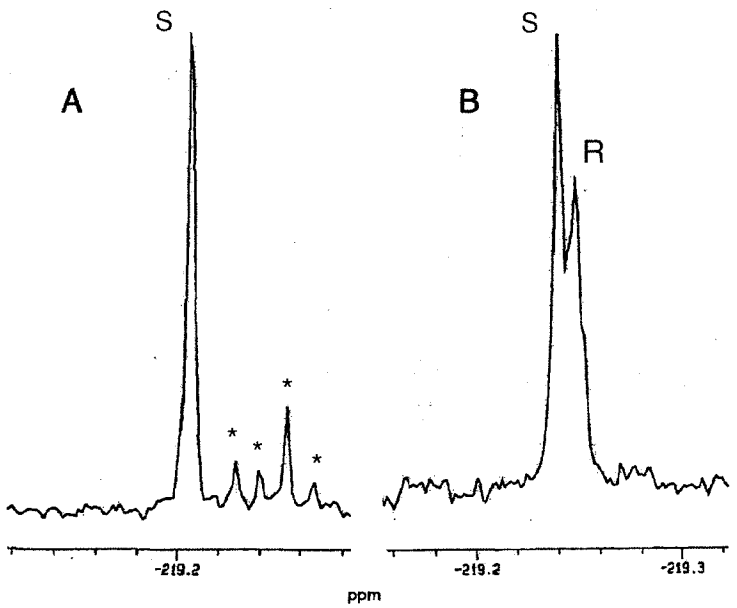


Figure 2.15 ^{19}F NMR spectra of biosynthetic methyl 15-fluoro-11-thiapentadecanoate, S-oxide before (A) and after (B) the addition of (*S*)-(+)-MPAA. *Unknown impurities.

Stereochemical analysis of biosynthetic sulfoxide was carried out by measuring the effect of (*S*)-(+)-MPAA (4 mg) addition on the ^{19}F reporter resonance (Figure 2.15B). The predominant enantiomer (32 % ee) was found to be (*S*) according to a Pirkle-type binding model (figure 2.12) that has been validated using a large number of sulfoxides of known configuration[47]. This stereochemical outcome is expected on basis of the known preference of the yeast Δ^9 desaturases to catalyze oxo transfer to sulfides in the manner shown in Fig 1.7 Introduction. The observed enantioselectivity of yeast desaturase-mediated sulfoxidation is consistent with that observed using a number of related substrates and corresponds to the known stereochemistry of hydrogen removal for the parent dehydrogenation reaction. The low enantiomeric enrichment of the biosynthetic sulfoxide obtained in this experiment was not unexpected and may correlate with our observation that the efficiency of Δ^9 desaturase-mediated oxo transfer is markedly

reduced for thiasubstrates bearing sulfur in positions other than C-9 and C-10 (recall Figure 2.8).

The ability for this method to measure % ee of the expected sulfoxide enantiomer is a testament to the sensitivity and effectiveness of the analytical probes, even down to μMolar levels. Compared to previous attempts[57] at determining enantiomerically enriched samples of bio-sulfoxidized product, only 25 mg of substrate was needed for incubation.

2.4 Chemoenzymatic synthesis of Various Potential Fluorine-Tagged Thia Fatty Acids: Potential Substrates for Δ12 Desaturation

This section describes a potential route to ω-fluoro-12- and 13-thia- 9-enoic fatty acid analogues - possible cryptoregio- and stereochemical probes for Δ^{12} desaturation. This represents the first step in applying our fluorine-based methodologies to the study of an *in vitro* membranous desaturase system. The overall scheme is shown in Figure 2.16.

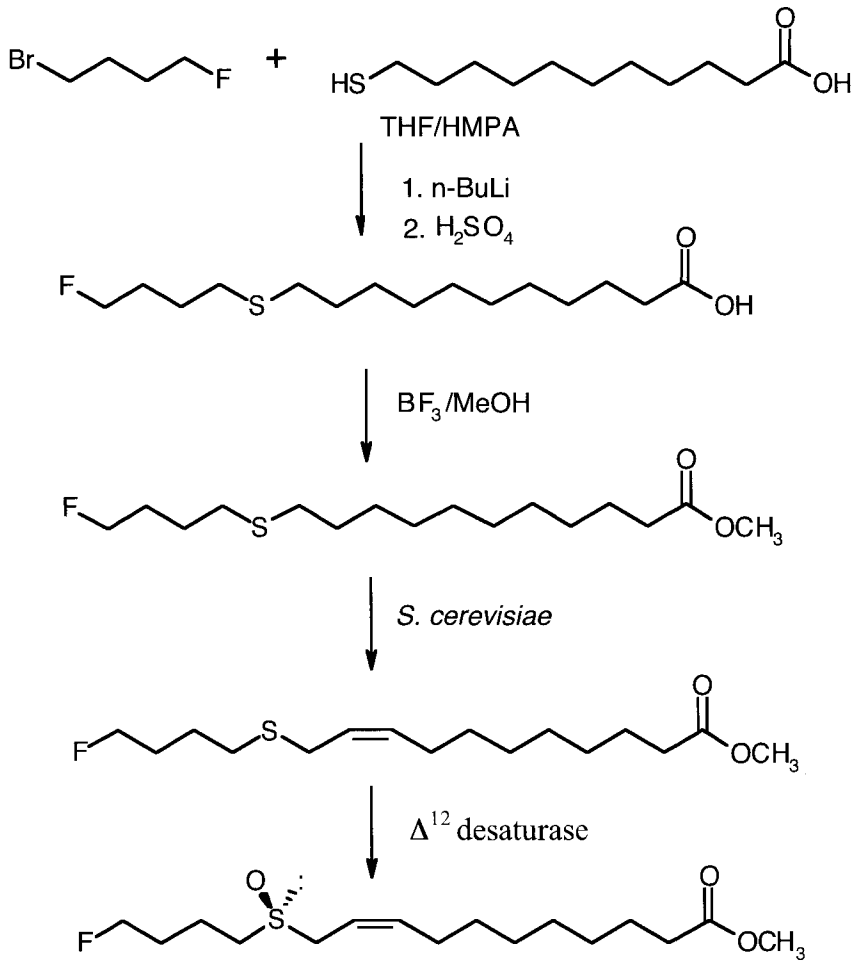


Figure 2.16 Synthesis and Δ^{12} desaturase-mediated sulfoxidation of a typical fluorine-tagged substrate: methyl 16-fluoro-12-thiaoctadecanoate.

2.4.1 Synthesis of Saturated Fluorine-tagged thia fatty acids

Synthesis of various fluorine-tagged saturated thia fatty acids was carried out via a route similar to that outlined in sections 2.2 and 2.3. The target fatty acid analogues were as follows: Methyl 16-fluoro-12-thiahexadecanoate, methyl 18-fluoro-12-thiaoctadecanoate and methyl 18-fluoro-13-thiaoctadecanoate.

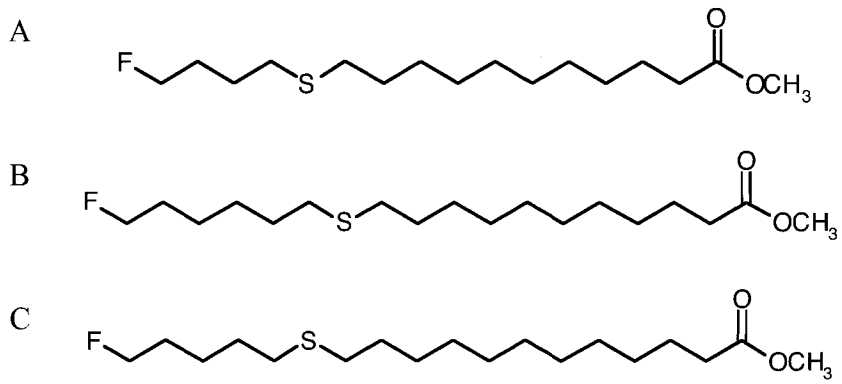


Figure 2.17 Structure of fluorine tagged fatty acid, precursors to potential probes for Δ^{12} desaturase. A) Methyl 16-fluoro-12-thiahexadecanoate, B) methyl 18-fluoro-12-thiaoctadecanoate C) methyl 18-fluoro-13-thiaoctadecanoate.

Methyl *cis*-16-fluoro-12-thia-9-hexadecenoate was initially chosen as a suitable candidate for *in vitro* Δ^{12} desaturation reactions due to the synthetic accessibility of the compound and the proximity of the fluorine reporter atom to the sulfur atom. The synthesis was achieved by coupling 11-thioundecanoic acid (derived from an ω-bromo-undecanoic acid) and 4-fluoro-1-bromo pentane with n-BuLi in THF/HMPA. Methylation of the resultant product was carried out using $MeOH/BF_3$ and the crude methyl ester was purified by flash chromatography (SiO_2, 10% AcOEt/hexanes) to afford the target compound in 7.4 % overall yield. GC-MS shows that purity of sulfide compound to be high (est. > 95%). The ^1H and ^{13}C NMR and mass spectrum of the product were similar to those obtained previously (Fig 2.10A and 2.4A) and were

consistent with the assigned structure. Diagnostic peaks include the doublet of triplets centered at 4.5 ppm ($C\underline{H}_2F$), partially resolved methylene resonances that neighbour the sulfur group (2.49 and 2.55 ppm, similar to figure 2.10A) and the methyl ester peak at 3.67 ppm. ^{13}C data displayed the very characteristic doublet of singlets centered at 83.7 ppm ($^{1}J_{CF}$ 164.9 Hz) for the fluorine bearing carbon and resonance for C11 and C13 at 31.6 and 32.1 ppm respectively. Identifiable fragment ions in the MS include; *m/z* 306 shows the presence of the parent ion, *m/z* 275 indicate a loss of a methoxy group (OCH_3) and *m/z* 199 indicates cleavage between the C11 and S12 positions.

Methyl 18-fluoro-12-thiaoctadecanoate and methyl 18-fluoro-13-thiaoctadecanoate were also synthesized based on a re-evaluation of the chain length dependence for Δ^{12} desaturation. Ongoing trial experiments at PBI Saskatoon suggested that C18 substrates were strongly preferred over C16 substrates. The synthetic route to the thia-octadecanoates were similar to that outlined above in section 2.2 and target compounds were characterized by GC- MS, ^{1}H and ^{13}C data, appear similar to those displayed in previous sections (figure 2.3A and 2.4A). The regioisomeric sulfides could be distinguished easily by MS: While the molecular ion for both 12- and 13-thia fatty acid methyl esters were the same (*m/z* 334), ions unique to the 12-thia analogue were *m/z* 199 ($CH_3OCO\text{-}(CH_2)_{10}$) and *m/z* 135 ($S\text{-}(CH_2)_5CH_2F$), while diagnostic ion fragments for the 13-thia analogue (*m/z* 213 ($CH_3OCO\text{-}(CH_2)_{11}$) and *m/z* 259 ($CH_3OCO\text{-}(CH_2)_{11}SCH_2$)) were present.

2.4.2 Δ^9 Desaturation of 12-thia Fatty Acid Analogues

Incubation of the saturated 12- and 13-thia fatty acid methyl esters with actively growing *S. cerevisiae* (Baker's yeast) cultures was carried out in order to prepare the corresponding thiaoctadeca-9-enoates. This step is required since Δ^{12} desaturation functions only with 9-enoic subtrates. Methyl 12-thiaoctadecanoate was included in this study as a non-fluorinated control substrate. Approximately 25 mg of substrate was incubated with yeast cultures as described in the Experimental section. The cellular fatty acid fraction was isolated by centrifuging the yeast cultures (10,000 RPM at 4°C for 15 minutes) and hydrolyzing the resultant cell pellets containing the incorporated synthetic fatty acid (~3 g wet weight) using procedures previously described.[58] GC-MS analysis was performed on the methylated (BF_3/MeOH) yeast extracts and showed relatively good incorporation of the thia fatty acids but variable amounts of desaturated product. A typical fatty acid profile is displayed in Fig 2.18 and the analytical data for each extract is given in Table 2.1.

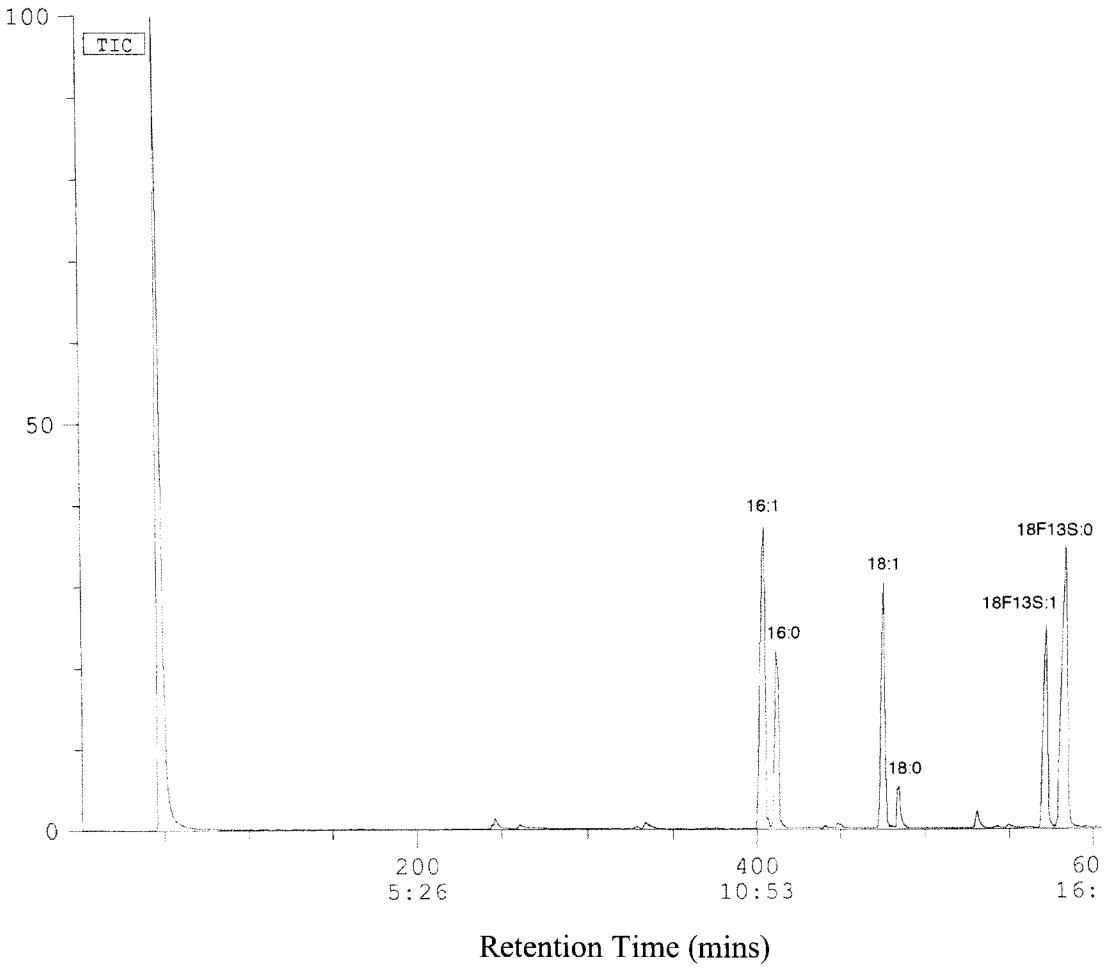


Figure 2.18 TIC chromatogram of cellular FAME yeast extract obtained from cultures incubated with methyl 18-fluoro-13-thiahexadecanoate.

Substrate	16:0	16:1	18:0	18:1	Thia:0	Thia:1	Incorp.	% Desat.
16F12S	13	34	5	18	28	2	30	7
18F12S	29	8	2	25	29	7	36	19
18F13S	9	21	3	17	32	18	50	36
C1812S	40	19	25	3	16	9	25	36
C1812S[a]	7	27	2	16	29	20	49	41
C1813S[a]	6	23	1	10	20	40	60	67

Table 2.1 GC analysis of fatty acid content from yeast cell extract. Displayed are the percentages of each component found from the yeast extract including the incorporation and % desaturation of the thia fatty acid substrates. *Unidentified impurity. [a]Results adapted from P.H. Buist et al., *Tetrahedron Lett.*, 1988, 29, 435

Each fatty acid component displayed in Figure 2.18 was identified on the basis of characteristic retention times and mass spectra. The mass spectrum of the desaturated fluorine-tagged thia product obtained from C16F12S is given below:

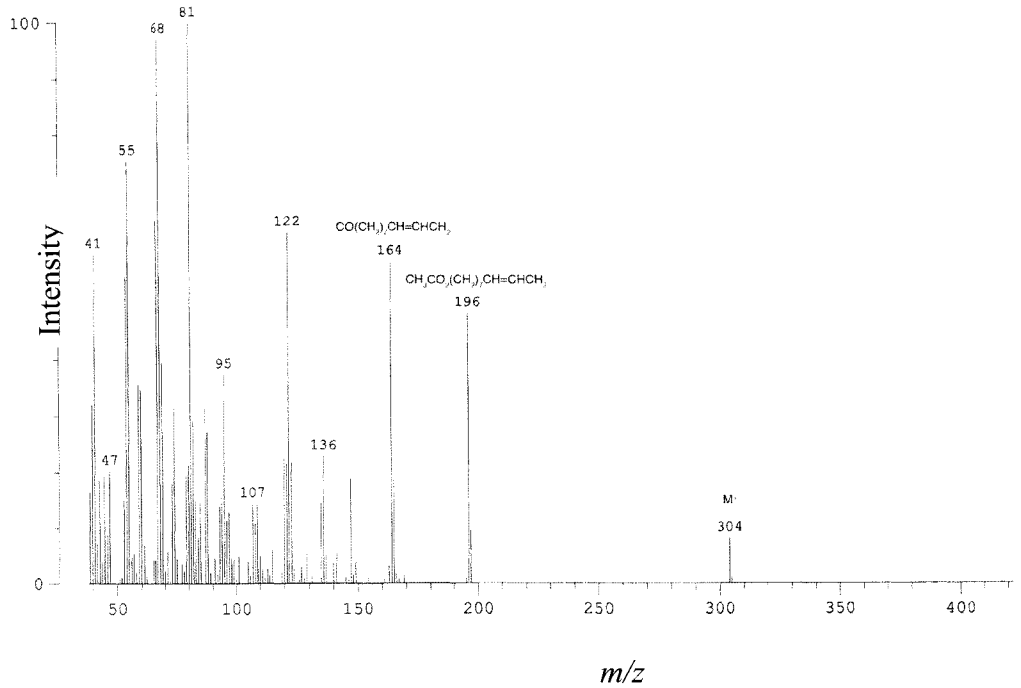


Figure 2.19 Mass spectrum of methyl 16-fluoro-12-thiaoctadec-9-enoate.

Diagnostic peaks include the molecular ion and fragment ions pertaining to chain cleavage alpha to sulfur. For C16 12-S-9-ene, identifiable ions include: *m/z* 196 ($CH_3CO_2(CH_2)_7CH{=}CHCH_2$ with loss of H), 164 ($CO(CH_2)_7CH{=}CHCH_2$ with loss of 2 H) and 122 ($(CH_2)_7CH{=}CH$ with loss of 2 H).

The thia fraction of the yeast cellular extract obtained from methyl 16-fluoro-12-thiahexadecanoate was isolated by flash chromatography (5% AcOEt/hexanes) and analyzed by GC-MS at PBI-Saskatoon using a higher resolution GC-column (DB-23 capillary column) (Figure 2.20).

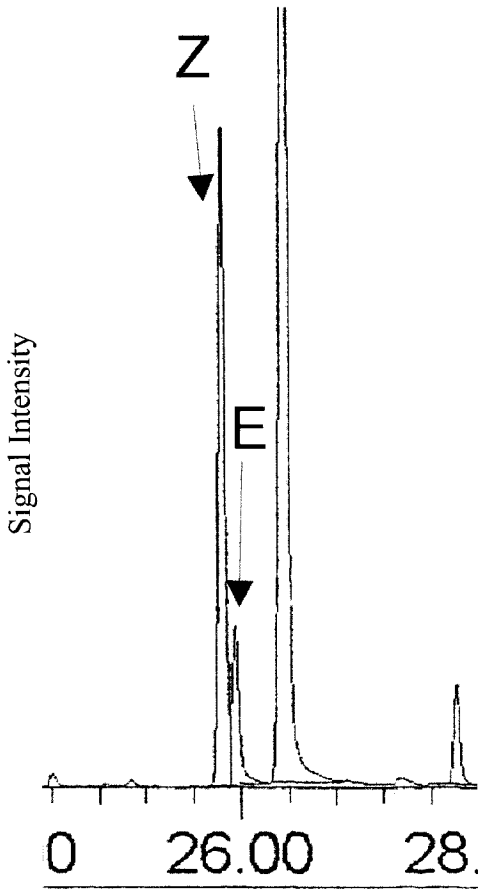


Figure 2.20 Chromatogram of methyl 16-fluoro-12-thiahexadecanoate displaying a shoulder on the expected *cis*-desaturated fatty acid. Indicated shoulder thought to be *trans*-geometric isomer of desaturated fatty acid.

