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INVESTIGATING THE
STEAROYL COA DESATURASE SYSTEM
OF *SACCHAROMYCES CEREVISIAE*
BY OXIDATION OF SULFIDE PROBES

by

DALE M. MARECAK, B.Sc.

A thesis

submitted to the Faculty of Graduate Studies

in partial fulfilment of the requirements

for the degree of

Doctor of Philosophy

Department of Chemistry

Carleton University

Ottawa, Canada

August, 1994

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**"INVESTIGATING THE STEAROYL COA DESATURASE SYSTEM
OF *SACCHAROMYCES CEREVISIAE* BY OXIDATION
OF SULFIDE PROBES"**

submitted by

DALE M. MARECAK, B.Sc.

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

Chair, Department of Chemistry

Thesis Supervisor

External Examiner

Carleton University, September , 1994

Abstract

A variety of sulfur-containing fatty acid analogues has been used to probe the Δ^9 -stearoyl CoA desaturase system in common Baker's yeast. We have shown that this enzyme can behave as a regio- and enantioselective oxygenating agent.

In this thesis, the validity of each probe as a substrate has been investigated by stereochemical analysis of the sulfoxide product resulting from incubation with growing cultures of *Saccharomyces cerevisiae*.

Quantitation of sulfoxide yield from a desaturase-deficient mutant clearly showed that sulfoxidation of our probe depended on the presence of an active desaturase system. This study allowed the discovery and subsequent evaluation of methoxyphenylacetic acid as a chiral NMR shift reagent.

Incubation of the 9-thiastearate probe with *S. cerevisiae* yielded between 10 and 20% isolated sulfoxide with a >96% enantiomeric excess (ee). The 10-thiastearate analogue incubation resulted in 5% sulfoxide with a 91% ee. Both major products were shown to possess the *R* configuration, through the use of (*S*)-(+)- α -methoxyphenylacetic acid as a chiral NMR shift reagent, and comparison to a suitable dialkyl standard. The assigned (*R*) configuration for our sulfoxide matches the previously suggested enzyme stereospecificity for pro-(*R*) hydrogen removal.

Methyl (*S*)-*S*-benzyl-8-mercaptooctanoate *S*-oxide (>98% ee) was obtained from incubation of the corresponding sulfide, in 50% isolated yield. A second benzyl analogue, (*S*-benzyl-9-mercaptononanoate), afforded 5% sulfoxide (88% ee). Absolute configuration was established by correlation of benzyl decyl sulfoxide standards to our biological products by polarimetry, circular dichroism and chiral shift reagent NMR.

Methyl *S*-phenyl-8-mercaptooctanoate, -9-mercaptononanoate, and methyl *S*-(2-naphthyl)-9-mercaptononanoate probes were not validated (2-3% yield of racemic sulfoxide). The naphthyl-mimic (methyl 10-(2-naphthyl)-9-thiadecanoate) of our best benzyl probe, afforded catalyzed sulfoxide (12% yield, 75% ee). Methyl 11-phenyl-9-thiaundecanoate produced 17% sulfoxide (>98% ee), while processing of methyl *S*-(*p*-trifluoromethyl)-benzyl-9-mercaptononanoate yielded 26% (96% ee).

Sulfoxidation at the 9-position is consistently more efficient than at the 10-position, which seems to indicate that dehydrogenation of stearate is initiated at C-9.

A substrate cleavage process, thought to be competing with sulfoxidation, was not detected.

A novel assay method for desaturase activity was explored which followed fluorine-tagged probe fates by ¹⁹F-NMR. Preliminary results support the preferred catalysis of sulfide probes with sulfur in the 9-position.

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I would like to thank Dr. Peter H. Buist for being a kind, helpful and patient supervisor filled with a cautious enthusiasm. I would also like to thank Anne Webb (NRC Sussex) for numerous elemental analyses, Barb Blackwell, John Nikiforuk, (Agriculture Canada), Raj Capoor, (Ottawa University), Keith Bourque, (Carleton University), and Brian Dawson (NRC Tunney's pasture) for high field NMR work, Clem Kazakoff (Ottawa University) for mass spectral analyses, Dr. Gordon Willick (NRC Montreal road) for circular dichroism spectroscopy, the staff at the Berkeley Yeast Genetic Stock Centre for the mutant, and Nataly Vo and Rod Livingstone for providing some synthetic materials.

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Terminology and Structures

9-mercapto and 10-thia refer to a compound in which sulfur serves as a methylene isostere replacing C-10 (as counted from the carboxylic carbon, C-1); names for substrates and products are occasionally shortened to refer to sulfur position and class of the group attached (eg. benzyl 10 sulfoxide, 9 thiaphenyl); numerical designations for probes follow that set in the experimental wherein a distinct ordering is maintained.