Importantly, using the PBI conditions, an additional minor GC peak (25.95 min) accompanied the major peak assigned to the 9-ene (25.85 min). This additional peak was not readily apparent using our standard method of analysis carried out at Ottawa U (Figure 2.18). Given the fact that the mass spectra of the two peaks in question were identical, it was concluded that the *trans*-isomer of the major *cis*- desaturated fatty acid had been produced. The apparent lack of stereoselectivity is considered highly unusual as the *cis* isomer was expected to be the sole product.

Additional evidence for the presence of a minor *trans*-olefinic contaminant in this fraction was obtained from an additional GC-MS chromatogram (polar 30 M INNOWAX column) as well the ^{1}H and ^{13}C NMR spectra (Figures 2.22-23).

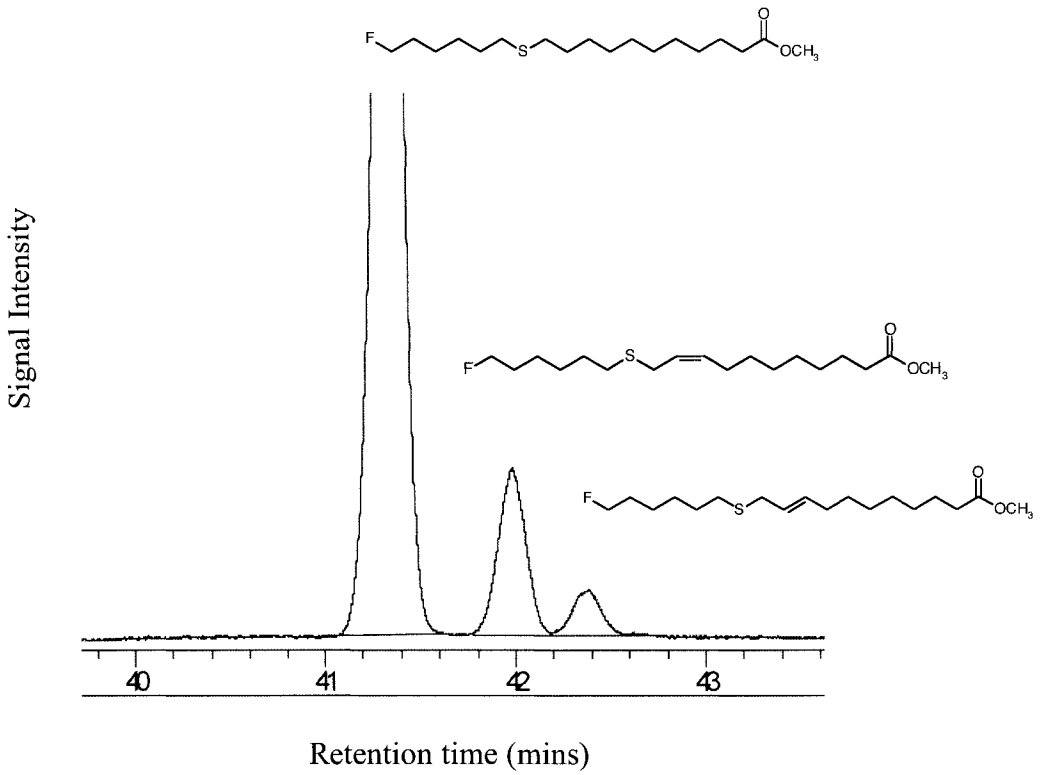


Figure 2.21 Partial GC-MS chromatogram of C16F12S fraction obtained using a polar GC Column (polar INNOWAX column).

Using the chromatogram displayed in Figure 2.21, the *cis*/*trans* ratio was estimated to be 4:1. Under these GC conditions, *trans*-olefins typically have longer retentions times than the corresponding *cis*-isomers because its linear profile allows for greater interaction with the column.

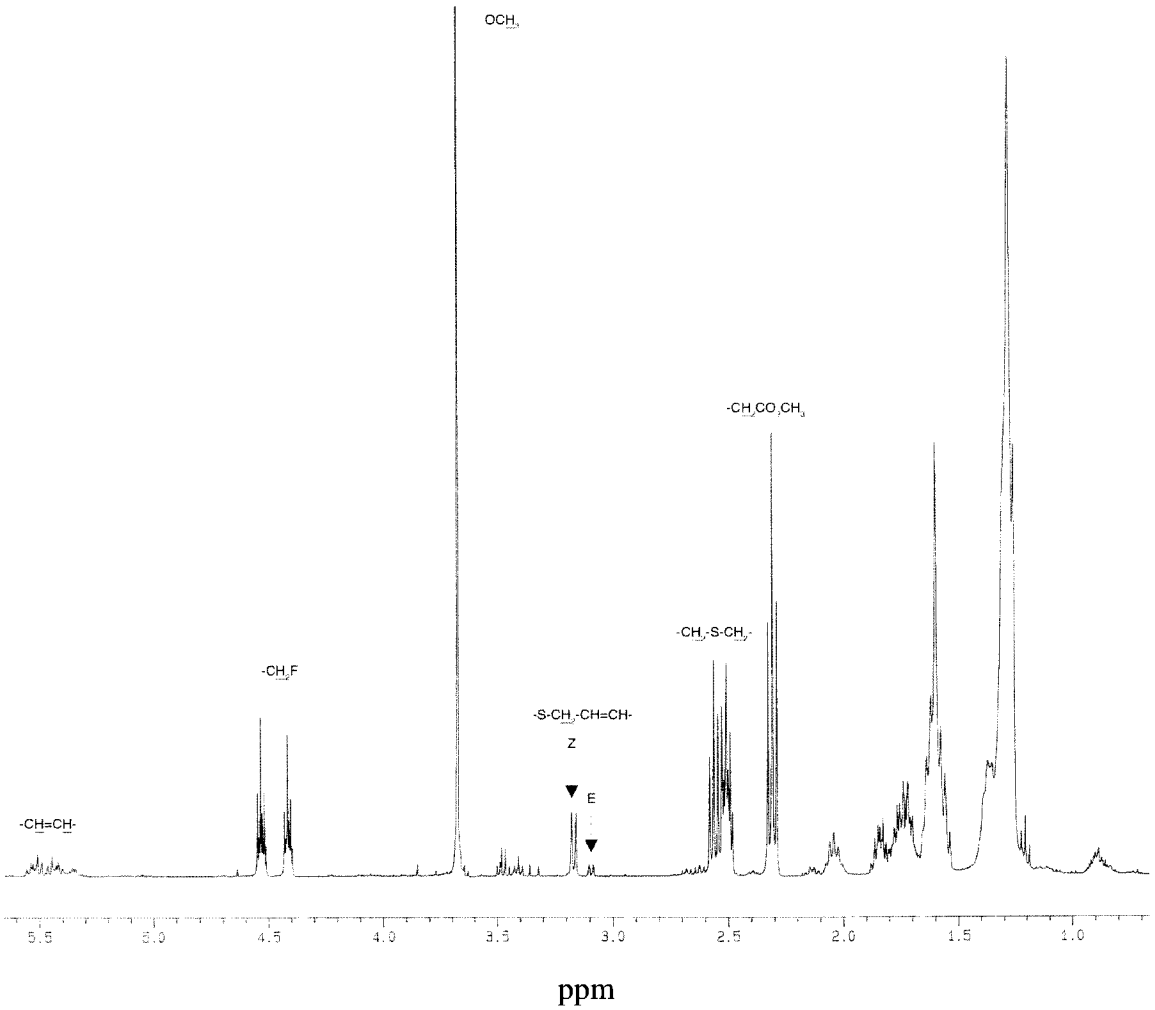


Figure 2.22 ^{1}H spectrum of a mixture of methyl 16-fluoro-12-thiahexadecanoate / methyl 16-fluoro-12-thia-9-decenoate (both stereoisomers).

Two set of doublets (vicinal coupling to C10 hydrogen) of unequal intensity could be assigned to the α-sulfide methylene group of the *cis*- and *trans*-9-ene product based on the GC data. From the literature[59], it is known that the allylic resonances for trans-olefins appears slightly upfield relative to those of the cis-isomer.

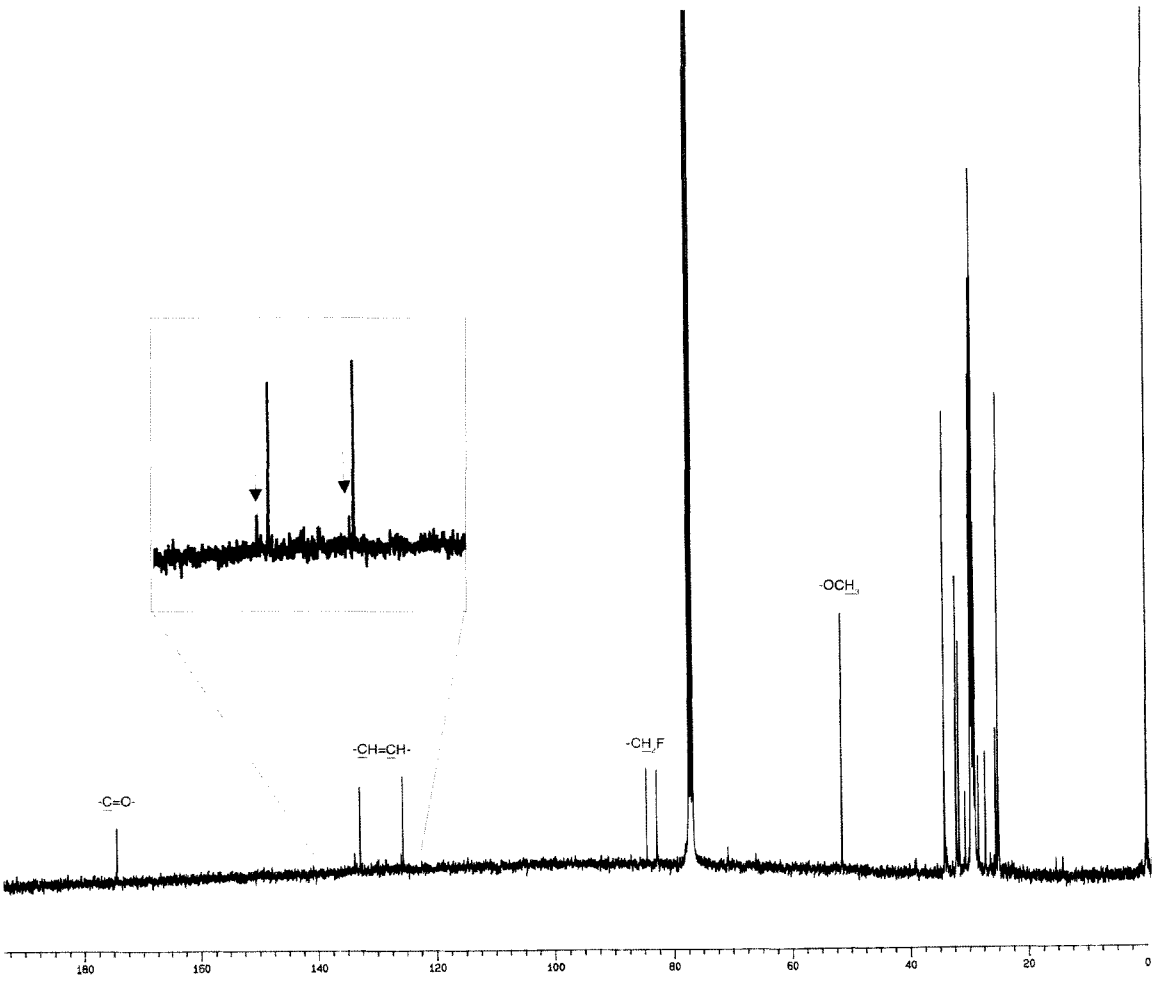


Figure 2.23 ^{13}C spectrum of a mixture of methyl 16-fluoro-12-thiahexadecanoate / methyl 16-fluoro-12-thia-9-decenoate (both stereoisomers). Arrows show putative signal of *trans*-olefinic carbon.

The presence of a minor amount of the *trans*-9-ene product was discernable in the olefinic carbon region of the ^{13}C NMR spectrum shown in Figure 2.23. The resonances were shifted downfield relative to the major product, this downfield shift was consistent with literature data on related compounds.[11] A detailed analysis of expanded sections of the ^{13}C NMR spectra of all of the yeast extracts, together with reference data allowed one to construct a table of tentative ^{13}C assignments for the fluorine-tagged *cis*- thiaolefinic

products shown in Table 2.2. Minor peaks due to *trans*-components are indicated as appropriate.

Allylic carbon resonances also have unique signals that are dependant on the geometric isomer of the fatty acid. Allylic signals of oleic acid tend to be around the 27 ppm range while elaidic acid has resonances closer to 32 ppm. Indeed, for the 16- and 18-fluoro-12-thia monounsaturates, an extra resonance was found around 32 ppm (see table 2.2). This provides further evidence for the significant presence of the trans isomer.

Carbon Position	Oleate (ppm)	C18-12S	16F-12S (ppm)	18F-12S (ppm)	18F-13S (ppm)
1	[174]	174.36	174.38	174.33	174.32
2	34.12	34.11	34.11	34.08	34.13
3	25.08	24.95	24.95	24.93	24.97
4	29.28	29.24	28.95	28.94	29.10
5	29.28	29.37	29.09	29.09	29.43
6	29.28	29.46	29.60	29.49	29.50
7	29.83	29.6	29.72	29.61	29.52
8	27.32	27.97	27.12 (32.18)	27.13 (32.49)	27.33
9	129.78	125.73	125.50 (125.88)	125.68 (126.04)	127.54
10	130.02	132.53	132.78 (133.74)	132.62	131.53
11	27.32	28.03	28.31	28.51	27.58
12	29.90	S	S	S	32.23
13	29.46	31.56	32.08	31.03	S
14	29.68	29.74	25.39	29.61	32.00
15	29.46	28.66	29.55	29.71	29.72
16	32.07	31.49	83.71	24.88	24.58
17	22.79	22.58	n/a	30.32	30.05
18	14.11	14.06	n/a	84.05	83.94
Me	51.21	51.46	51.48	51.43	51.46

Table 2.2 ^{13}C assignment of monounsaturated *cis*- thia fatty acid chemical shifts. Chemical shifts in brackets are resonances assigned to the minor trans component. C18-12S:methyl 12-thiaoctadecanoate; 16F-12S : methyl16-fluoro-12-thiahexadecanoate; 18F-12S: 18-fluoro-12-thiaoctadecanoate; 18F-13S: 18-fluoro-13-thiaoctadecanoate.

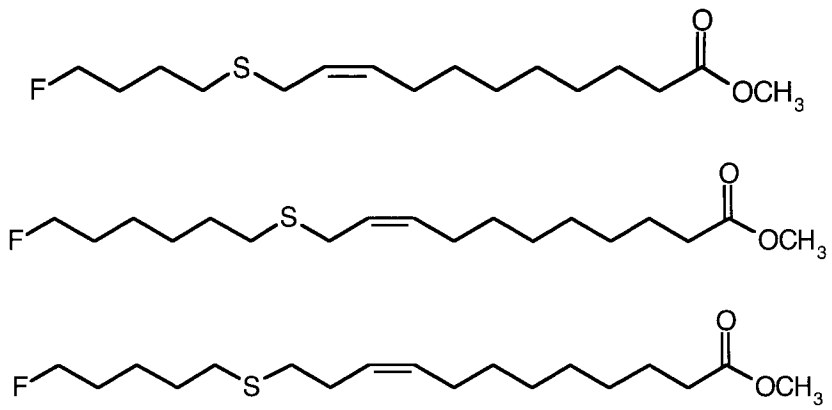


Figure 2.24 Structure of the fluorine-tagged *cis*-thia-fatty acids produced by yeast Δ^9 desaturation.

The % *trans* content of each thia 9-ene as well as endogenous olefinic fatty acid produced by yeast Δ^9 desaturation was quantitated by GC-MS (PBI-Saskatoon) and the results are listed in Table 2.3. In the case of the 12-thia compounds, corroborating evidence from ^{1}H NMR was available (See Table 2.3).

Substrate	% *trans* content of desaturated fatty acid
16FC16-12S	17 ± 3*
18FC18-12S	21.9 ± 0.7*
18FC18-13S	1.7**
C18-12S	23 ± 15
C16	3.3 ± 2.2***
C18	1.6 ± 1.4***

Table 2.3 *Trans*-9-ene olefin content as a percentage of total desaturated fatty acid using GC (and in some cases ^{1}H NMR). *Error calculated based on average of *trans* content calculated from NMR and GC analysis. **Error not calculated due to lack of unique ^{1}H resonance.*Standard deviation calculated from GC data alone (3 trials).**

As seen from table 2.3, the largest proportion of *trans*-fatty acid production occurs with the 12-thia analogues while the *trans*-content of the 13-thia analogue as well as the endogenous fatty acids was quite low. If *trans*-content can be attributed to a

perturbing influence of a sulfur substituent on substrate conformation in the active site, then a possible explanation for the low level of *trans*-olefin production in the case of the 13-thia analogue may be the increased distance of sulfur atom from the incipient double bond. The results of the control experiment and other data in the literature[1] indicate that the ω-fluorine substituent does not influence *trans*-fatty acid production.

It should be noted that conversion of saturated fatty acid to cis- or *trans*-monounsaturates is itself not unprecedented. The Δ^{11} desaturase enzyme that exists in certain insects desaturate fatty acids produces a mixture of cis- and *trans*- 11-tetradecenoates[60]. The substrate is capable of interconverting between two different conformers which sets up for desaturation that leads to either cis- or *trans*-product. It could be conceivable that such a mechanism may exist in the yeast system due to the perturbing influence of the 12-thia sulfur atom close to the site of desaturation. Other examples of desaturase-linked "error" relate to the FAD2 desaturase (Δ^{12}), producing low levels of hydroxy products[61]. The enzyme found in *Arabidopsis thaliana* was found to exhibit this behaviour.

Despite the lack of totally stereoselective production of the required *cis*-9-enoates chemoenzymatic formation remains the more convenient manner of obtaining monounsaturated fatty acids in a selective fashion. Yields of chemoenzymatic synthesis was fairly low, out of a 25 mg incubation, recovery was only approximately 3 mg (> 1 mg of desaturated material) of synthetic fatty acid mixture. However, these amounts are suitable for in vitro experiments that only require μMolar amounts. Based on ^{19}F NMR data, these amounts could be easily detected.

Future work in this area would involve HPLC purification of the yeast extracts to resolve the two stereoisomeric 9-enes. Experiments designed to investigate to rule out non-desaturase related causes for *trans*-olefin formation are also required. Thus all saturated substrates should be incubated at the same time to ensure consistency with respect to such factors as possible bacterial contamination, temperature control, etc. In addition, incubation of methyl 7-thiastearate would be interesting to test if the amount of *trans*-product obtained would be similar to that generated from the 12-thia analogues.

2.4.3 Chemical Sulfoxidation of Methyl 18-fluoro-12-Thia -9-octadecenoate

In preparation for future *in vitro* mechanistic studies involving Δ^{12} desaturase-mediated sulfoxidation of our fluorine-tagged thia probes, it was necessary to prepare synthetic standards by chemical oxidation.

Fractionation of the thia-fraction of yeast FAME mixture derived from a 18F12S incubation by flash chromatography (5% AcOEt/hexanes, SiO_2), yielded a (7:1) mixture of methyl 18-fluoro-12-thiaoctadecanoate and its 9-desaturated product as determined by GC-M. Oxidation of this mixture using *m*-CPBA (5 mg, 1 equivalent) were conducted on a 5 mg portion in an NMR tube and the progress monitored by ^{1}H-decoupled-^{19}F NMR (282 MHz) (Figure 2.24).

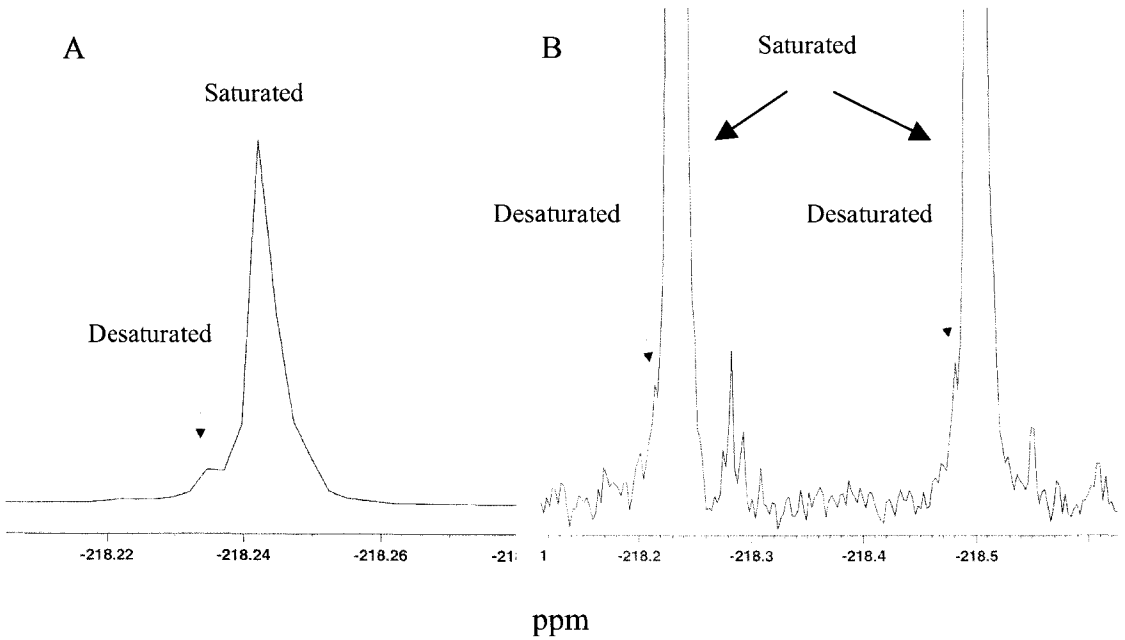


Figure 2.25 ^{1}H-decoupled ^{19}F spectra of A) methyl 18-fluoro-12-thiaoctadecanoate/9ene and B) oxidation product using 0.5 equiv. of *m*-CPBA. Desaturated product shown as indicated.

Prior to oxidation (Panel A), the ^{1}H-decoupled ^{19}F NMR spectrum exhibited a major peak with a downfield shoulder which can be attributed to the saturated and unsaturated product respectively. The chemical shift difference between the saturated and desaturated material (*cis/trans* mix) was $\Delta\delta < 0.01$ ppm. A similar result was obtained with the 16F12/9ene mix where the $\Delta\delta = 0.03$ ppm. After partial oxidation (Panel B), a major new ^{19}F signal was apparent which corresponded to what was expected for the sulfoxide of methyl 18-fluoro-12-thiaoctadecanoate S- oxide ($\Delta\delta_{S0\text{-}S} = 0.27$ ppm). This result was expected based on the trends documented previously in Section 2.2, Figure 2.6 since the fluorine reporter atom is closer compared to the sulfur atom than the 18-fluoro-11-thia analogue ($\Delta\delta_{S0\text{-}S} = 0.1$ ppm). The possibility that the double bond in the unsaturated component of the 18F-12S/9-ene mixture was converted to an epoxide product is unlikely since the minor desaturated component could still be observed as a

shoulder of the major sulfoxy product. However, this conclusion must be regarded as tentative due to the appearance of other ^{19}F resonances (Panel B) in the oxidized mixture. Further work with purified 9-enes is required. To complement the data in Figure 2.6, Table 2.4 was compiled to summarize the data gathered of the new sulfoxy species.

Substrate	Sulfide (ppm)	Sulfoxide (ppm)	Δδ (ppm)
18F12S	-218.44	-218.70	0.26
18F13S	-218.43	-219.00	0.57

Table 2.4 ^{19}F NMR data for methyl 18-fluoro-12(13)-thiaoctadecanoates and their corresponding sulfoxides. Chemical shifts were referenced to methyl 15-fluoro-11-thiaoctadecanoate-S,S dioxide (-219.73 ppm) as an internal standard.

The large $\Delta\delta_{S\text{-}SO}$ associated with the 13S analogue was expected since the sulfoxide moiety is closer to the fluorine reporter group leading to a large change in chemical shift. The Δδ seem to fit with the trend outlined in Figure 2.6 and bodes well for *in vitro* experiments using these substrates as probes.

2.4.4 Trial des A-mediated sulfoxidation of 16-Fluoro-12-Thiahexadec-9-enoic acid

In collaboration with the Covello (Plant Biotechnology Institute, NRC) group, we attempted to utilize the synthetic substrates in an *in vitro* reaction with a Δ^{12} desaturase (desA). Using previously reported procedures[67] the group has provided the enzyme by overexpressing the desA gene that encodes the Δ^{12} desaturase in *E. coli*. This gene was originally from the cyanobacterium desaturation mutant *Synechocystis* sp. Strain PCC6803. Using oleic acid as a substrate, it was demonstrated that enzyme activity was suitable for *in vitro* experiments. It was not known at the time exactly what form of the substrate was bound by the desaturase enzyme, therefore derivatization to a phospholipids or acyl CoA thioester was not performed.

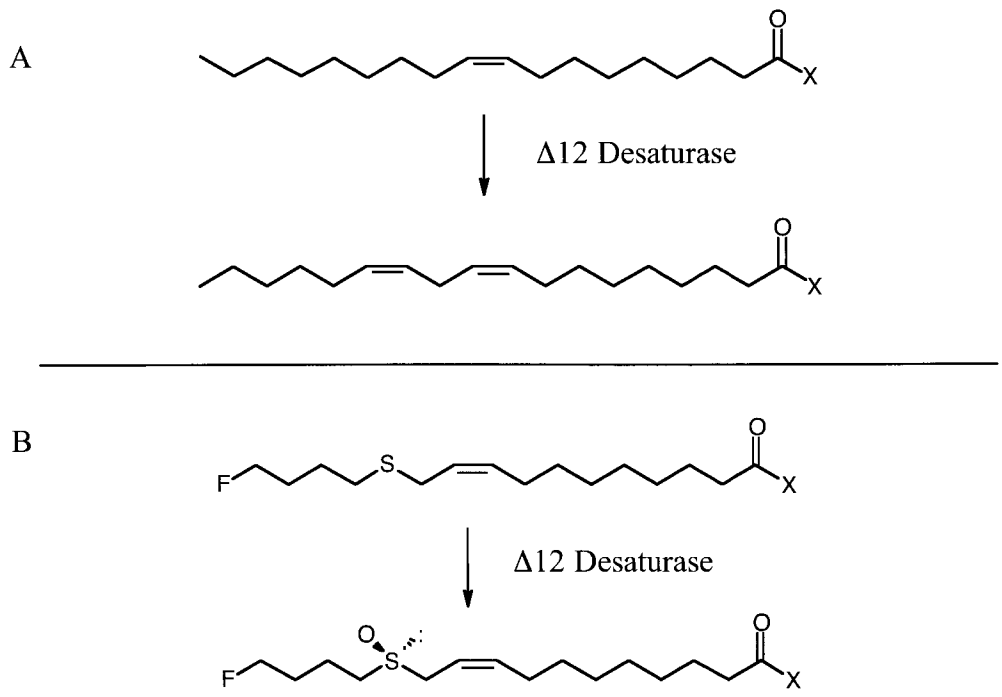


Figure 2.26 *In vitro* Desaturase-mediated oxidation; in vitro reaction of A) oleic acid leads to the conversion of linoleic acid and reaction of B) methyl 16-fluoro-12-thia-hexadecanoic acid is expected to lead to sulfoxide formation. "X" is a possible phospholipid derivative as the active form of the substrate.

Incubation of a mixture of 16-fluoro-12-thiahexadec-9-enoic acid and its saturated precursor (~1.1 mg/ml) was carried out as described in the Experimental section. Trial runs on the protein homogenate were tested on oleic acid to ensure acceptable enzyme activity. The reaction was halted by quenching the reaction with MeOH/KOH solution and heated at 80 °C for 1.5 hours. Subsequent methylation after acidification was performed by heating the solution in acidic methanol at 60 °C for 30 mins. The organic extract was analyzed by GC-MS and no starting sulfide could be observed under these conditions. Denatured enzyme experiments and vector control experiments were conducted in order to assess whether the synthetic substrate was detectable after the reaction. Saturated substrate was still not detectable which indicated that the harsh workup probably decomposed the sulfide analogues possibly through autoxidation. A

final experiment was designed to scale up substrate concentration and prepare the reaction mixture for direct analysis for ^{19}F NMR.

^{19}F signals obtained displayed the expected resonance from the saturated and desaturated thia fatty acid. In addition to the expected signals, an unidentifiable broad peak was found within close proximity to the free acid and can be seen in figure 2.27. This signal does not match any of the standards previously described; therefore positive identification is not possible at this time. The presence of sulfoxy product was not detected at the expected chemical shift.

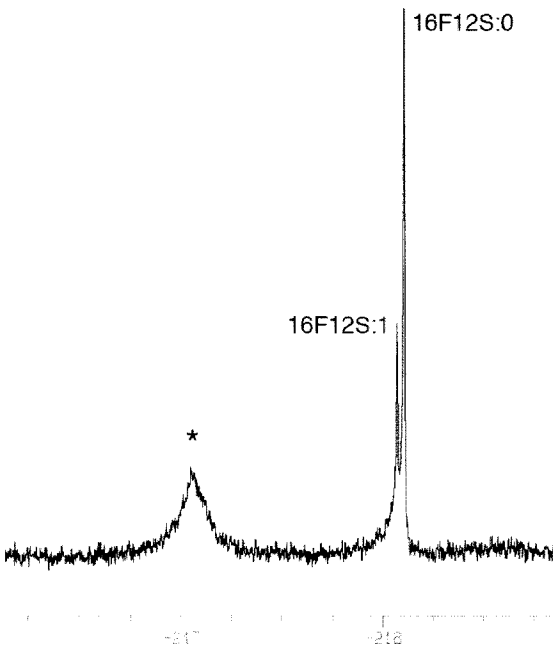


Figure 2.27 ^{19}F spectrum of desA reaction matrix in D_2O containing synthetic fatty acid substrates and unknown peak(*).

The lack of success with respect to desA reaction with the synthetic substrate may be primarily due to the inappropriate chain length. At the time the trials were carried out, it was not known whether the enzyme would accept a palmitic acid substrate derivative.

However, the choice of substrate length was chosen out of necessity if stereochemical analysis would be performed as in section 2.3. Presence of the sulfur and fluorine atom may also play a negative effect that inhibits uptake of the substrate, but similar *in vitro* experiments were conducted with much success using ACP Δ^9 desaturase[1]. New information from the Covello lab has lead to the decision to utilize stearate derivatives instead, and compensating for the increased distance of the fluorine reporter group by employing a different NMR shift reagent than the planned (*S*)-(+)-MPAA.

2.5 Conclusions and Future Work

In this project, a methodology was designed to take advantage of the versatility of ^{19}F NMR with respect to biological probing of desaturase-mediated sulfoxidation. It has been shown that chemical shifts of ^{19}F resonances were sensitive to changes in its chemical environment for as far as 11 carbon atoms away. Changes in chemical shift due to sulfoxidation have been shown to be adequately resolved in order to minimize interference from other fatty acid analogues. It has also been shown that stereochemical analysis was possible using this approach with a suitable chiral NMR shift reagent. In order to go forward with in vitro experiments with the membrane bound Δ^{12} desaturase enzyme, the chemoenzymatic route was found to be an adequate means to obtain sufficient amounts of monounsaturated fatty acid. Despite low yields, the fluorine approach for detection eliminates the need to obtain large quantities of the oleate substrates.

Future work would have to involve the evaluation of various chiral NMR shift reagents suitable for stereochemical analysis of 18-fluoro-13- and 12-thia sulfoxide derivatives. Current reports[62] reveal that alternatives such as (*S*)-9-AMA (9-anthrylmethoxyacetic acid) would be a good candidate as the presence of an anthryl group would increase the ability of the shift reagent to induce non-equivalence beyond 4 carbons (current limit of MPAA).

Further investigation of the *trans*-fatty acid production by yeast Δ^9 desaturase should be pursued in order to assess the cause of the reduced stereoselectivity with 12-thia-substrates. Synthesis of a 7-thia fatty acid and other analogues might give further insight into a probable cause. It would be interesting to know if in fact, the sulfur atom at the C12 position was responsible in inducing the stereochemical error in Δ^9 desaturation.

Chapter 3: Experimental

3.1 Materials and Methods

^{1}H NMR (400, 300 MHz) and ^{13}C NMR (100, 75 MHz) spectra were obtained on either a Bruker AMX 400 or Bruker Avance 300 spectrometer. Some ^{1}H NMR (200 MHz) spectra were obtained on a Varian Gemini 200 spectrometer. ^{1}H-decoupled ^{19}F (376 MHz) NMR was performed by Dr. Derek Hodgson on a Bruker AM 400 spectrometer at Health Canada (Centre for Biologics Research, Biologics and Genetic Therapies Directorate). In the later stages of the thesis work, the Bruker Avance 300 spectrometer was used to obtain ^{1}H-decoupled ^{19}F decoupled (282 MHz) NMR spectra. All compounds were dissolved in $CDCl_3$, that was dried by passing the solvent through a short column packed with neutral alumina (Activated aluminum oxide, Brockmann I standard grade, 150 mesh, 58 A). Chemical shifts for ^{1}H and ^{13}C NMR spectra are reported in ppm (δ) relative to TMS (0.00 ppm) and normalized to a chemical shift value of 7.2650 ppm (^{1}H) and 77.0275 ppm (^{13}C) for residual $CHCl_3$ (^{1}H) and $CDCl_3$ (^{13}C). ^{19}F chemical shifts were measured relative to $CFCl_3$ (0.00 ppm, external standard). When the spectra were obtained at Carleton University on the Bruker Avance 300 spectrometer, chemical shifts were measured against a known internal standard (Methyl 15-fluoro-11-thiapentadecanoate) previously recorded at Health Canada. Abbreviations used to assign multiplicity of peaks are as follows: s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet and b = broad.

GC-MS data were obtained courtesy of the University of Ottawa's mass spectrometry facility (Dr. Clem Kazakoff) using a Kratos Concept 1 H mass spectrometer

at 70 eV interfaced with a J & W 30 m x 0.21 mm, DB-5 capillary column. The temperature program started at 120 °C and increased at 10 °C/min until 320 °C and held for 2 minutes. Mass spectra of sulfoxides and sulfones were obtained using an EI direct probe.

GC data at the Plant Biotechnology Institute in Saskatoon (Dr. Darwin Reed) was obtained using a Hewlett-Packard 5890 GC-FID. GC-MS was obtained using an Agilent 6890 GC connected to a 5973 Agilent MS detector operating in EI mode. The column used for both GC systems was a DB-23 capillary column (30 m x 0.25 mm with a film thickness of 0.25 μm). Temperature programs used for both systems started at 160 °C for 1 min. then was increased to 240 °C at a rate of 4 °C/min and held for 15 minutes. Helium flow was set to 30 m/s.

Purification by flash chromatography was done using Merck silica gel (60 A), grade 9385 (230-400 mesh) with an air pressure of ca. 5 psi. Thin layer chromatography was done on a glass backed plate coated with Merck silica gel (60 A). Visualization was achieved by I_2 vapour or water spray.

All reagents obtained commercially were acquired from Sigma-Aldrich and used without purification unless indicated. Methylation via diazomethane was done by activating ca. 300 mg of N-nitrosomethyl urea in a test tube containing 15 mL of diethyl ether and 3 mL of 50% KOH. The yellow ethereal solution was dried over Na_2SO_4 and used immediately. Pyridine was dried over NaOH pellets, p-toluene sulfonyl chloride was recrystallized using hexanes and chloroform[63]; THF was distilled over Na metal. Organic extracts of reaction mixtures were dried by passing the extracts through a funnel containing Na_2SO_4. Solvents were removed in vacuo using a Büchi RE 111 Rotovapor.

ω-Thiooctanoic and ω-thiodecanoic acids were prepared from the corresponding, commercially available bromoacids. In a similar manner, ω-thioheptanoic and ω-thiononanoic acid were obtained from ω-bromoheptanoic acid and ω-bromononanoic acid respectively; the latter two compounds were available from the corresponding ω-bromoalkan-1-ol via Jones oxidation.

Early sulfoxidation experiments made use of *Saccharomyces cerevisiae S288C* cultures obtained from the Yeast genetic Stock Center at the University of California at Berkeley. The strain of baker's yeast (NRRL Y-2574) used in the chemoenzymatic experiments was obtained from Dr. C.R. Kurtzman, USDA Peoria, Illinois, USA. These organisms were maintained by monthly transfer to solid agar slants and stored at 4 ° C.

Sterilization of culture media was accomplished using an AMSCO 2021 gravity autoclave using the "Liquids" setting over 20 minutes with an atmospheric pressure of about 1.2 kg/cm^2. Yeast cultures were incubated with substrate in a New Brunswick Scientific Co. Model G25 Incubator Shaker in 500 mL mL Erlenmeyer flasks which were agitated at 150 RPM with a temperature setting between 27-29 °C. Oxygen was supplied to the cultures by passive diffusion through sterilized foam plugs.

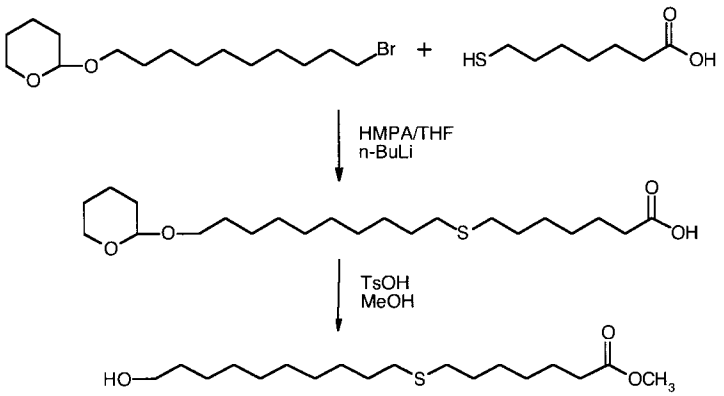
Reagents for biological experiments were purchased from VWR Canlab. Distilled deionized water was used in all cases and was obtained using a Millipore Milli-Q water filtration system. A Sorval superspeed RC2-B automatic refrigerated centrifuge (Carleton Univeristy, TB 132) was used to centrifuge biological cell cultures at 10,000 rpm.

ω-Thiooctanoic and ω-thiodecanoic acids were prepared from the corresponding, commercially available bromoacids. In a similar manner, ω-thioheptanoic and ω-

thiononanoic acid were obtained from ω-bromoheptanoic acid and ω-bromononanoic acid respectively; the latter two compounds were available from the corresponding ω-bromoalkan-1-ol via *J*ones oxidation. Protection of the required ω-bromoalkan-1-ols as the THP ethers was carried out according to a standard procedure

3.2 Synthesis of Thia Fatty Acid analogues

3.2.1 Methyl 18-hydroxy-8-thiaoctadecanoate

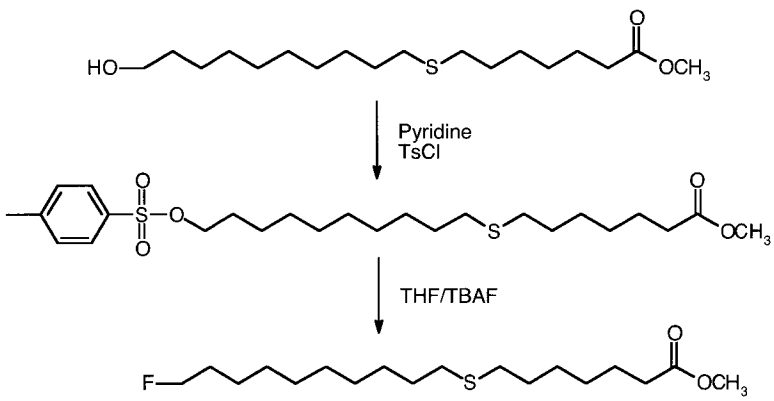


To a soln of ω-thioheptanoic acid (1.63 g, 10.0 mmol) in 3:1 THF/HMPA (66 ml) was added a 1.6 M n-butyl lithium (14.0 ml) reagent via syringe, under N_2 at 0 °C. After stirring the reaction mixture at 0 °C for 30 min., a solution of 10-bromodecan-1-ol THP ether (3.23 g, 10.1 mmol) was added via syringe. The reaction mixture was stirred at r.t. for 22 h and quenched with the addition of H_2O (60 ml), acidified to a pH of 2 with 3 M HCl (15 ml) and extracted with hexanes (4 x 60 ml). The combined organics were washed with sat. NaCl, dried (Na_2SO_4) and evaporated to give crude ω-hydroxy-8-thiastearic acid, THP ether (3.19 g). This compound was deprotected and methylated with TsOH (115 mg) /MeOH (30 ml, refluxing) and after evaporation of the reaction solvent, the crude product (1.99 g) was purified by flash chromatography using 20 %

EtOAc/hexanes to give the title compound (558 mg, 17 % based on ω-thioheptanoic acid) as a white solid.