(*R*)-(-)-(3,5-dinitrobenzoyl)- α -methylbenzylamine is replaced by "the Kagan reagent", which refers to the discoverer of this ¹H-NMR shift reagent; the use of the (*S*)-enantiomer of this shift reagent occurs only in the Wickerman's media experiment for the purposes of splitting the clearer (left) half of the AB quartet of concern.

ee refers to the enantiomeric excess of a chirally enriched compound, and is calculated by subtracting the percentage molar composition of the lesser enantiomer from that of the enantiomer in abundance (eg. 90% (*R*) - 10% (*S*) = 80% ee).

YPD is a media-type for culturing yeast, consisting of 1% Yeast extract, 2% bacto-Peptone, and 2% Dextrose.

FIGURE #1 Structures, names and numbers for probes.

Structure	Full Name	Designated Compound #
	Methyl 9-thiastearate	22
	Methyl 10-thiastearate	23
	Methyl S-benzyl-8-mercaptooctanoate	25
	Methyl S-benzyl-9-mercaptononanoate	26
	Methyl S-phenyl-8-mercaptooctanoate	27
	Methyl S-phenyl-9-mercaptononanoate	28
	Methyl 10-(2-naphthyl)-9-thiadeconoate	31
	Methyl S-(2-naphthyl)-9-mercaptononanoate	30
	Methyl 11-phenyl-9-thiaundecanoate	NV o
	Methyl 11-(p-trifluoromethyl)-phenyl-9-thiaundecanoate	32
	Methyl S-(p-trifluoromethyl)-benzyl-9-mercaptononanoate	33

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1.1 General Introduction

The biosynthesis of monounsaturated fatty acids involves the "syn" removal of two hydrogen atoms from adjacent carbons of a saturated precursor. ⁽¹⁾ The 9,10-biodehydrogenation (desaturation) of stearoyl CoA is of particular interest, as it occurs in a wide variety of organisms and plays a vital role in the aerobic production of unsaturated lipids. ⁽²⁾ The catalyst in this conversion is a non-haem iron protein, called Δ -9 desaturase. It binds a molecule of oxygen to form an active iron-oxo species, a proposed requirement for hydrogen removal. ⁽³⁾ It has been postulated by Bloch, ⁽⁴⁾ on the basis of his isotope study using *Corynebacterium diphtheriae*, that removal of the pro-(*R*) hydrogen at carbon position 9 occurs first in this process, (Scheme 1).

Scheme 1 : Δ -9 Desaturation of Stearoyl CoA

The coenzyme A thioester of stearic acid is thus converted to the oleic acid thioester. The intimate mechanistic details of this process have remained obscure, mostly due to the unstable nature of the enzyme. ⁽⁵⁾ Based on work with a variety of desaturase systems and on comparison to cytochrome P-450-catalyzed processes, three major possibilities have been put forth. ^(1,6)

The first, involving hydroxylation followed by dehydration (Scheme 2), accepts the notion of a discrete alcohol intermediate. ⁽⁷⁾ Although Bloch has shown that the synthetic alcohol intermediate is not processed to the olefin product, ⁽⁸⁾ this scheme is not fully dismissable due to the possibility of the enzyme not being able to bind the pre-formed intermediate.

Scheme 2 : C-9 Hydroxy/Dehydration

A second scheme, postulates that the olefin is formed as a result of sequential hydrogen atom abstraction. This "disproportionation" sequence (Scheme 3), may be common to chemistry, but there exists some skepticism about the use of a free hydroxyl radical by an enzyme.

Scheme 3 : Radicals/Disproportionation

The third possibility, supported by our group, involves formation of a carbocation by an electron transfer process, after initial hydrogen atom abstraction, (Scheme 4).⁽⁶⁾ Subsequent deprotonation of the C-10 methylene then completes the desaturation. The rationale for such a scheme is based on an analogy with the better-studied cytochrome P-450 enzyme system, which has, in many cases,⁽⁷⁾ been implicated in dehydrogenation processes like our own.

Scheme 4 : C-9 Carbocation/Deprotonation

The mechanisms all have in common the notion that one hydrogen is removed first, the pro-(*R*) at C-9. Thus, in designing a probe system that would eventually differentiate between the different theories, one must first confirm this initial assumption.

The thrust of this work, therefore, was to devise a suitable set of probes to locate the position of the oxidant relative to the substrate. The information gathered from the probes would add evidence to support or weaken the proposal that the C-9 pro-(*R*) hydrogen is removed first, and would validate the use of such probes in future mechanistic studies of desaturase activity.