TLC (hexane/EtOAc 40:60): R_f 0.25. M.p. 43 - 45° (amorphous crystals, recrystallized from hexane). IR (KBr): 3340, 2931, 2851, 1738, 1471,1462, 1437, 1254, 1176, 1171, 1073. ^{1}H-NMR (400 MHz, $CDCl_3$): 3.66 (*s*, 3 H); 3.63 (*t*, *J* = 6.6, 2 H); 2.489, 2.487 (partially resolved overlapping *t*, *J* = 7.4, 4 H); 2.30 (*t*, *J* = 7.5, 2 H); 1.53 –1.67 (*m*, 9 H); 1.25-1.45 (*m*, 16 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 174.23; 63.10; 51.47; 34.03; 32.82; 32.23; 32.11; 29.73; 29.53; 29.53; 29.45; 29.41; 29.24; 28.94; 28.78; 28.55; 25.74; 24.84. EI-MS: 332 (8, M^+), 301 (11, $[M - 31]^+$), 283 (2), 259 (4), 231 (3), 203 (6), 189 (16, $[HO(CH_2)_{10}S]^+$), 171 (51, $[CH_2{=}CH(CH_2)_8S]^+$), 159 (10), 143(89, $[(CH_2)_6CO_2CH_3]^+$), 129 (8), 111 (26),101 (25), 87 (54), 69 (60) , 55 (100), 41 (66). HR-EI-MS: 332.2392 ($C_{18}H_{36}O_3S$, $[M]^+$; calc. 332.2385).

3.2.2 Methyl 18-fluoro-8-thiaoctadecanoate

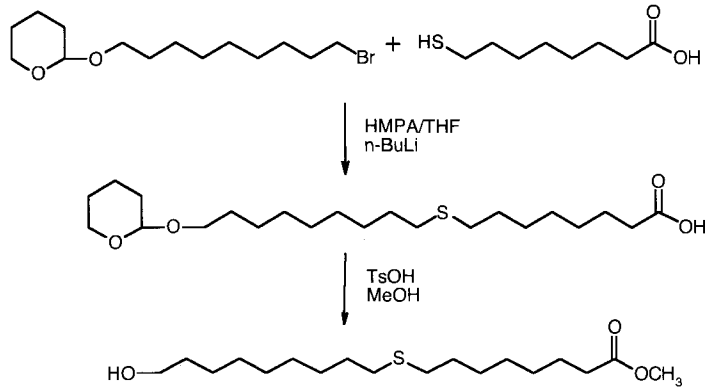


To a soln of methyl ω-hydroxy-8-thiaoctadecanoate (0.307 g, 0.92 mmol) in dry pyridine (2 ml) was added *p*-toluenesulfonyl chloride (0.352 g) under N_2 at room temperature. After stirring at room temperature for 3 h, the reaction was quenched with the addition of dH_2O (20 ml), and extracted with ether (4 x 30 ml). The combined

organic layers were washed with 2M HCl (2 x 20 ml), dried (Na_2SO_4) and evaporated to give the crude tosylate (0.266 g, 59 % est.): TLC (hexane/EtOAc 30:70): R_f 0.35. ^{1}H-NMR (200 MHz , $CDCl_3$): 7.79 (*d*, *J* = 8.3, 2H); 7.35 (*d*, *J* = 8.3, 2H); 4.01 (*t*, *J* = 6.3, 2H); 3.67 (*s*, 3 H); 2.49 (overlapping *t*, *J* = 6.7, 4 H); 2.45 (s 3H), 2.31 (*t*, *J* = 7.3, 2 H); 1.45 – 1.7 (*m*, 8H); 1.1 –1.4 (*m*, 14H). This compound was stirred at room temperature with 6 equivalents of TBAF in dry THF (50 ml) over molecular sieves (0.5g), for 3 h. THF was removed in vacuo and the residue partitioned between water (10 ml) and hexane (4 x 20 ml). The hexane layer was washed with sat. NaCl (1 x 50 ml), dried (Na_2SO_4) and evaporated. The crude product was purified by flash chromatography using 5 % EtOAc/hexanes to give the title compound (111 mg, 36 % based on methyl ω-hydroxy-8-thiaoctadecanoate) as a colourless oil.

TLC (hexane/EtOAc 90:10): R_f 0.34. M.p. 24 – 24.5°. IR (film): 2928, 2855, 1741, 1463,1436, 1248, 1198, 1171. ^{1}H-NMR (400 MHz , $CDCl_3$): 4.44 (*dt*, *J* = 47.4, 2 H); 3.67 (*s*, 3 H); 2.497, 2.494 (partially resolved overlapping *t*, *J* = 7.4, 4 H); 2.31 (*t*, *J* = 7.5, 2 H); 1.53-1.80 (*m*, 8 H); 1.25-1.45 (*m*, 16 H). ^{13}C-NMR (100 MHz , $CDCl_3$): 174.23; 84.25 (*d*, *J* = 163.9); 51.49; 34.01; 32.04; 32.20; 30.41 (*d*, *J* = 19.3); 29.71; 29.50; 29.45; 29.42; 29.22; 29.22; 28.94; 28.76; 28.55; 25.15 (*d*, *J* = 5.5); 24.83. ^{19}F NMR (376.5 MHz, $CDCl_3$): -218.18. EI-MS: 334 (24, M^+), 303 (10, $[M - 31]^+$), 191(49, $[F(CH_2)_{10}S]^+$), 171 (22, $[CH_2{=}CH(CH_2)_8S]^+$, 143 (100, $[(CH_2)_6CO_2CH_3]^+$), 111 (38), 87 (45), 69(62), 55 (95), 41 (71). HR-EI-MS: 334.2323 ($C_{18}H_{35}O_2FS$, $[M]^+$; calc. 334.2342).

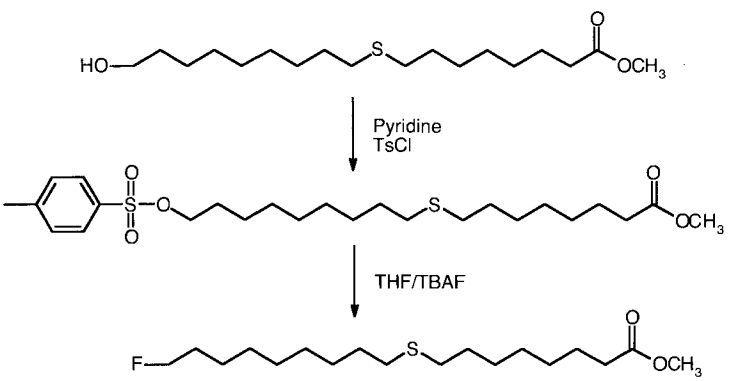
3.2.3 Methyl 18-hydroxy-9-thiaoctadecanoate



Title compound was obtained in a similar manner to that described in 3.2.1, from ω-thiooctanoic acid and 9-bromononan-1-ol THP ether, as a colourless solid.

TLC (hexane/EtOAc 40:60): R_f 0.25. M.p. 42 - 43° (plates, recrystallized from hexane). IR (KBr): similar to that of **3.2.1**. ^{1}H-NMR (400 MHz, $CDCl_3$): 3.66 (*s*, 3 H); 3.63 (*t*, *J* = 6.6, 2 H); 2.49 (unresolved overlapping *t*, *J*~ 7, 4 H); 2.30 (*t*, *J* = 7.4, 2 H); 1.53-1.66 (*m*, 9 H); 1.5- 1.45 (*m*, 16 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 174.31; 63.08; 51.49; 34.07; 32.80; 32.19; 32.14; 29.70; 29.64; 29.47; 29.36; 29.18; 29.03; 28.91; 28.89; 28.74; 25.72; 24.89. EI-MS: 332 (5, M^+), 301 (10, $[M - 31]^+$), 175 (8, $[HO(CH_2)_9S]^+$), 157 (100, $[CH_2{=}CH(CH_2)_7S]^+$, $[(CH_2)_7CO_2CH_3]^+$), 87 (47), 69 (37) , 55 (100), 41 (53). HR-EI-MS: 332.2389 ($C_{18}H_{36}O_3S$, $[M]^+$; calc. 332.2385).

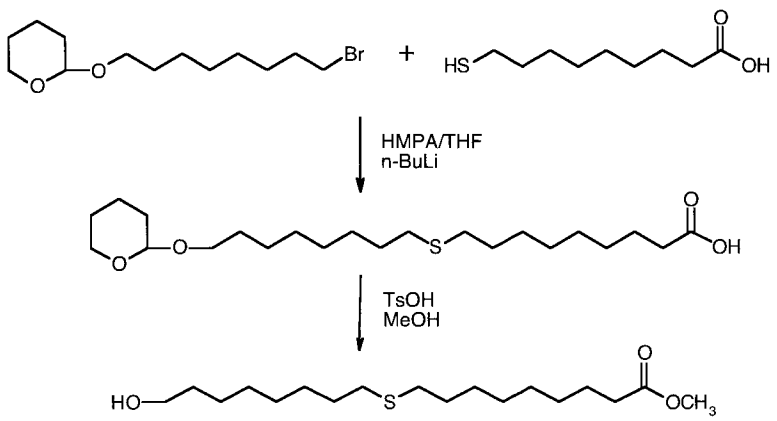
3.2.4 Methyl 18-fluoro-9-thiaoctadecanoate



Title compound was obtained in a similar manner to that described in 3.2.1, from methyl 18-hydroxy-9-thiaoctadecanoate, as a colourless oil.

TLC (hexane/EtOAc 90:10): R_f 0.34. M.p. 22.5-23.5°. IR (film): similar to that of **3.2.2**. ^{1}H-NMR (400 MHz , $CDCl_3$): 4.43 (*dt*, J = 47.4, 2 H); 3.67 (*s*, 3 H); 2.50 (unresolved overlapping *t*, J = 7.4, 4 H); 2.30 (*t*, J = 7.5, 2 H); 1.53-1.83 (*m*, 8 H); 1.25-1.45 (*m*, 16 H). ^{13}C-NMR (100 MHz , $CDCl_3$): 174.25; 84.21 (*d*, J = 164.0); 51.45; 34.04; 32.17; 32.12; 30.39 (*d*, J = 19.3); 29.68; 29.62; 29.37; 29.16; 29.13; 29.01; 28.89; 28.87; 28.72; 25.13 (*d*, J = 5.5); 24.87. ^{19}F NMR (376.5 MHz, $CDCl_3$): -218.18. EI-MS: 334 (37, M^+), 303 (20, $[M - 31]^+$), 189 (28, $[S(CH_2)_7CO_2CH_3]^+$ 177 (63, $[F(CH_2)_9S]^+$), 157 (100, $[CH_2{=}CH(CH_2)_7S]^+$, $[(CH_2)_7CO_2CH_3]^+$), 125 (56), 87 (62), 55 (92), 41 (63). HR-EI-MS: 334.2352 ($C_{18}H_{35}O_2FS$, $[M]^+$; calc. 334.2342).

3.2.5 Methyl 18-hydroxy-10-thiaoctadecanoate

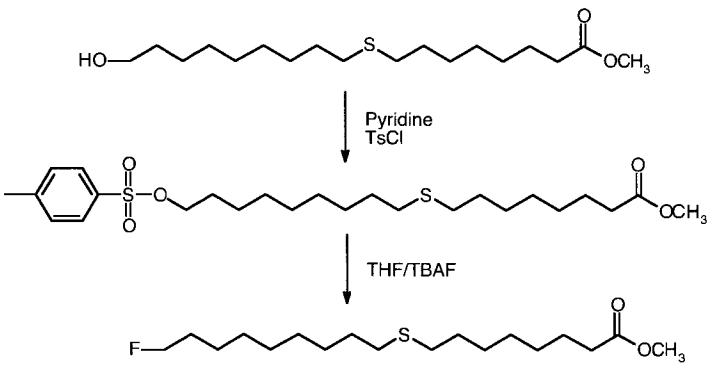


Title compound was obtained in a similar manner to that described in 3.2.1, from ω-thiononanoic acid and 8-bromooctan-1-ol THP ether, as a colourless solid.

TLC (hexane/EtOAc 40:60): R_f 0.25. M.p. 41.5 – 42.5° (amorphous crystals, recrystallized from hexane). IR (KBr): similar to that of **3.2.1**. ^{1}H-NMR (400 MHz,

$CDCl_3$): 3.66 (*s*, 3 H); 3.63 (*t*, J = 6.6, 2 H); 2.49 (unresolved overlapping *t*, J~ 7, 4 H); 2.30 (*t*, J = 7.5, 2 H); 1.53-1.66 (*m*, 9 H); 1.25-1.45 (*m*, 16 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 174.36; 63.05; 51.48; 34.09; 32.77; 32.17; 32.17; 29.69; 29.69; 29.30; 29.21; 29.14; 29.08; 29.05; 28.86; 28.86; 25.68; 24.92. EI-MS: 332 (4, M^+), 301 (11, [M - 31]$^+$), 171(42, [$(CH_2)_8CO_2CH_3$]), 161(6, [$HO(CH_2)_8S]^+$), 143 (64, [CH_2=$CH(CH_2)_6S]^+$), 87 (54), 69 (59), 55 (100), 41 (57). HR-EI-MS: 332.2383 ($C_{18}H_{36}O_3S$, $[M]^+$; calc. 332.2385).

3.2.6 Methyl 18-fluoro-10-thiaoctadecanoate

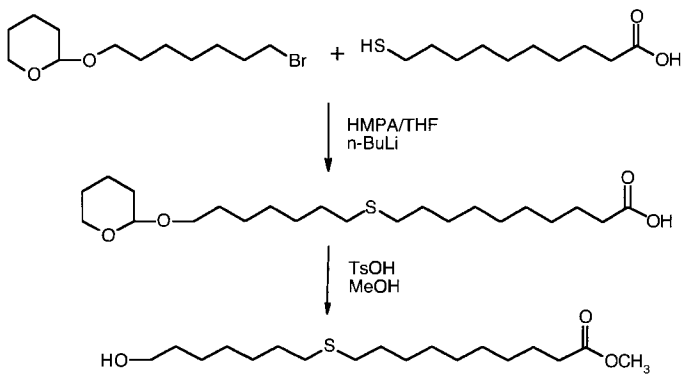


Title compound was obtained in a similar manner to that described in 3.2.1, from methyl 18-hydroxy-10-thiaoctadecanoate, as a colourless oil.

TLC (hexane/EtOAc 90:10): R_f 0.34. M.p. 19.5-20°. IR (film): similar to that of **3.2.2**. ^{1}H-NMR (400 MHz , $CDCl_3$): 4.44 (*dt*, J = 47.4, 2 H); 3.67 (*s*, 3 H); 2.500, 2.498 (partially resolved, overlapping *t*, J = 7.4, 4 H); 2.30 (*t*, J = 7.5, 2 H); 1.53- 1.80 (*m*, 8 H); 1.25-1.45 (*m*, 16 H). ^{13}C-NMR (100 MHz , $CDCl_3$): 174.24; 84.06 (*d*, J = 164.1); 51.39; 34.04; 32.14; 32.14; 30.33 (*d*, J = 19.3); 29.65; 29.64; 29.10; 29.09; 29.04; 29.01; 28.87; 28.82; 28.78; 25.07 (*d*, J = 5.4); 24.88. ^{19}F NMR (376.5 MHz, $CDCl_3$): -218.24. EI-MS: 334 (47, M^+), 303 (32, [M - 31]$^+$), 203 (22, $S(CH_2)_8CO_2CH_3$), 171 (100,

$[(CH_2)_8CO_2CH_3]^+$), 163 (60, $[F(CH_2)_8S]^+$), 143 (65, $[CH_2{=}CH(CH_2)_7S]^+$) 109 (32) 87 (69), 69 (73), 55 (72), 41 (51). HR-EI-MS: 334.2330 ($C_{18}H_{35}O_2FS$, $[M]^+$; calc. 334.2342).

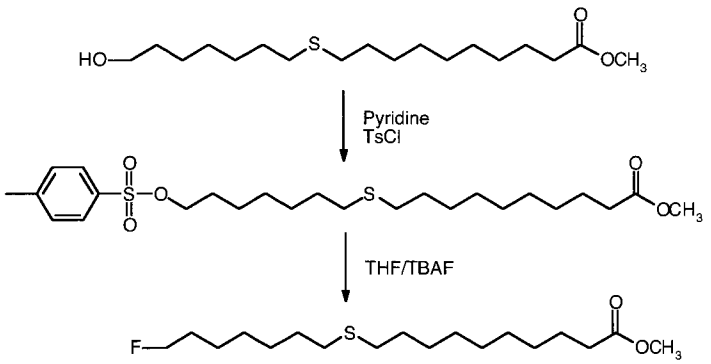
3.2.8 Methyl 18-hydroxy-11-thiaoctadecanoate



Title compound was obtained in a similar manner to that described in 3.2.1, from ω-thiodecanoic acid and 7-bromoheptan-1-ol THP ether, as a colourless solid.

TLC (hexane/EtOAc 40:60): R_f 0.25. M.p. 40 - 41° (amorphous crystals, recrystallized from hexane). IR (KBr): similar to that of **3.2.1**. ^{1}H-NMR (400 MHz, $CDCl_3$): 3.66 (*s*, 3 H); 3.63 (*t*, J = 6.6, 2 H); 2.495, 2.501(partially resolved, overlapping *t*, J = 7.4, 4 H); 2.30 (*t*, J = 7.5, 2 H); 1.53-1.66 (*m*, 9 H); 1.25-1.45 (*m*, 16 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 174.38; 63.02; 51.48; 34.11; 32.73; 32.19; 32.15; 29.70; 29.64; 29.32; 29.20; 29.20; 29.13; 29.05; 28.91; 28.89; 25.64; 24.95. EI-MS: 332 (4, M^+), 301 (13, [M - 31]$^+$), 185 (28, $[(CH_2)_9CO_2CH_3]$), 147 (5, $[HO(CH_2)_7S]^+$), 129 (65, $[CH_2{=}CH(CH_2)_6S]^+$), 87 (52), 69 (47) , 55 (100), 41 (51). HR-EI-MS: 332.2386 ($C_{18}H_{36}O_3S$, $[M]^+$; calc. 332.2385).

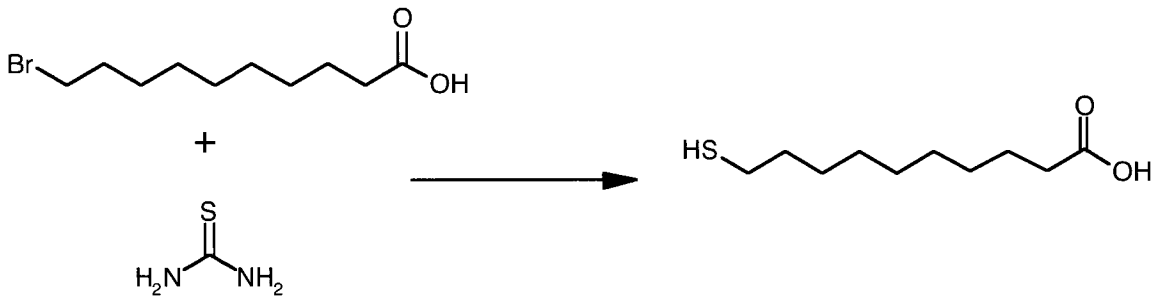
3.2.9 Methyl 18-fluoro-11-thiaoctadecanoate



Title compound was obtained in a similar manner to that described in 3.2.1, from methyl 18-hydroxy-11-thiaoctadecanoate, as a colourless oil.

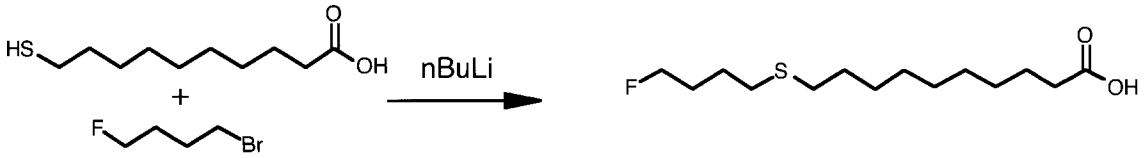
TLC (hexane/EtOAc 90:10): R_f 0.34. M.p. 24 -25°. IR (film): similar to that of **3.2.2**. ^{1}H-NMR (400 MHz , $CDCl_3$) 4.44 (*dt*, $^2J_{HF}$ = 47.4, 2 H); 3.67 (*s*, 3 H); 2.503, 2.496 (unresolved, overlapping *t*, *J* = 7.4, 4 H); 2.30 (*t*, *J* = 7.5, 2 H); 1.53- 1.80 (*m,* 8 H); 1.25-1.45 (*m,* 16 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 174.34; 84.16 (*d*, *J* = 164.1); 51.46; 34.22; 32.22; 32.15; 30.35 (*d*, *J* = 19.6); 29.72; 29.60; 29.33; 29.19; 29.19; 29.13; 28.92; 28.87; 28.79; 25.08 (*d*, *J* = 5.4); 24.95. ^{19}F NMR (376.5 MHz, $CDCl_3$): -218.30. EI-MS: 334 (40, M^+), 303 (37, $[M - 31]^+$), 185 (69, $[(CH_2)_9CO_2CH_3]^+$), 149 (45, $[F(CH_2)_7S]^+$), 129 (18, $[CH_2{=}CH(CH_2)_7S]^+$) 95 (37) 87 (69), 55 (100), 41 (69). HR-EI-MS: 334.2329 ($C_{18}H_{35}O_2FS$, $[M]^+$; calc. 334.2342).

3.2.10 10-Thiodecanoic acid



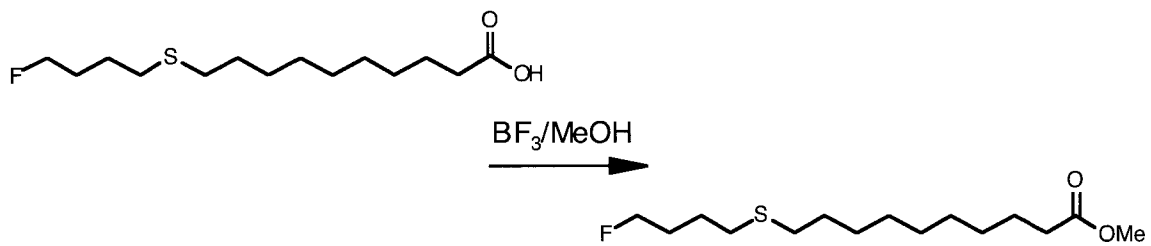
A solution of 10-bromo-decanoic acid (2.48 g, 9.87 mmol) and thiourea (0.82 g, 10.8 mmol) 95% ethanol (50 mL) was refluxed for 3 hours under N_2, after which the reaction mixture was cooled to room temperature and a solution of 2 M NaOH (30 mL) added. After an additional 1.5 hours at reflux temperature, the reaction mixture was cooled and diluted with dH_2O (50 mL). The cloudy solution was transferred to a separatory funnel and dripped into a stirring solution of 6 M H_2SO_4 (36 mL) and CH_2Cl_2 (80 mL). The organic phase was separated and set aside while the aqueous fraction was extracted with CH_2Cl_2 (3 x 50 mL). The combined organic extracts were washed with 25 mL of acidified (3 drop of 6M H_2SO_4) saturated NaCl, dried over anhydrous Na_2SO_4 and concentrated in *vacuo* to give a colourless solid (1.9 g). ^{1}H-NMR (200 MHz, $CDCl_3$) δ 10.51 (s, 1 H, COO**H**); 3.73 (q, 2 H, HS-**CH_2**-); 2.34 (t, 2 H, -**CH_2**-COOH); 1.10-1.75 (m, HS-CH_2 –**$(CH_2)_7$**-CH_2-COOH).

3.2.11 15-Fluoro-11-thiapentadecanoic Acid



To a stirred solution of 10-thiodecanoic acid (1.58 g, 7.73 mmol) in freshly distilled THF (40 mL) and HMPA (8 mL), 1.6 M n-butyl lithium (10.6 mL, 2.2 eq.) was added under N_2 and 0 °C. After 30 minutes, 4-bromo-1-fluorobutane (~1.2 g, 7.74 mmol) was added and the cloudy beige solution stirred at room temperature overnight (ca. 17 hours). The reaction was quenched with dH_2O (60 mL) followed by 3 M HCl (60 mL). The solution was extracted with hexanes (4 x 40 mL) and the combined organic phase dried over anhydrous Na_2SO_4 and concentrated in *vacuo* to give a colourless solid (1.51 g). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.74 (broad s, R-COO**H**), 4.48 (dt, $^2J_{HF}$ = 48.0, F-C**H**$_2$-R); 2.56 (t, 2 H, -C**H**$_2$-S-CH$_2$-); 2.51 (t, 2 H, -S-C**H**$_2$-CH$_2$-); 2.34 (t, 2 H, R-C**H**$_2$-COOH); 1.50-1.88 (m, 4 H, F-CH$_2$-(**CH**$_2$)$_2$-CH$_2$-S-R), 1.30 (m, 14 H, R-S-CH$_2$-(**CH**$_2$)$_7$-CH$_2$-COOH).

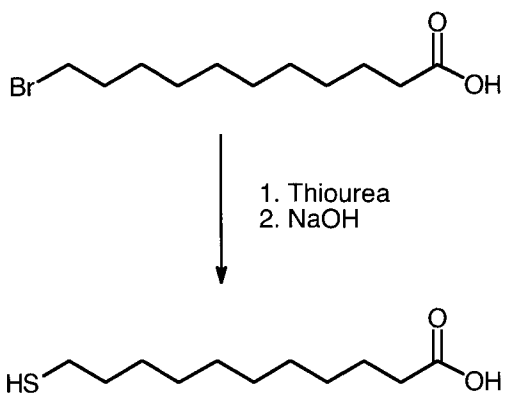
3.2.12 Methyl-15-Fluoro-11-thiapentadecanoate



A solution containing crude 15-fluoro-11-thiapentadecanoic acid (1.47 g), methanol (100 mL) and BF_3 etherate (20 mL) was refluxed under N_2 for 4 hours. The solution was cooled to room temperature and quenched with dH_2O (100 mL). The reaction mixture was extracted with CH_2Cl_2 (4 x 25 mL) and the combined organic

extracts were washed with saturated NaCl (2 x 25 mL), dried over anhydrous Na_2SO_4 and concentrated in *vacuo* to give an orange solid (1.38 g). This material was purified by flash chromatography (10% ethyl acetate/hexanes) to give a colourless oil (178 mg). R_f = 0.38 (SiO_2, hexane/EtOAc 90:10); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 4.47 (dt, $^2J_{HF}$ = 47.3, $^3J_{HH}$ = 7.5, 2 H, -CH_2F), 3.67 (*s*, 3 H), 2.56 (*t*, $^3J_{HH}$ = 7.0, 2 H), 2.50 (*t*, $^3J_{HH}$ = 7.4, 2 H), 2.30 (*t*, $^3J_{HH}$ = 7.5, 2 H), 1.53 - 1.86 (*m*, 8 H), 1.25-1.40 (*m*, 10 H); ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ 174.33, 83.68 (*d*, $^1J_{CF}$ = 164.8), 51.46, 34.09, 32.06, 31.66, 29.64, 29.55 (*d*, $^2J_{CF}$ = 19), 29.31, 29.19, 29.17, 29.11, 28.88, 25.39 (*d*, $^3J_{CF}$ = 4.7), 24.93; ^{19}F NMR (376.5 MHz, $CDCl_3$) δ -218.82; IR (film): 2928, 2855, 1740 (C=O), 1436,1436, 1253, 1197, 1172 cm^{-1}; EI-MS: *m/z* 292 (22, M^+), 261 (17, [M - 31]$^+$), 185 (56, [$(CH_2)_9CO_2CH_3$]$^+$), 121 (25), 87 (61, [CH_2=$CH(CH_2)_2S$]$^+$), 74 (64), 55 (100); HR-EI-MS *m/z* 292.1859 ([M]$^+$, $C_{15}H_{29}O_2FS$; calc. 292.1872).

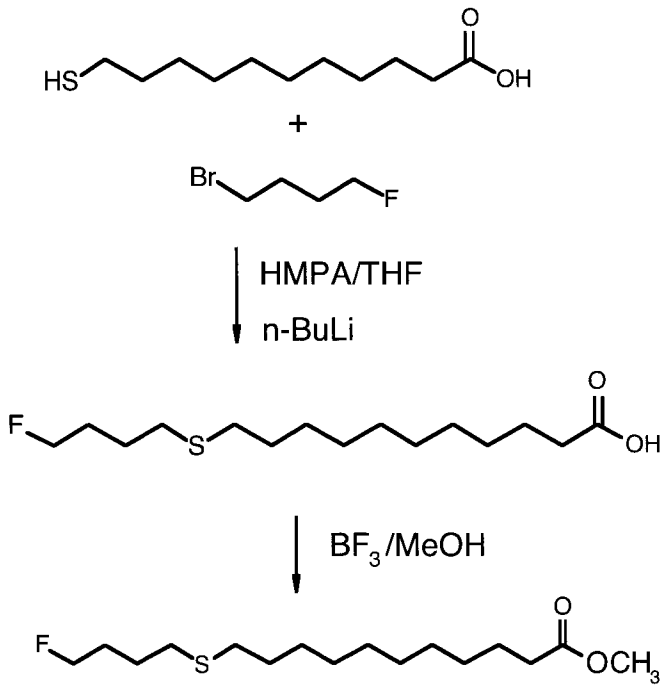
3.2.13 11-Thioundecanoic Acid



The title compound was obtained by a procedure similar to that described in **3.2.10**, as a crude colourless solid (7.27g). ^{1}H NMR (200 MHz): δ 1.28 & 1.60 (b, 16H, R-(CH_2)$_8$-(CH_2)-SH); 2.35 (t, 2H, R-C**H**$_2$-COOH); 2.52 (q, 2H, SH-C**H**$_2$-(CH_2)$_8$-R);

11.45 (b, 1H, R-COO**H**). ^{13}C NMR (75.5 MHz): δ 24.7; 28.4; 29.0; 29.2; 29.3; 29.4; 34.0; 180 (C1).

3.2.14 Methyl 16-Fluoro-12-Thiahexadecanoate

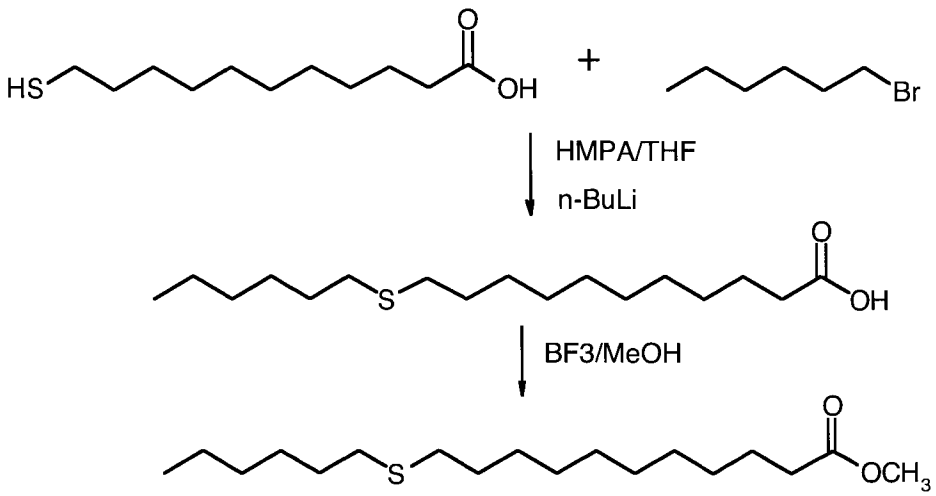


The title compound was obtained in a manner similar to that described in **3.2.10-3.2.12** as a colourless oil at room temperature (1.11 g, 2.6 mmol).

TLC (hexane/AcOEt 90:10): R_f 0.33. M.p. 18-20°. IR (film): 2926, 2854, 1739, 1172, 1035. ^{1}H NMR (400 MHz): δ 1.27-1.35 (m, 14 H, CH_3OCO-(CH_2)-$(C\mathbf{H}_2)_7$-(CH_2)-S-R); 1.59 (m, 2 H, CH_3OCO-(CH_2)-$C\mathbf{H}_2$-$(CH_2)_4$-CH_2-S-R); 1.72 (m, 2 H, R-S-CH_2-$C\mathbf{H}_2$-CH_2-CH_2F); 1.83 (m, 2 H, R-$C\mathbf{H}_2$-CH_2F); 2.29 (t, 2 H, CH_3OCO-$C\mathbf{H}_2$-R); 2.49 (t, 2 H, R-$C\mathbf{H}_2$-S-$(CH_2)_3$-CH_2F); 2.55 (t, 2 H, R-CH_2-S-$C\mathbf{H}_2$-$(CH_2)_2$-CH_2F); 3.66 (s, 3 H, $C\mathbf{H}_3OCO$-R); 4.46 (dt $^2J_{HF}$ = *47.3 Hz*, 2 H, R-$C\mathbf{H}_2F$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.33 (C1); 34.08; 24.92; 28.88; 29.10; 29.19; 29.19; 29.34; 29.42; 29.63; 32.05; 31.63; 25.38 (C14,

d, $^{3}J_{CF}$ = 4.8 Hz); 29.52 (C15, d, $^{2}J_{CF}$ = 21.0); 83.67 (C16, d, $^{1}J_{CF}$ = 164.9 Hz); 51.43 (Me). ^{19}F NMR (282 MHz): -219.81 EI-MS: 306 (M^+), 275 (M^+-OCH_3), 199 ($CH_3OCO(CH_2)_{10}^+$), 245, 121, 55. HR-EI-MS: 306.2023 (M^+, $C_{18}H_{36}O_2S$; calc. 306.2029).

3.2.15 Methyl 12-thiaoctadecanoate

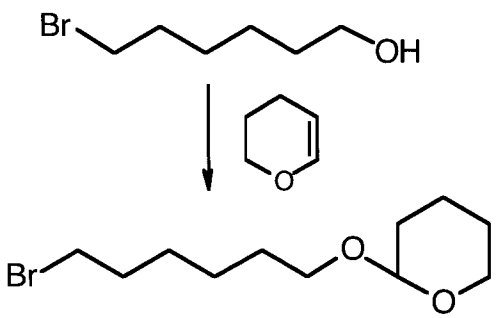


The non-fluorinated thia fatty acid was synthesized similar to that described in 3.2.14. Crude methyl ester product was purified by flash chromatography using 20% ethyl acetate/hexanes to yield a colourless oil (0.47 g, 1.48 mmol).

TLC (hexane/AcOEt 90:10): R_f 0.46. M.p. 12-14°. IR (film): 2925, 2854, 1742, 1171. ^{1}H NMR (400 MHz): δ 0.89 (t, 3 H, C**H**$_3$-CH_2-R), 1.28-1.42 (m, 14 H, CH_3OCO-$(CH_2)_2$-($C\mathbf{H}_2$)$_7$-(CH_2)-S-R); 1.60 (m, 6 H, CH_3OCO-CH_2-C**H**$_2$-$(CH_2)_6$-C**H**$_2$-CH_2-S-CH_2-C**H**$_2$-R); 2.30 (t, 2 H, CH_3OCO-C**H**$_2$-R); 2.501 or 2.498 (t, 2 H, R-C**H**$_2$-S-$(CH_2)_3$-CH_2F); 2.501 or 2.498 (t, 2 H, R-CH_2-S-C**H**$_2$-$(CH_2)_2$-CH_2F); 3.67 (s, 3 H, C**H**$_3$OCO-R). ^{13}C NMR (100 MHz): δ 174.35; 34.11; 24.95; 29.23; 29.38; 29.46; 29.14; 29.23; 28.95; 29.71; 29.73;

32.20; 32.18; 28.66; 31.49; 22.58; 14.06; 51.46. EI-MS: 316 (M^+), 285 (M^+-OCH_3), 199 ($CH_3OCO(CH_2)_{10}^+$), 117, 55. HR-EI-MS: 316.2424 (M^+, $C_{18}H_{36}O_2S$; calc. 316.2436).

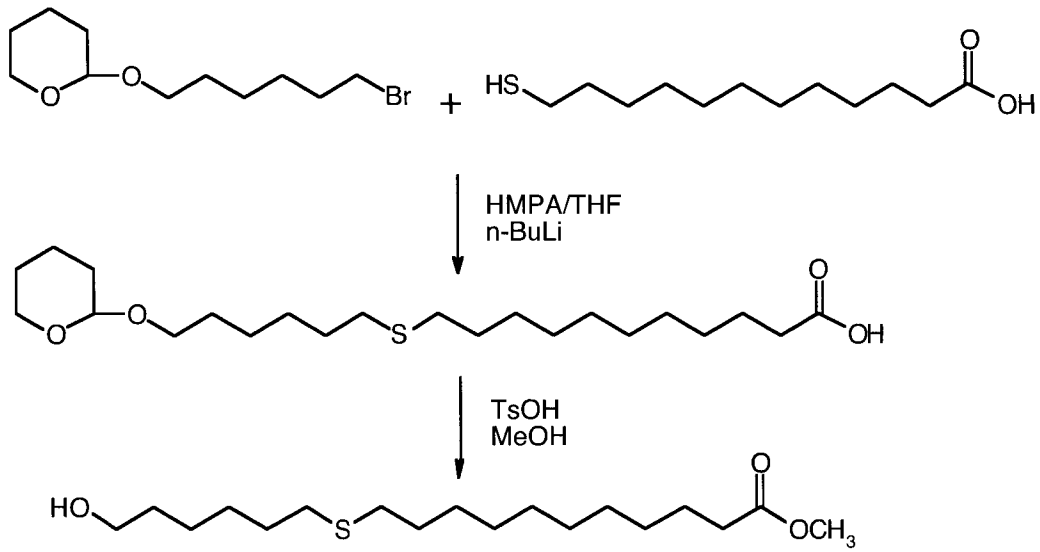
3.2.16 6-Bromo-hexanol-tetrahydropyranyl (THP) ether



A solution of 6-bromo-hexan-1-ol (3.0 g, 16.6 mmol) and ca. 2 mL of DHP (slightly acidified with 3 droplets of conc. HCl) was stirred under N_2 for 4 hours at room temperature. A spatula tip of $NaHCO_3$ was added at the end of the reaction and the reaction mixture was dissolved in CH_2Cl_2 (5 mL). The organic mixture was dried over Na_2SO_4 and concentrated *in vacuo* to produce a dark brown liquid (3.9 g, 14.7 mmol est). This material was used directly in the next step of the synthesis without purification.

^{1}H NMR (200 MHz): δ 1.37-1.87 (m, 14 H, Br-CH_2-($C\mathbf{H}_2$)$_4$-CH_2-O-THP[($C\mathbf{H}_2$)$_3$]); 3.41 (t, 2 H, Br-$C\mathbf{H}_2$-R); 3.72 (t, 2 H, THP-O-$C\mathbf{H}_2$-$(CH_2)_4$-CH_2Br); 3.86 (m, 2 H, THP[-O-$C\mathbf{H}_2$-R]); 4.56 (m, 1 H-axial, R-O-C**H**-THP); 4.95 (m, 1 H-equatorial, R-O-C**H**-THP).