The suspected ability of a desaturase to exhibit regio- and enantioselective oxygenase activity towards appropriately modified substrates ⁽¹⁰⁾ was targeted, through the use of sulfide substrates. Resulting enzymatic oxidation of these substrates or probes (Scheme 5), supported the regio- and stereochemical aspects of our proposal, validated the use of certain probes as substrate analogues, and provided some novel insights about the active site topology.

Scheme 5 : The Desaturase Functioning as an Oxygenase

1.2 Historical Introduction

1.2.1 Monounsaturated fatty acids, distribution and function

Monounsaturated fatty acids are ubiquitous in nature. ^(11,12) They are found in vast variety serving an equally wide array of purposes, (Figure #2). ⁽¹³⁾ They show up as essential components in the living structure of all cellular life forms. More specifically, *cis*-unsaturated fatty acids are of special importance as building blocks for lipids in membranes. ⁽¹⁴⁾ The precise structural fluidity of any membrane is largely determined by the amount of *cis*-double bonded fatty acid that was used to create the lipids making up that membrane. ^(15,16) Maintaining a specific membrane fluidity is essential to many uni- and multi-cellular organisms. ^(13,17) Such life forms must therefore either have the ability to incorporate external sources of unsaturated fatty acids or be able to synthesise them *in situ*. ^(16,18) As a result, the viability of a great number of species depend on a fully functional *cis*-fatty acid desaturation system. The most common desaturase by far is the *cis* Δ^9 -desaturase. ⁽¹⁹⁾

FIGURE #2 Monounsaturated fatty acids;

A few examples illustrating the variety of roles they play in nature.

a) Oils (free acids)

eg. vegetable oil (oleic acid)

b) Membrane components

eg. phospholipid

glycolipid

c) Energy storage

eg. triglyceride

d) Intracellular messengers

eg. diacylglycerol

e) Polyunsaturated fatty acid precursors (in turn precursors to some hormones)

eg. arachidonic

acid precursor

f) Pheromones, their precursors and degradative products

eg. dog tick mounting pheromone

1.2.2 Biosynthesis of monounsaturates and enzymology of desaturases

The soluble desaturases common to plants require acyl carrier protein (ACP) derivatives of fatty acids as substrates, while most other systems are membrane-bound and can act only on coenzyme A (CoA) derivatives. ⁽²⁰⁾ In a typical case, the CoA thioester of stearic acid becomes transformed into the cis-monounsaturated oleic acid CoA ester, (Scheme 6).

Scheme 6 : Desaturation, and its Requirements

The regulation of this enzyme was in part found to involve changes in ambient temperature, ⁽¹⁷⁾ and the effect of unsaturated fatty acids on both m-RNA levels ⁽²¹⁾ and stearic acid (precursor) uptake. ⁽²²⁾

The enzyme as described above, is in fact a set of three proteins, collectively named the Δ^9 -stearoyl CoA desaturase system (Figure #3a). The desaturase protein acts in conjunction with a single electron shuttle, called cytochrome b_5 . The cytochrome b_5 coenzyme works alongside an electron delivery enzyme, cytochrome b_5 reductase, which starts each catalytic cycle by acquiring two electrons from the NADH cofactor. Working together, the components perform a unique regiospecific biological oxidation.

FIGURE #3 The stearoyl CoA desaturase system

The catalytic portion, now termed the terminal desaturase enzyme, was initially dubbed the "cyanide-sensitive factor". ⁽²⁵⁾ However, sensitivity to cyanide has since been found to be species-dependent. ⁽²⁶⁾ The method by which this enzyme positions the substrate to allow the observed strict regiospecificity is unknown. It is suspected that the thioester end is tightly held in place so as to allow the oxidant access to only positions 9 and 10 along the fatty acid carbon backbone. ⁽²⁷⁾ The assumed cisoid conformation along these positions could be due to steric hindrance in the enzyme active pocket. The flexible nature of the long substrate suggests grooves are available to hold the carbon

backbone steady during catalysis. These ideas about requirements allow one to put forth a crude representation of what the enzyme may look like. ⁽²⁸⁾

In addition to such speculation, there is some physical evidence to suggest grooves or recognition regions at the alkane end of the substrate. The James group has shown for numerous aerobic Δ^9 -desaturases that C-16 and C-17 analogues of the C-18 native substrate become desaturated with high efficiency, C-15 and C-19 analogues are less efficiently processed, while C-14 and C-20 analogues are essentially not accepted by the system. ^(29,30) The implication here is that there exists recognition regions that deter desaturation of too long or too short a substrate.