3.2.17 Methyl 18-Hydroxy-12-Thiaoctadecanoate

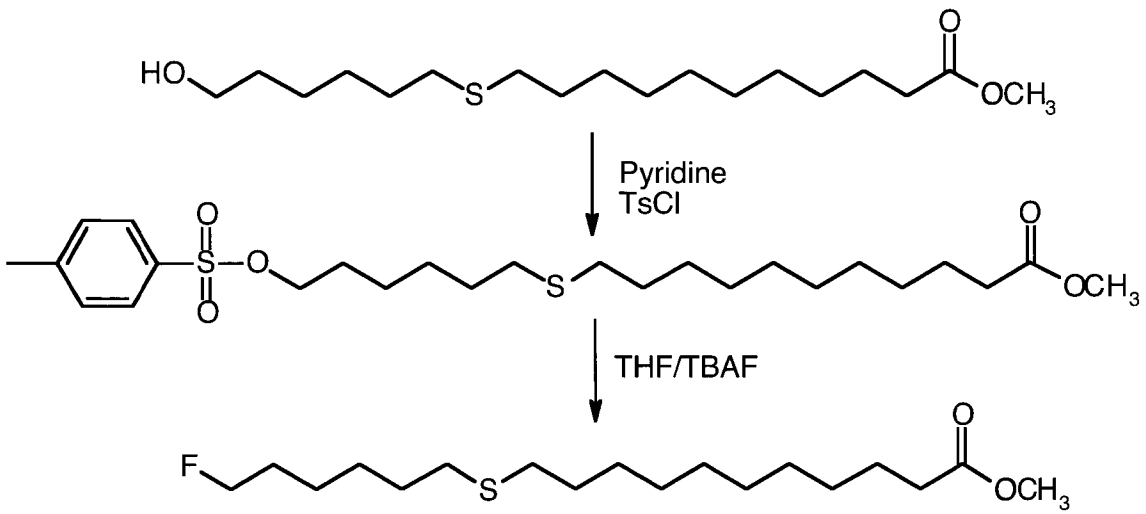


The coupling synthesis was carried out in a manner similar to that described in **3.2.11** using commercially available 11-thioundecanoic acid (2.1 g, 9.6 mmole) and crude THP ether (2.54 g, 9.58 mmole est.). The coupled THP ether (3.09 g) was deprotected by treatment with a solution of p-toluene sulfonic acid monohydrate (0.12 g, 0.70 mmol) in 30 mL of dry methanol. The mixture was refluxed under N_2 for 1 hour. The cooled solution was quenched with dH_2O (35 mL) and extracted with CH_2Cl_2 (3 x 30 mL). The crude product (2.50 g) was purified by flash chromatography (20% AcOEt/hexanes) to give a white solid (0.48 g, 1.45 mmol, 15% yield based on thioacid).

TLC (hexane/AcOEt 40:60): R_f 0.58. IR (film, cm^{-1}): Similar to that of 3.2.1. ^1H NMR (400 MHz): δ 1.28-1.46 (m, 18 H, $CH_3OCO\text{-}CH_2\text{-}(C\mathbf{H}_2)_6\text{-}(CH_2)_2\text{-}S\text{-}(CH_2)_2\text{-}(C\mathbf{H}_2)_3\text{-}R$); 1.58 (m, 6 H, $CH_3OCO\text{-}CH_2\text{-}C\mathbf{H}_2\text{-}(CH_2)_6\text{-}C\mathbf{H}_2\text{-}CH_2\text{-}S\text{-}CH_2\text{-}C\mathbf{H}_2\text{-}R$); 2.30 (t, 2 H, $CH_3OCO\text{-}C\mathbf{H}_2\text{-}R$); 2.51 & 2.49 (partially resolved overlapping t, 4 H, $R\text{-}C\mathbf{H}_2\text{-}S\text{-}C\mathbf{H}_2\text{-}R$); 3.65 (t, 2 H, $R\text{-}C\mathbf{H}_2OH$); 3.67 (s, 3 H, $C\mathbf{H}_3OCO\text{-}R$). ^{13}C-NMR (100 MHz, $CDCl_3$): δ

174.37, 34.12, 24.96, 25.40, 62.92, 28.69, 28.93, 29.14, 29.23, 29.38, 29.45, 29.64, 29.72, 32.66, 32.21, 51.46.

3.2.18 Methyl 18-Fluoro-12-thiaoctadecanoate

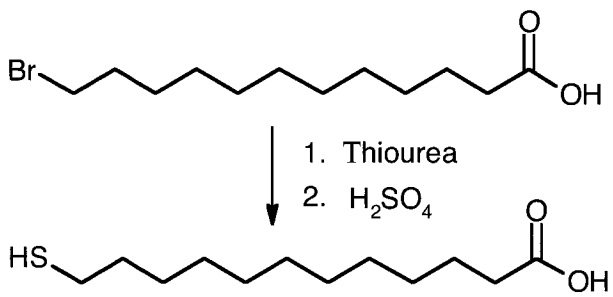


The ω-hydroxy product (0.483 g, 1.45 mmol), prepared as described in **3.2.17**, was dissolved in pyridine (ca. 2 mL) and 0.3 g of freshly crystallized TsCl was added to this solution. After stirring under N_2 for 3 hours, the reaction mixture was quenched with dH_2O (20 mL) and extracted with ether (3 x 30 mL). The combined organic extracts were washed with 3 M HCl (20 mL), sat. NaCl (25 mL), dried over Na_2SO_4 and concentrated in vacuo producing a colourless solid (0.47 g, 0.96 mmol est). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.79 (d, 2 H); 7.35 (d, 2 H); 4.02 (t, 2 H, -C**H**$_2$O-SO_4-Tol); 3.67 (s, 3 H, -OC**H**$_3$); 2.48 (t, 4 H, -C**H**$_2$-S-C**H**$_2$-); 2.45 (s, 3 H, -C_6H_5-C**H**$_3$); 2.30 (t, 2 H, -C**H**$_2$-CO_2CH_3); 1.68-1.25 (m, 24 H, $CH_3CO_2CH_2$(C**H**$_2$)$_8$$CH_2SCH_2$(C**H**$_2$)$_4$$CH_2OTs$).

The tosylate (0.47 g, 0.96 mmole est) was added to a stirred solution of TBAF (1.8 g, 5.7 mmol) in THF (10 mL). After 3 hours of stirring under N_2, the reaction was diluted with 10 mL dH_2O and extracted with hexanes (3 x 20 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated *in vacuo*. The fluorinated

product was purified by flash chromatography (5% AcoEt/hexanes) yielding a colourless oil (135.1 mg, 0.40 mmol, 27.6 % overall yield based on methyl 18-hydroxy-12-thiaoctadecanoate). TLC (hexane/AcOEt 90:10): R_f 0.36. IR (film) 2925 & 2854 (C-H stretch), 1742 (C=O stretch), 1171 (C-O stretch). 1H NMR (300 MHz): δ 1.28-1.45 (m, 18 H, CH_3OCO-$(CH_2)_2$-$(CH_2)_6$-$(CH_2)_2$-S-$(CH_2)_2$-$(CH_2)_3$-CH_2F); 1.60 (m, 6 H, CH_3OCO-CH_2-CH_2-$(CH_2)_6$-CH_2-CH_2-S-CH_2-CH_2-R); 2.30 (t, 2 H, CH_3OCO-CH_2-R); 2.50 (partially resolved overlapping t, 2 H, CH_3OCO-$(CH_2)_9$-CH_2-S-R); 2.51 (partially resolved overlapping t, 2 H, R-S-CH_2-$(CH_2)_4$-CH_2F); 3.67 (s, 3 H, CH_3OCO-R); 4.44 (dt $^2J_{HF}$ = 48 Hz, 2 H, -CH_2F). ^{13}C NMR (100 MHz): δ 174.36, 34.11, 24.95, 29.23, 29.37, 29.45, 29.14, 29.23, 28.94, 29.71, 32.21, 32.05, 29.61, 28.51, 24.88, 30.32, 84.08 ($^1J_{CF}$ 164.2 Hz). ^{19}F NMR (282 MHz): -218.44. EI-MS: 334 (M^+), 303 ([M - OCH_3]$^+$), 199 (CH_3OCO-$(CH_2)_{10}^+$), 135 ([S-$(CH_2)_5CH_2F$]$^+$), 55. HR-EI-MS: 334.2361 (M^+, $C_{18}H_{35}O_2SF$; calc. 334.2342).

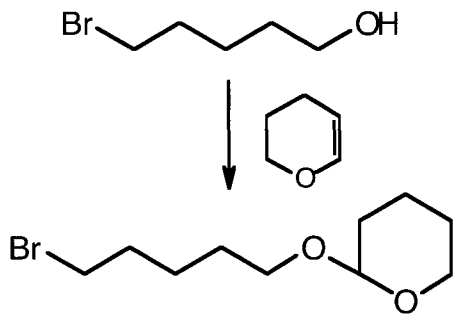
3.2.19 12-Thiododecanoic acid



Similar to 3.2.1 giving a colourless solid (4.1 g, 17.6 mmol), a. 99% crude recovery.

^{1}H NMR (300 MHz): δ 1.26 & 1.57 (b, 16 H, R-$(CH_2)_9$-(CH_2)-SH), 2.32 (t, 2 H, R-CH_2-COOH), 2.50 (q, 2 H, SH-CH_2-$(CH_2)_8$-R), 11.30 (b, 1 H, R-COOH).

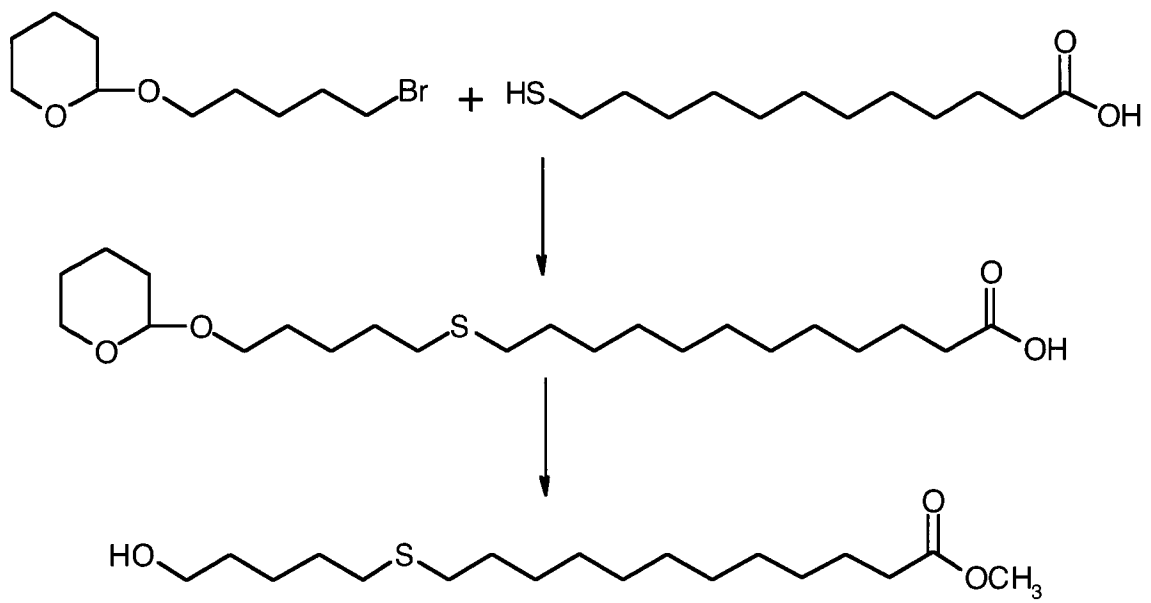
3.2.20 5-Bromo-1-pentanol-tetrahydropyranyl (THP) ether



Similar to 3.2.7 giving a dark brown liquid (8.75 g, 35 mmol).

^{1}H NMR (300 MHz, $CDCl_3$): δ, 1.47-2.01 (m, Br-CH_2-(CH_2)$_3$-CH_2-OTHP[(CH_2)$_3$]), δ 3.42 (t, 2 H, BR-CH_2-R) , δ 3.78 (dt, 2 H, R-THP[R-CH-OCH_2-R], δ 3.88 (m, BrCH_2-(CH_2)$_3$-CH_2-OTHP), δ 4.58 (m, 1 H-axial, R-O-CH-THP), δ 4.95 (m, 1 H-equatorial, R-O-CH-THP).

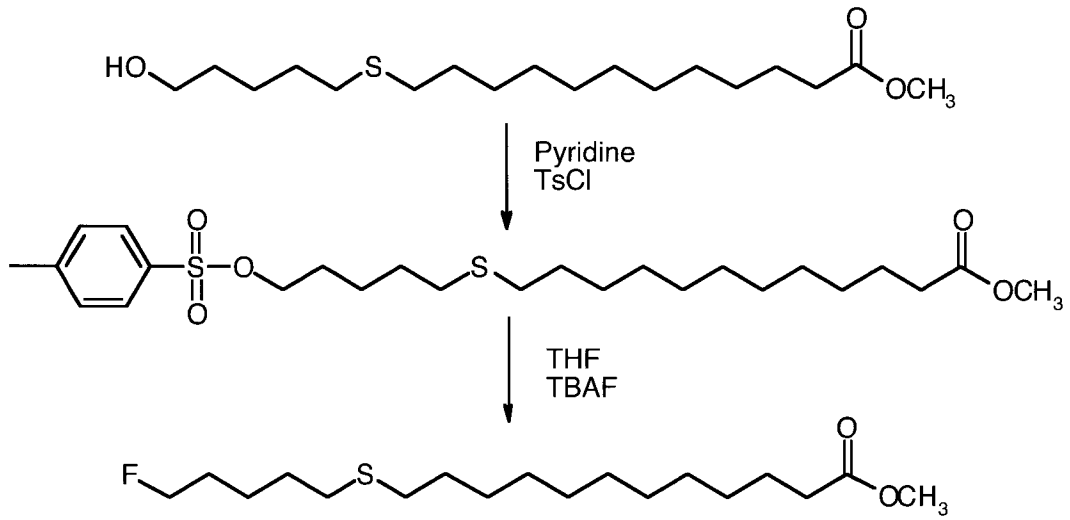
3.2.21 Methyl 18-Hydroxy-13-thiaoctadecanoate



Coupling and deprotection synthesis similar to 3.2.8 with purification by flash chromatography (20 % AcOEt/hexanes) to give a colourless solid (0.45 g, 1.35 mmol).

TLC (hexane/AcOEt 40:60): Rf 0.66. IR (film, cm^{-1}): Similar to that of 3.2.1. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.27-1.47 (m, 18 H, CH_3OCO-$(CH_2)_2$-$(CH_2)_7$-$(CH_2)_2$-S-$(CH_2)_2$-$(CH_2)_2$-CH_2OH), δ 1.60 (m, 6 H, CH_3OCO-CH_2-CH_2-$(CH_2)_7$-CH_2-CH_2-S-CH_2-CH_2-R), δ 2.30 (t, CH_3OCOCH_2-R), 2.51 (overlapping t, 4 H, R-CH_2-S-CH_2-R), δ 3.65 (t, 2 H, R-CH_2OH), δ 3.67 (s, 3 H, CH_3OCO-R). ^{13}C NMR (75 MHz, $CDCl_3$): δ 62.82; 51.47; 34.12 (C2), 32.35 (C12 or C14), 32.20 (C12 or C14), 32.07 (C17), 29.71, 29.51, 29.49, 29.46, 29.24, 29.15, 28.94, 25.06, 24.96.

3.2.22 Methyl 18-Fluoro-13-thiaoctadecanoate



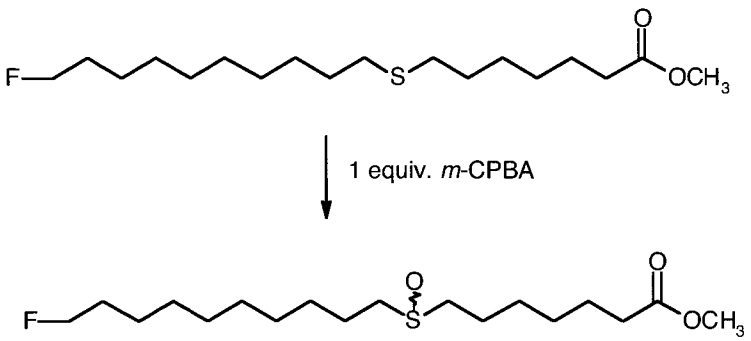
Synthesis of fluorinated fatty acid was similar to that described in 3.2.9. Purification by flash chromatography (10% AcOEt/hexanes) gave a colourless liquid (105 mg, 0.31 mmol) at room temperature.

TLC (hexane/AcOEt 90:10): R_f 0.38. IR (film, cm^{-1}): 2926 & 2854 (C-H stretch), 1740 (C=O stretch), 1435 (C-H bend), 1171 (C-O stretch). ^1H NMR (300 MHz, $CDCl_3$): δ 1.27 (b, 18 H, CH_3OCO-$(CH_2)_2$-$(CH_2)_7$-$(CH_2)_2$-S-$(CH_2)_2$-$(CH_2)_2$-CH_2F), δ 1.45-1.78 (m, 6 H, CH_3OCO-CH_2-CH_2-$(CH_2)_7$-CH_2-CH_2-S-CH_2-CH_2-R), δ 2.30 (t, 2H, CH3OCO-CH_2-R), δ 2.52 & 2.55 (overlapping t, 4 H, R-CH_2-S-CH_2-R'), δ 3.66 (s, 3 H, CH_3OCO-R), δ 4.44 (dt J = 47.4 Hz, 2 H, R-CH_2F). ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.26 (C1), 34.06 (C2), 24.91 (C3), 29.10, 29.20, 29.27, 29.37, 29.45, 29.47, 29.67, 28.89, 32.33 (C12), 32.08 (C14), 24.52 (C16, $^3J_{CF}$ = 5.3 Hz), 30.16 (C17, $^2J_{CF}$ = 19.6 Hz), 83.97 (C18, $^1J_{CF}$ = 163.6). ^{19}F NMR (282 MHz, $CDCl_3$): -218.43. EI-MS: *m/z* 334 (M^+), 303 ([M – OCH_3]$^+$), 259 (CH_3OCO-$(CH_2)_{11}SCH_2^+$), 213 (CH_3OCO-$(CH_2)_{11}^+$). HR-EI-MS: 334.2337 (M+, $C_{18}H_{35}O_2SF$; calc. 334.2342).

3.3 Synthesis of S-Oxides

The following section describes the oxidation of methyl 18-fluoro-8-thiaoctadecanoate using *m*-CPBA. The same procedure was used to oxidize the other thia-fatty acid methyl esters.

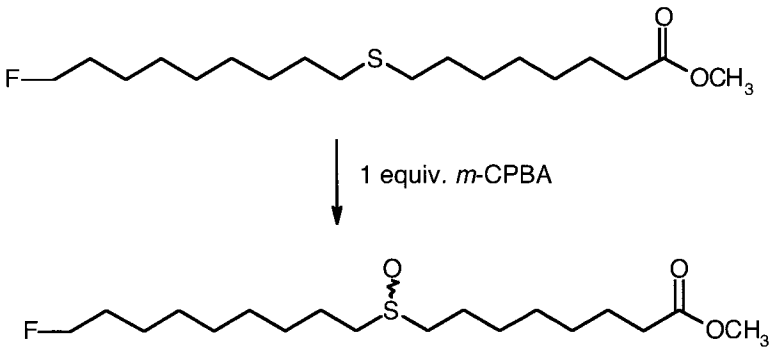
3.3.1 (*R,S*)-Methyl 18-Fluoro-8-thiaoctadecanoate, S-Oxide



To a soln of methyl ω-fluoro-8-thiaoctadecanoate (16.6 mg, 0.044 mmol) in CH_2Cl_2 (1 ml) was added *meta*-chloroperbenzoic acid (0.5 equiv., 8.0 mg) at 0°. After standing at 0° for 30 min, the reaction mixture was filtered to remove a precipitated white solid (*meta*-chlorobenzoic acid) and the filtrate washed with 3M NaOH (2 x 2.5 ml), dried (Na_2SO_4) and evaporated to give the crude sulfoxide which was purified by recrystallization from EtOAc/hexanes to give the title compound (6.4 mg, 42 %) as a white solid.

TLC (EtOAc): R_f 0.40. M.p. 66-66.5° (amorphous crystals, recrystallized from hexane/EtOAc). IR (KBr): 2921, 2850, 1737, 1464,1437, 1254, 1207, 1174, 1011. ^{1}H-NMR (400 MHz , $CDCl_3$): 4.44 (*dt*, $^2J_{HF}$ = 47.4, 2 H); 3.67 (*s*, 3 H); 2.57-2.73 (*m*, 4 H); 2.32 (*t*, *J* = 7.5, 2 H); 1.78,1.76 (overlapping *m*, 4 H); 1.6-1.68 (*m*, 4H), 1.24-1.56 (*m*, 16 H). ^{13}C-NMR (100 MHz , $CDCl_3$): 174.07; 84.23 (*d*, $^1J_{CF}$ = 164.0); 52.51; 52.32; 51.53; 33.90; 30.40 (*d*, $^2J_{CF}$ = 19.3); 29.38; 29.24; 29.18; 29.18; 28.88; 28.71; 28.54; 25.14 (*d*, $^3J_{CF}$ = 5.4); 24.64; 22.62; 22.48. ^{19}F NMR (376.5 MHz, $CDCl_3$): -218.23. EI-MS: 333 (100, $[M - OH]^+$), 303(3), 191(18), 160 (57), 143 (32, $[(CH_2)_6CO_2CH_3]^+$), 111 (32), 55 (95). HR-EI-MS: 333.2263 ($C_{18}H_{34}O_2FS$, $[M - OH]^+$; calc. 333.2264).

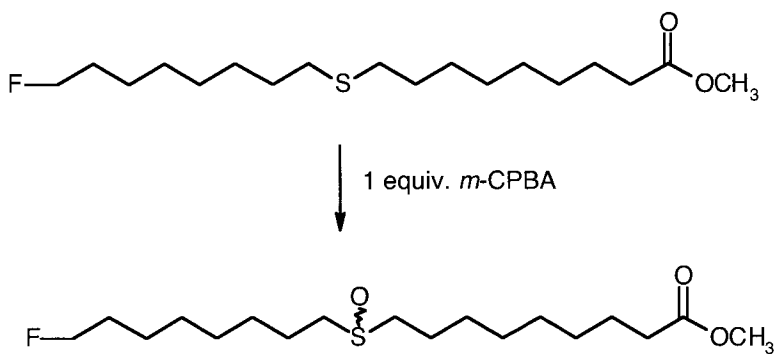
3.3.2 (*R,S*)-Methyl 18-Fluoro-9-thiaoctadecanoate, S-Oxide



Title compound obtained in a similar manner to that of 3.3.1.

TLC (EtOAc): R_f 0.40. M.p. 66-66.5° (amorphous crystals, recrystallized from hexane/EtOAc). IR (KBr): similar to that of 3.3.1. ^{1}H-NMR (400 MHz , $CDCl_3$): 4.44 (*dt*, $^2J_{HF}$ = 47.4, 2 H); 3.67 (*s*, 3 H); 2.57-2.73 (*m*, 4 H); 2.31 (*t*, *J* = 7.5, 2 H); 1.78, (*m*, 4 H); 1.6-1.68 (*m*, 4H), 1.24-1.56 (*m*, 16 H). ^{13}C-NMR (100 MHz , $CDCl_3$): 174.20; 84.20 (*d*, $^1J_{CF}$ = 164.0); 52.48; 52.41; 51.42; 33.99; 30.38 (*d*, $^2J_{CF}$ = 19.2); 29.23; 29.11; 28.86; 28.86; 28.85; 28.85; 28.69; 25.13 (*d*, $^3J_{CF}$ = 5.4); 24.80; 22.62; 22.57. ^{19}F NMR (376.5 MHz, $CDCl_3$): -218.25. EI-MS: 333 (26, $[M - OH]^+$), 303 (8), 177 (49), 174 (16), 157 (73, $[(CH_2)_7CO_2CH_3]^+$), 125 (41), 55 (100). HR-EI-MS: 333.2256 ($C_{18}H_{34}O_2FS$, $[M - OH]^+$; calc. 333.2264).

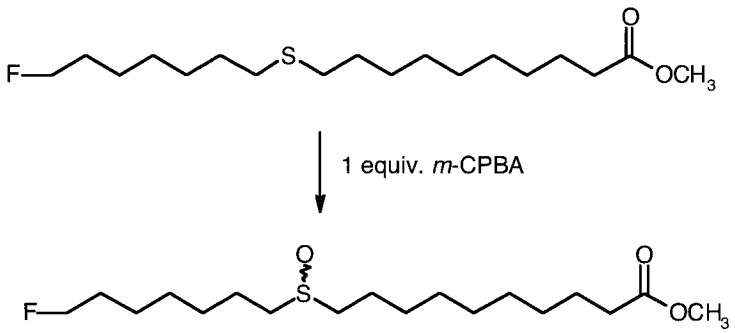
3.3.3 (*R,S*)-Methyl 18-Fluoro-10-thiaoctadecanoate, S-Oxide



Title compound obtained in a similar matter to that of 3.3.1.

TLC (EtOAc): R_f 0.41. M.p. 65.5-66.5° (amorphous crystals, recrystallized from hexane/EtOAc). IR (KBr): similar to that of 3.3.1. ^{1}H-NMR (400 MHz , $CDCl_3$): 4.44 (*dt*, $^2J_{HF}$ = 47.4, 2 H); 3.67 (*s*, 3 H); 2.57-2.73 (*m*, 4 H); 2.30 (*t*, *J* = 7.5, 2 H); 1.77,1.76 (two overlapping *m*, 4 H); 1.6-1.68 (*m*, 4H), 1.24-1.56 (*m*, 16 H). ^{13}C-NMR (100 MHz , $CDCl_3$): 174.27; 84.14 (*d*, $^1J_{CF}$ = 164.3); 52.64; 52.44; 51.48; 34.05; 30.35 (*d*, $^2J_{CF}$ = 19.2); 29.11; 29.01; 29.01; 28.99; 28.96; 28.82; 28.79; 28.69; 25.10 (*d*, $^3J_{CF}$ = 5.4); 24.87; 22.61; 22.61. ^{19}F NMR (376.5 MHz, $CDCl_3$): -218.37. EI-MS: 333 (26, $[M - OH]^+$), 303 (9), 188(12), 171 (49, $[(CH_2)_8CO_2CH_3]^+$), 163 (26), 139 (30), 55 (100). HR-EI-MS: 333.2247 ($C_{18}H_{34}O_2FS$, $[M - OH]^+$; calc. 333.2264).

3.3.4 (*R*,*S*)-Methyl 18-Fluoro-11-thiaoctadecanoate, S-Oxide

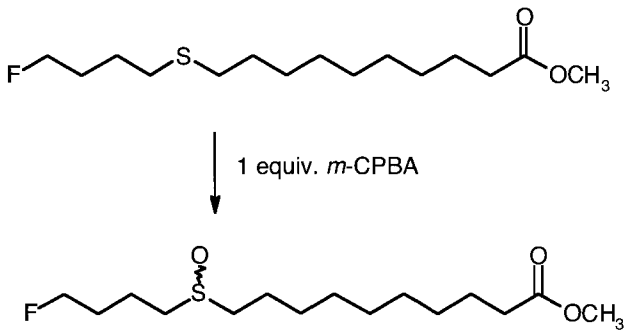


Title compound obtained in a similar matter to that of 3.3.1.

TLC (EtOAc): R_f 0.41. M.p. 59-60° (amorphous crystals, recrystallized from hexane/EtOAc). IR (KBr): similar to that of 3.3.1. ^{1}H-NMR (400 MHz , $CDCl_3$): 4.44 (*dt*, $^2J_{HF}$ = 47.3, 2 H); 3.67 (*s*, 3 H); 2.57-2.73 (*m*, 4 H); 2.30 (*t*, *J* = 7.5, 2 H); 1.78,1.76 (two overlapping *m*, 4 H); 1.58-1.68 (*m*, 4H); 1.58-1.68 (*m*, 4H); 1.38-1.52 (*m*, 8 H); 1.24-1.38 (*m*, 8 H). ^{13}C-NMR (176 MHz , $CDCl_3$): 174.34; 84.07 (*d*, $^1J_{CF}$ = 164.4); 52.50; 52.38; 51.49; 34.09; 30.29 (*d*, $^2J_{CF}$ = 19.2); 29.15; 29.14; 29.08; 28.86; 28.86; 28.78; 28.69; 25.01 (*d*, $^3J_{CF}$ = 5.7); 24.92; 22.62; 22.57.^{19}F NMR (376.5 MHz, $CDCl_3$): -

218.46. ^{19}F NMR (376.5 MHz, $CDCl_3$): -218.37. EI-MS: 333 (44, $[M - OH]^+$), 303 (3), 202 (25), 185 (6, $[(CH_2)_9CO_2CH_3]^+$), 153 (19), 149 (7), 55 (100). HR-EI-MS: 333.2271 ($C_{18}H_{34}O_2FS$, $[M - OH]^+$; calc. 333.2264).

3.3.5 (*R,S*)-Methyl 15-Fluoro-11-thiapentadecanoate, S-Oxide



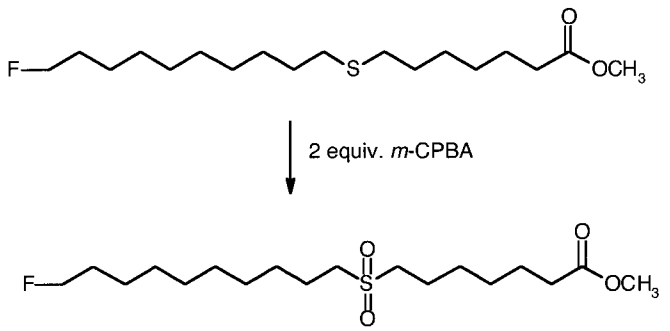
Title compound obtained in a similar matter to that of 3.3.1.

TLC (AcOEt) R_f 0.13. IR (KBr, cm^{-1}): 2920 & 2848 (C-H stretch), 1731 (C=O stretch), 1176 (C-O stretch). ^1H NMR (400 MHz): δ 1.25-1.98 (m, 18 H, $CH_3OCO-CH_2-(CH_2)_7-CH_2-S=O-CH_2-(CH_2)_2-CH_2F$), δ 2.30 (t, 2 H, $CH_3OCO-CH_2-R$), δ 2.59-2.75 (m, 4 H, $R-CH_2-S=O-CH_2-R'$), δ 3.67 (s, 3 H, CH_3OCO-R), δ 4.50 (dt $^2J_{HF}$ = 47.8 Hz, 2 H, $R-CH_2F$). ^{13}C NMR (100 MHz): δ 174.33, 83.4 (d, $^1J_{CF}$ = 165.6), 52.5, 51.8, 51.5, 34.1, 29.5 (d, $^2J_{CF}$ = 20.0), 29.12, 29.06, 28.82, 24.9, 22.6, 19.08 (d, $^3J_{CF}$ = 4.7). ^{19}F NMR (376 MHz): -219.24. EI-MS: m/z 291 ($[M-OH]^+$), 277 ($[M-OCH3]^+$), 69, 55. HR-EI-MS m/z 291.1787 ($[M-OH]^+$, $C_{15}H_{28}O_2FS$; calc. 291.1794).

3.4 Synthesis of Sulfones

The following section describes the oxidation of methyl 18-fluoro-8-thiaoctadecanoate using *m*-CPBA to the corresponding sulfone product. The same procedure was used to oxidize the other thia-fatty acid methyl esters.

3.4.1 Methyl 18-fluoro-8-thiaoctadecanoate S, S-dioxide

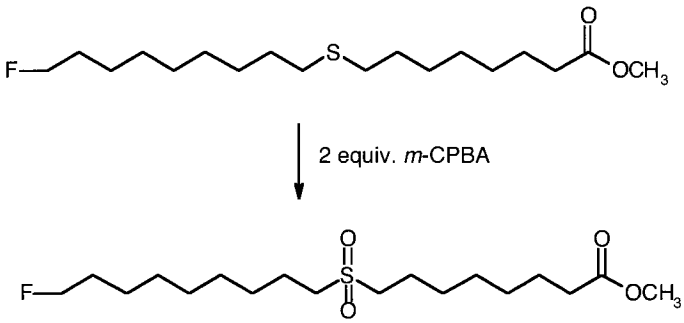


To a soln of methyl ω-fluoro-8-thiaoctadecanoate (14.8 mg, 0.044 mmol) in CH_2Cl_2 (1 ml) was added metachloroperbenzoic acid (2 equiv., 31.4 mg) at 0°. After standing at 0° for 30 min, the reaction mixture was filtered to remove a precipitated white solid (*meta*-chlorobenzoic acid) and the filtrate washed with 3M NaOH ((2 x 2.5 ml),), dried (Na_2SO_4) and evaporated to give the crude sulfone which was purified by flash chromatography using 25 % EtOAc/hexanes to give the title compound (14.8 mg, 92%) as a white solid.

TLC (Hexane/EtOAc 75:35): R_f 0.19. M.p. 67-68° (amorphous crystals, recrystallized from hexane/EtOAc). IR (KBr): 2938, 2850, 1738, 1474, 1438, 1262, 1213, 1174, 1137. ^{1}H-NMR (400 MHz , $CDCl_3$): 4.44 (*dt*, $^2J_{HF}$ = 47.4, 2 H); 3.67 (*s*, 3 H); 2.940 (*m*, 4 H); 2.32 (*t*, *J* = 7.5, 2 H); 1.85, 1.83 (overlapping *m*, 4 H); 1.6 –1.78 (*m*, 4 H); 1.28 –1.52 (*m*, 16 H). ^{13}C-NMR (100 MHz , $CDCl_3$): 173.98; 84.21 (*d*, $^1J_{CF}$ = 164.1); 52.82; 52.61; 51.55; 33.82; 30.39 (*d*, $^2J_{CF}$ = 19.3); 29.35; 29.15; 29.15; 29.04; 28.56; 28.51; 28.20;

25.14 (*d*, $^{3}J_{CF}$ = 5.4); 24.52; 21.94; 21.78. ^{19}F NMR (376.5 MHz, $CDCl_3$): -218.26. EI-MS: 335 (7, $[M - OCH_3]^+$), 315 (3), 293 (9), 143 (85, $[(CH_2)_6CO_2CH_3]^+$), 111 (73), 83 (100), 55 (91).HR-EI-MS: 335.2039 ($C_{17}H_{32}O_3FS$, $[M - OCH_3]^+$; calc. 335.2056).

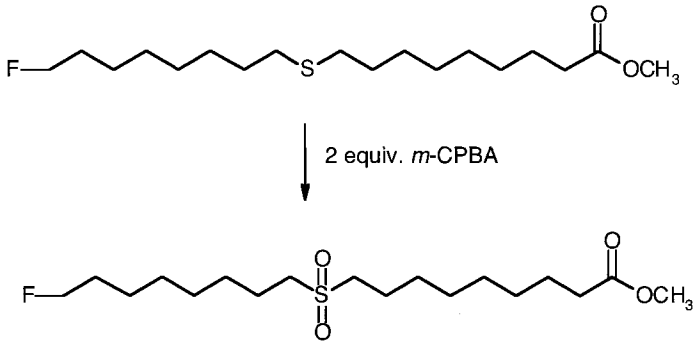
3.4.2 Methyl 18-fluoro-9-thiaoctadecanoate S, S-dioxide



Title compound obtained in a similar manner described in section 3.4.1.

TLC (hexane/EtOAc 75:25): R_f 0.22. M.p. 69-70° (plates, recrystallized from hexane/EtOAc). IR (KBr): similar to that of 3.4.1. ^{1}H-NMR (400 MHz, $CDCl_3$): 4.44 (*dt*, $^{2}J_{HF}$ = 47.4, 2 H); 3.67 (*s*, 3 H); 2.94 (*m*, 4 H); 2.31 (*t*, *J* = 7.5, 2 H); 1.83(*m*, 4 H); 1.6 – 1.78 (*m*, 4 H); 1.28 –1.52 (*m*, 16 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 174.14; 84.17 (*d*, $^{1}J_{CF}$ = 164.1); 52.77; 52.70; 51.51; 33.95; 30.37 (*d*, $^{2}J_{CF}$ = 19.6); 29.13; 29.01; 28.73; 28.73; 28.73; 28.49; 28.32; 25.13 (*d*, $^{3}J_{CF}$ = 5.4); 24.87; 21.93; 21.86. ^{19}F NMR (376.5 MHz, $CDCl_3$): -218.33. EI-MS: 335 (9, $[M - OCH_3]^+$), 315 (13), 293 (19), 157 (59, $[(CH_2)_7CO_2CH_3]^+$), 125 (89), 55 (100). HR-EI-MS: 335.2037 ($C_{17}H_{32}O_3FS$, $[M - OCH_3]^+$; calc. 335.2056).

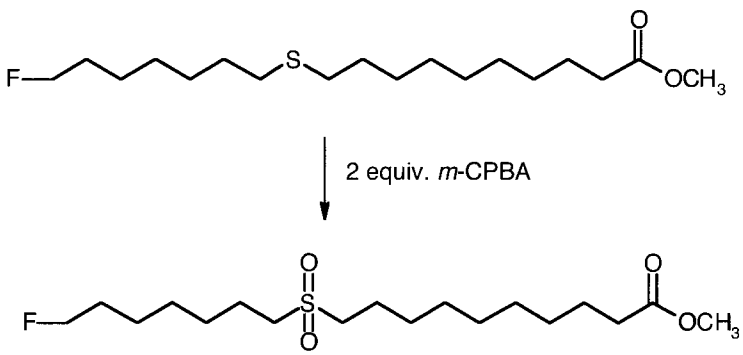
3.4.3 Methyl 18-fluoro-10-thiaoctadecanoate S, S-dioxide



Title compound obtained in a similar manner described in section **3.4.1**.

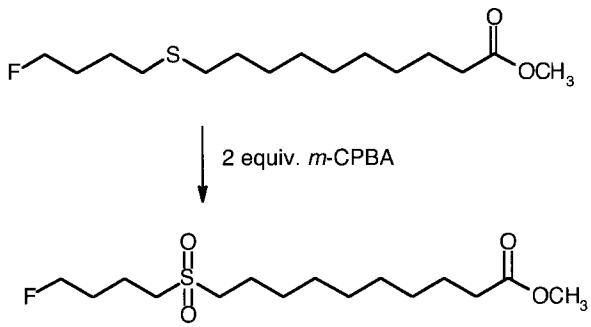
TLC (hexane/EtOAc 75:25): R_f 0.24. M.p. 66-67° (plates, recrystallized from hexane/EtOAc). IR (KBr): similar to that of 3.4.1. ^{1}H-NMR (400 MHz, $CDCl_3$): 4.44 (*dt*, J = 47.4, 2 H); 3.67 (*s*, 3 H); 2.94 (*m*, 4 H); 2.30 (*t*, J = 7.5, 2 H); 1.84,1.83(overlapping *m*, 4 H); 1.6 –1.78 (*m*, 4 H); 1.28 –1.52 (*m*, 16 H). ^{13}C-NMR (100 MHz , $CDCl_3$): 174.23; 84.10 (*d*, J = 164.1); 52.76; 52.72; 51.48; 34.02; 30.32 (*d*, J = 19.4); 28.96; 28.96; 28.88; 28.87; 28.87; 28.45; 28.42; 25.09 (*d*, J = 5.2); 24.83; 21.92; 21.90. ^{19}F NMR (376.5 MHz, $CDCl_3$): -218.43. EI-MS: 335 (11, $[M - OCH_3]^+$), 334 (14, $[M - HOCH_3]^+$), 315 (9), 293 (8), 171 (37, $[(CH_2)_8CO_2CH_3]^+$), 139 (89), 55 (100). HR-EI-MS: 334.1964 ($C_{17}H_{31}O_3FS$, $[M - HOCH_3]^+$; calc. 335.1978).

3.4.4 Methyl 18-fluoro-11-thiaoctadecanoate S, S-dioxide



TLC (hexane/EtOAc 75:25): R_f 0.22. M.p. 66.5-67° (plates, recrystallized from hexane/EtOAc). IR (KBr): similar to that of 3.4.1. ^{1}H-NMR (400 MHz , $CDCl_3$): 4.44 (*dt*, J = 47.3, 2 H); 3.67 (*s*, 3 H); 2.944, 2.938 (overlapping *m*, 4 H); 2.30 (*t*, J = 7.5, 2 H); 1.6 –1.78 (*m*, 4 H); 1.28 –1.52 (*m*, 16 H). ^{13}C-NMR (100 MHz , $CDCl_3$): 174.30; 83.99 (*d*, J = 164.2); 52.81; 52.66; 51.47; 34.07; 30.25 (*d*, J = 19.4); 29.09; 29.04; 29.04; 28.99; 28.72; 28.50; 28.43; 24.95 (*d*, J = 5.4); 24.89; 21.94; 21.85. ^{19}F NMR (376.5 MHz, $CDCl_3$): -218.43. ^{19}F NMR (376.5 MHz, $CDCl_3$): -218.56. EI-MS: 335 (11, $[M - OCH_3]^+$), 315 (1), 293 (13), 185 (27, $[(CH_2)_9CO_2CH_3]^+$), 153 (48), 55 (100). HR-EI-MS: 335.2058 ($C_{17}H_{31}O_3FS$, $[M - OCH_3]^+$; calc. 335.2056).

3.4.5 Methyl 15-Fluoro-11-thiapentadecanoate S, S-dioxide



TLC (hexane/EtOAc 75:25) R_f 0.23. M.p. 59-60 °C (amorphous white solid). IR (film): 2915, 2848, 1735, 1466,1416, 1328, 1267, 1246, 1124 (SO_2) cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400.1 MHz) δ 4.51 (*dt*, $^2J_{HF}$ = 47.2, $^3J_{HH}$ = 7.5, 2 H), 3.67 (*s*, 3 H), 3.02 (*m*, 2 H), 2.96 (*m*, 2 H), 2.31 (*t*, J = 7.5, 2 H), 1.8 –2.06 (*m*, 6 H), 1.61 (*m*, 2 H), 1.44 (*m*, 2 H), 1.25 1.38 (*m*, 8H); ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ 174.30, 83.23 (*d*, $^1J_{CF}$ = 165.8), 52.89, 52.10, 51.49, 34.06, 29.15 (*d*, $^2J_{CF}$ = 19), 29.09, 29.03 (2 C's), 28.98, 28.46, 24.88, 21.91, 18.55 (*d*, $^3J_{CF}$ = 4.2); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ -219.73. EI-MS *m/z* 293 (9, $[M - OCH_3]^+$), 273 (<1), 251 (4), 185 (9, $[(CH_2)_9CO_2CH_3]^+$), 153 (18), 141 (20), 98 (81), 69

(74), 55 (100); HR-EI-MS: *m/z* 293.1587 ([M - OCH_3]$^+$, $C_{14}H_{26}O_3FS$ requires 293.1587).

3.5 *Incubation of Substrates with S. cerevisiae*

The following section describes the methods used to incubate the synthetic thia fatty acids with actively growing Baker's yeast cultures.

3.5.1 Maintenance of Yeast cultures

S. cerevisiae (Baker's yeast) and colonies were stored on agar medium contained in a Petri plate at 4 °C. The agar medium was prepared as follows: a solution of BACTO peptone (20 g), BACTO yeast extract (10 g) and granulated agar (15 g) was suspended? in 900 mL of ddH_2O. A solution of D-glucose (20 g) in ddH_2O (100 mL) was prepared. The flasks containing the two solutions were stoppered with a foam plugs, capped with aluminum foil and then autoclaved for 20 minutes. While still hot, the two solutions were mixed thoroughly under asceptic conditions, poured into Petri plates and allowed to cool for ca. 1 hour prior to storage at 4 °C. *S. cerevisiae* cultures were transferred using a wire loop under asceptic conditions.

3.4.2 Incubation of Substrates with *Saccharomyces Cerevisiae*

The medium for the liquid cultures was prepared as follows: BACTO peptone (20 g) and BACTO yeast extract (10 g) was dissolved in ddH_2O (900 mL) contained in a 2 L Erlenmeyer flask that was then capped with a foam plug, covered with foil and

autoclaved. The golden YP stock solution was cooled and stored (4 °C). Growth medium for a starter culture was prepared by autoclaving a portion of the YP liquid medium (9 mL) in a 25 mL capped Erlenmeyer flask and a D-glucose solution (1 g in 5 mL ddH_2O) in another capped 25 mL Erlenmeyer flask. For experiments where the desaturated product was isolated from the yeast cells, 0.1g/10 mL of tergitol Type (Aldrich), (~2 mL) was included in the medium. A set of Pasteur pipettes wrapped in tin foil was also autoclaved. After the solutions cooled, a platinum loop was flamed and a sample of yeast was transferred from the maintenance culture to the YP liquid medium along with ca. 1 mL of the D-glucose solution. The culture was incubated in a rotary shaker for 24 hours at 150 RPM and 27 - 29 °C. At this point, the cultures were turbid. A 500 mL Erlenmeyer flask containing a solution of YP liquid medium (180 mL) and D-glucose (5 g in 20 mL ddH_2O) (separately autoclaved) was innoculated with approximately 0.5 mL of freshly grown out yeast starter culture. A sample of the sulfur-containing fatty acid methyl ester (25 mg) was dissolved in 100 μL of 95 % ethanol and introduced to the liquid medium via sterile pasteur pipette. After incubation for 24 hours at ca. 27 °C and 150 RPM, the resultant turbid cultures were centrifuged at 10,000 RPM for 15 minutes and the supernatant medium was decanted from the yeast cell pellet.

To isolate fatty acid sulfoxides, the supernatant (~180 mL) was acidified using AcOH (ca. 15 mL, pH ~ 3.5) and extracted with CH_2Cl_2 (3 x 80 mL). The combined organic extracts (along with any emulsions formed) were dried over Na_2SO_4 and concentrated *in vacuo*. The crude yellow extract (25 mg) was methylated in a fume hood by the following method: approximately 300 mg (2 spatula tips) of N-nitrosomethylurea was added to a test tube containing anhydrous diethyl ether (15 mL) and 50% KOH (3

mL) at room temperature. The solution quickly turned yellow due to diazomethane formation with the evolution of gas bubbles (Caution: diazomethane gas is toxic and explosive). After 10 minutes, an aliquot of the yellow solution was transferred to a second test tube containing anh. Na_2SO_4 and then added to the crude yeast extract via paster pipette. The mixture was agitated for 1 minute at room temperature and the ethereal layer concentrated *in vacuo*. The crude methylated sulfoxide (25 mg) was analyzed by TLC and purified by flash chromatography (100% EtOAc).

To analyze the extent of % substrate desaturation, the cell pellets were washed with a 1% saline solution (~100 mL) and centrifuged once more. The saline solution was immediately decanted.

Saponification of the combined cell pellets was carried out by refluxing with 5 % KOH solution (60 mL, 1:1 dH_2O:95% EtOH) with stirring for 3 hours under N_2. The cooled reaction mixture was filtered by vacuum and the filtrate was diluted with 50 mL dH_2O. The fatty acids were precipitated out from the filtrate using 3 M H_2SO_4 (~pH 2-3). The aqueous solution was extracted with CH_2Cl_2 (3 x 40 mL) and the combined organic extracts were washed with sat. NaCl (2 x 40 mL). The organic phase was dried over Na_2SO_4 and concentrated *in vacuo*.

The resultant crude fatty acid fraction (30 mg) was methylated by refluxing the extracts in dry MeOH (10 mL) and BF_3 (1 mL) for 3 hours under N_2. After the reaction mixture was cooled, it was concentrated to 2 mL in vacuo and then diluted with dH_2O (30 mL). The aqueous solution was then extracted with CH_2Cl_2 (3 x 20 mL) and the combined extracts were dried over Na_2SO_4 and concentrated *in vacuo*. The crude thia

fatty acid fraction (x mg) was analyzed by GC-MS and TLC and purified by flash chromatography (2.5% AcOEt/hexanes).

3.6 Trial in vitro incubation experiments using E coli microsomes containing DesA

3.6.1 Small Scale Hydrolysis of Methyl 16-Fluoro-12-Thiahexadec-9-eneoic acid/ mixture

The monounsaturated fatty acid methyl ester was dissolved in a solution containing 10% MeOH/KOH in dH_2O (~3 mL). After the mixture was heated to 80 °C for 1 hour, the aqueous layer was acidified with 50% AcOH (~1.5 mL) and extracted with hexanes (2 x 2 mL) to give a colourless solid. The precipitate was dissolved in a solution of ammonia giving a total concentration of 3.3 mg/mL of thia fatty acid.

3.6.2 DesA Reaction with 16-Fluoro-12-Thiahexadec-9-eneoic acid

Expression of the desA gene (Δ^{12} desaturase) and preparation of membranes were performed based on previously reported procedures[64]. Wet weight of starter culture was 0.73 g while the control was found to be 0.57 g. Cell membrane was lyzed by employing a French press under a pressure of approximately 900 psi. The homogenate was centrifuged (6000 RPM at 4 °C) for 15 minutes, the supernatant was collected and centrifuged once more (40,000 RPM at 4 °C) for 30 minutes. The supernatant was discarded and the remaining protein fraction was washed (twice) with Buffer A (5 mL) containing 200 mM of KCl, the resultant mixture was centrifuged (40,000 RPM at 4 °C) for 30 minutes. The concentration of the washed membrane was determined by the Bradford method[65]. The desaturase protein was determined with a concentration of 3.5 mg/mL and the vector control with a concentration of 3.3 mg/mL. For each reaction

performed, 800 μg of protein would be mixed with various components to make up the asssay[67]. The following constituents were included in the mixture to give the final concentration indicated and was made up in a total volume of 250 μL: 40 mM Tricine – KOH, pH 8.0 (N-tris[hydroxymethyl]methyl-glycineKOH), 10 mM $MgCl_2$, 100 μg ferredoxin, 5 mM NADPH, 50405 units of catalase and 200 mU of ferredoxin-NADP+oxidoreductase. The difference between protein homogenate, substrate solution and reaction constituents to the total volume was made up by diluting with dH_2O. The reaction was incubated at room temperature and agitated at a speed of 90 RPM for 15 minutes.

3.5.3 Extraction and Methylation of Thia fatty acids from for ^{19}FNMR and GC- analysis..

The substrate was extracted from the reaction mixture by quenching the reaction with 10% MeOH/KOH (3 mL). The solution was transferred to a screw capped tube and heated to 80 °C for 1.5 hours. After the solution was cooled, pre-extraction of the aqueous solution was carried out with hexanes (2 x 2 mL). The aqueous solution was acidified using 50% AcOH (2 mL) and was extracted with hexanes (2 x 2 mL). The combined organic extracts were blown down under a stream of N_2 to give a colourless solid.

The crude extract was dissolved in 1% H_2SO_4 in MeOH (~1.5 mL) and heated to 60 °C for 30 minutes. The aqueous phase was extracted by adding hexanes and dH_2O (2 mL each) to the reaction mixture. The hexane layer was separated and blown down under N_2 to give a colourless film.

Appendix

A.1 Calculation of sulfoxide content of ω-fluorooctadecanoates after yeast incubation.

To determine sulfoxide content of yeast extracts, flunisolide was used with known concentration as an external standard to calculated the amount of 18-fluoro-8-thiaoctadecanoate, S,S-oxide that will be used as a standard. The synthetic fatty acid will then be used as an external standard to calculated synthetic fatty acid content of yeast extract.

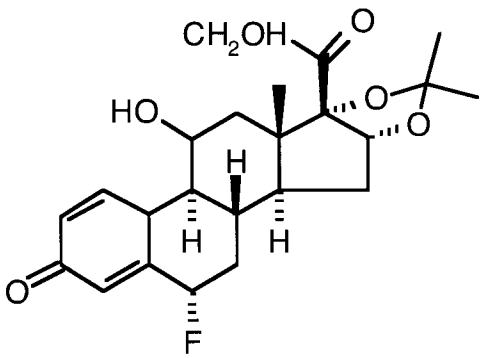


Figure A.1 Flunisolide standard used to calibrate a standard that will be used to calculate sulfoxide content.

In 500 μL of CFCl3, 500 μg of the fluorinated standard was dissolved with a final concentration of 2.73 x 10^{-3} $molL^{-1}$. Fluorine spectrum was acquired simultaneously with the standard and the integrations (standard = 1.0) were compared. In the case of methyl 18-fluoro-8-thiaoctadecanoate, S,S-dioxide, integration ratios were found to be 0.22:1. Based on calculations of the known standard, the concentrations of sulfone standard was 5.99 x 10-4 $molL^{-1}$.

Based on integrations of the ^{19}F resonance from the yeast extracts and external standard, the amounts of sulfoxide concentration and content could be calculated. The amounts of the 8, 9, 10 and 11-thia oxides found were: 3.02×10^{-4} g, 1.75×10^{-3} g, 1.12×10^{-3} g and 5.05×10^{-4} g respectively. As a percentage, this was calculated based on a 25 mg sample used for yeast incubation, percent conversion was found to be: 1.2%, 7%, 4.5% and 2.0% for 8, 9, 10 and 11-thia fatty acids respectively.

A.2 List of Abbreviations

RBF – Round bottom flask
dH2O – distilled water
ddH2O – deionized distilled water
THF – tetrahydrofuran
HMPA – hexamethylphosphoramide
THP – tetrahydropyran
RT- room temperature
n-BuLi – n-butyllithium
BF_3 – boron trifluoride diethyl etherate
TsCl – p-toluenesulfonyl chloride
TBAF – tetrabutylammonium fluoride trihydrate
m-CPBA – meta-chloroperbenzoic acid
EtOH – ethanol
MeOH – methanol
AcOH – acetic acid
AcOEt – ethyl acetate
(S)-MPAA – (S)-methylphenylacetic acid
KIE – kinetic isotope effect

References

1. J. D Weete; *Lipid Biochemistry of Fungi and Other Organisms*, New York: Plenum Press, **1980**.

2. M. Inaba, I. Suzuki, B. Szalontai, Y. Kanesaki, D. A. Los, H. Hayashi and N. Murata, *J. Biol. Chem.*, **2003**, 278, 12191.

3. F. J. van de Loo, B. G. Fox and C. Somerville, *Lipid Metabolism in Plants*, ed. T. S. Moore, jr., CRC Press, Boca Raton, **1993**, pp. 91-126.

4. N. J. Oldham and W. Boland, *Naturwissenchaften*, **1996**, 83, 248.

5. ref

6. E. Aaes-Jorgensen, *J. Agric. Food Chem.*, **1959**, 7(4), 246

7. S. L. Pereira, A. E. Leonard and P. Mukerji, *Prostaglandins, Leukotrienes Essent. Fatty Acids*, **2003**, 68, 97.

8. K. Hofmann, R.A. Lucas and S.M Sax, J. Biol. Chem. 1952, 195, 473

9. M.I. Gurr and A.T. James, Lipid Biochemistry: An Introduction. 1975, New York, John Wiley & Sons Inc., pp. 19.

10. K.L. Mikolajczak, C.R. Smith, M.O. Bagby and I.A. Wolff, *J. Org. Chem.*, **1964**, 29 (2), 318

11. A.R. Johnson, J.A. Pearson, F.S.Shenstone and A.C. Fogerty. *Nature*, **1967**, 214, 1244

12. J. Browse and Z. Xin. *Curr. Opin. Plant Biol.*, **2001**, 4, 241

13. J.G. Wallis and J. Browse, *Prog. Lipid Res.*, **2002**, 41, 254

14. F.J. van de Loo, B.G. Fox and J.C. Somerville, Lipid Metabolism in Plants, ed. T.S. Moore, Jr., CRC Press, Boca Raton, **1993**, pp. 91-126

15. A.C. Fogert, A.R. Johnson and J.A. Pearson. *Lipids*, **1972**, 7, 335

16. I. Prost, S. Dhondt, G. Rothe, et. al., *Plant Physiol.*, **2005**, 139, 1902

17. N.J. Oldham and W. Boland, *Naturwissenschaften*, **1996**, 83, 248

18. J. Napier, L.V. Michaelson and T.M. Dunn, *Trends Plant Sci.*, **2002**, 7, 475

19. S. Pyne and N. Pyne, *Biochem. J.*, **2000**, 67, 27

20. L.B. Bjostad and W.L. Roelofs, *J. Biol. Chem.*, **1981**, 256, 7936

21. W. Liu, H. Jiao, M. O'Connor and W.L. Roelofs, *Insect Biochem. Mol. Biol.*, **2002**, 32, 1489.

22. P.H. Buist, *Nat. Prod. Rep.*, **2004**, 21, 249

23. A.J. Fulco, *Annu. Rev. Biochem.*, **1974**, 43, 215.

24. M.I. Gurr, MTP International Review of Science, Biochemistry Series One, T.W. Goodwin, Butterworth, London, 1974, vol. 4, pp. 181-235

25. J. Shanklin, E. Whittle and B.G. Fox, *Biochemistry*, **1994**, 33, 12787

26. J. Shanklin, C. Achim, H. Schmidt, B.G. Fox and E. Munck, Proc. *Natl. Acad. Sci. USA*, **1997**, 94, 2981

27. J. Shanklin and E. Whittle, *FEBS Lett.*, **2003**, 545 188

28. G.A. Thompson, D.E. Scherer, S. Foxall-Van Aken, J.W. Kenny, H.L. Young, D.K. Shintani, J.C. Kridl and V.C. Knauf, *Proc. Natl. Acad. Sci. USA*, **1991**, 88, 2578

29. B.G. Fox, J. Shanklin, J. Y. Ay, T.M. Loehr and J. Saunders-Loehr, *J. Biochem*, **1994**, 33, 12776

30. Y. Lindqvist, W. Huang, G. Schneider and J. Shanklin, *EMBO J.*, **1996**, 15, 4081

31. G.J. Schroepfer and K. Bloch, *J. Biol. Chem.*, **1965**, 240, 54

32. L.J. Morris, R.V. Harris, W. Kelly and A.T. James, *Biochem. J.*, **1968**, 19, 673

33. G.K. Cook and J.M. Mayer, *J. Am. Chem. Soc.*, **1994**, 116, 1855

34. J.A. Broadwater, E. Whittle and J. Shanklin, *J. Biol. Chem.*, **2002**, 277, 15613

35. J.R. Collins, D.I. Camper and G.H. Loew, *J. Am. Chem.*, **1991**, 113, 2736

36. P.H. Buist and D.M. Marecak, *Can. J. Chem.*, **1994**, 72, 176

37. G.J. Schroepfer and K. Bloch, *J. Biol. Chem.*, **1965**, 240, 54

38. P.H. Buist and D.M Marecak, *Can. J. Chem*, **1994**, 72, 176

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

40. L.J. Morris, R.V. Harris, W. Kelly and A.T. James, *Biochem. J.*, **1968**, 109, 673

41. F. Carvalho, L.T. Gauthier, D.J. Hodgson, B. Dawson and P.H. Buist, *Org. Biomol. Chem.*, **2005**, 3, 3979

42. B. Behrouzian, L. Fauconnot, F. Daligault, C. Nugier-Chauvin, H. Patin and P.H. Buist, *Eur. J. Biochem.*, **2001**, 268, 3545

43. P.H. Buist, *Nat. Prod. Rep.*, 2004, 21, 249-262

44. L. Fauconnot and P.H. Buist, *J. Org. Chem*, **2001**, 66, 1210

45. B. Behrouzian, D. Hodgson, C.K. Savile, B. Dawson, P.H. Buist and J. Shanklin, *Magn. Reson. Chem.* **2002**, 40, 524

46. W.H. Pirkle, S.D. Beare and R.L. Muntz, *Tetrahedron Lett.*, **1974**, 26, 2295

47. P.H. Buist and D. Marecak, *Tetrahedron: Asymmetry*, **1995**, 6, 7

48. M. Kimura, A. Kuboki and T. Sugai, *Tetrahedron: Asymmetry*, **2002**, 13, 1059

49. Y.G. Gakh, A.A. Gakh, A.M. Gronenborn, *Magn. Reson. Chem.*, **2000**, 38, 551

50. R.J. Sasata, D.W. Reed, M.C. Loewen and P.S. Covello, *J. Biol. Chem.*, **2004**, 279, 39296

51. H.W. Cook, Biochemistry of Lipids, Lipoproteins and Membranes, D.E. Vance & J. Vance Elsevier, New York, 1991

52. T. Yabuuchi, T. Kusumi, *J. Am. Chem. Soc.*, **1999**, 121, 10646

53. P.H. Buist and D.M. Marecak, *J. Am. Chem. Soc.*, **1991**, 113, 5877

54. P. H. Buist, H. G. Dallmann, R. R. Rymerson, P. M. Seigel, P. Skala, *Tetrahedron Lett.*, **1988,** *29*, 435.

55. P.H. Buist and D.M. Marecak, *J. Am. Chem. Soc.*, **1992**, 114, 5073

56. P.H. Buist, H.G. Dallmannm R.R. Rymerson, P.M. Seigel, P. Skala, *Tetrahedron Lett.* **1987**, 28, 857

57. D. Hodgson and P.H. Buist, *Tetrahedron: Asymmetry*, **2003**, 14, 641

58. P. H. Buist and B. Behrouzian, *J. Am. Chem. Soc.*, **1998**, 120, 871

59. F.D. Gunstone, M.R. Pollard, C.M. Scrimgeour and H.S. Vedanayagam, *Chem. Phys. Lipids*, **1997**, 18, 115

60. A. Pinilla, F. Camps, and G. Fabrias, *Biochemistry*, **1999**, 38, 15272

61. J.A. Broadwater, E. Whittle and J. Shanklin, *J. Biol. Chem.*, **2002**, 277, 15613

62. S.K. Latypov, J.M. Seco, E. Quinoá and R. Riguera, *J. Org. Chem.*, **1995**, 60, 504

63. L. F. Fieser and M. Fieser, Reagents for Organic Synthesis, **1967**, John Wiley and Sons Inc., pp. 1179-1180

64. S. Panpoom, D.A. Los, N. Murata, *Biochim. Biophys. Acta*, **1998**, 1390, 323

65. M.M. Bradford, *Anal. Biochem.*, **1976**, 72, 248

66. Pavia, D. L., Lampman, G. M., Kriz, G. S., Introduction to Spectroscopy 3rd ed., Thomson Learning Inc., Toronto, ON, Canada, **2001**, pp 323.

67. H. Wada, M-H. Avelange-Macherel and N. Murata, J. Bacteriol., 1993, 175, 6056

68. B. Behrouzian and P. H. Buist, *Phytochemistry Rev.*, **2003**, 2, 103