A more detailed look at oxidant positioning inside the active site came about from an elaborate experiment in 1964. ⁽⁴⁾ Bloch and co-workers, through the use of both tritium and deuterium isotope effects and relative ratios of radioactivity, ($^{14}\text{C}:\text{}^3\text{H}$), suggested that position 9 was attacked first before position 10, and that it was the pro-*R* hydrogen being removed in each case. The results allowed the formation of a crude model or enzyme map (Figure #3b). Surprisingly, little has been done since to follow-up or substantiate this model, except for partial repetition of similar experiments on other systems. ⁽³¹⁾ All such similar experiments supported the work of Bloch, except for those on plant systems wherein no notable isotope effect was found.

1.2.3 Mechanism of Δ^9 -stearoyl CoA desaturase

Information was missing not only on the structural aspects of this enzyme, but also on the mechanism of catalysis; how do the electrons flow through this oxidative olefin formation? Knowledge of the electron and oxygen requirements ⁽³²⁾ and the sequential nature of the hydrogen removal,⁽⁴⁾ has helped in formulating numerous hypotheses, but further work on the subject is greatly needed. The biggest factor which has seemed to slow the work was finding a suitable enzyme system. There existed many sources from which analogous enzymes could be obtained, but most were crude isolates due to the difficulties of purification of such tightly membrane-bound enzymes.

Extensive studies are not limited to fatty acid desaturases however. An equally great volume of work has been done on desaturases with steroid substrates (Examples; microbial steroid 1-dehydrogenase, concerning oestrogen ⁽³³⁾ and yeast Δ^{22} sterol desaturase, concerning ergosterol ⁽³⁴⁾). These undoubtedly have a unique active site topology which differs from our fatty acid system, but the finer details of mechanism may be sufficiently similar to allow direct comparisons.

Some of the earliest work on the Δ^9 -desaturase, was done using the system we still employ today, namely that found in the common Baker's yeast, *Saccharomyces cerevisiae*. Resnick's group, in 1966, was able to locate a region of DNA suspected to hold the gene for the desaturase enzyme. ^(35,36) Their work was based on a genetic comparison between wild type cells and those of a mutant (strain KD 115) known to be deficient in the ability to biosynthesize oleic acid.

Despite this headstart, it was the enzyme from rat liver, that in 1974, was the first to be purified. ⁽³⁾ This enzyme was found to be a single, largely non-polar polypeptide, with a mass of 53,000 daltons, containing 1 atom of iron. The first ever *in vivo* reconstitution of the desaturase system was ground-breaking research by Strittmatter's group. ⁽³⁷⁾ This system, assayed using spectrophotometric methods, enabled them to provide good evidence in support of the cisoid substrate-conformation requirement, concerning the active site structure. Further studies employing group-specific reagents suggested that tyrosyl groups may be involved with chelation of the iron atom and that arginyl residues may play a specific role in binding the CoA moiety of the substrate. ⁽³⁸⁾

Five years later, the $\Delta 9$ -desaturase from a hen liver microsomal fraction was purified. ⁽³⁹⁾ This enzyme had a lower molecular weight than that of the rat, and had an antibody that did not recognize the rat enzyme.

The next substantial effort was the elucidation of the DNA sequence and the primary protein structure for the rat system, in 1985. ⁽⁴⁰⁾ Then, in 1989, the isolation of the gene (named OLE 1) from yeast was announced by Martin's group. ⁽⁴¹⁾ The amino acid sequences of the rat- and yeast-derived enzymes were compared and showed substantial homology (36%, Figure #4). It was demonstrated that the gene from the rat could be inserted into the genome of the yeast mutant and thereby restore it to a wild-type phenotype. ⁽⁴²⁾ Just two years later, in 1992, transposition was again demonstrated by the expression of the OLE 1 gene in tobacco plants. ⁽⁴³⁾ In the same year, using the novel system of the housefly, Reitz's group demonstrated that there may well be significantly different versions of this common $\Delta 9$ -desaturase enzyme. ⁽²⁶⁾

The work was based on enzyme inhibitor studies as compared to other systems, and suggested that there may be three forms of desaturase, each sensitive to a different inhibitor.

Despite the many possible structural differences (re: antibodies, molecular weights, inhibitors, etc.), the amino acid sequence homology is high in some, non-hydrophobic, centrally-located areas ⁽⁴⁰⁾ and readily allows for a unified mechanism.

Protein structure determination, through the use of X - ray crystallography, would be the next obvious step in this research. The difficulty however, in growing a sufficient crystal for study from such a deeply membrane-imbedded enzyme, has been to date, insurmountable. The problem may have a solution involving the soluble desaturase from plants, which has also been recently isolated and characterized. ^(44,45,46) Undoubtedly easier to crystallize due to its solubility, an important question will remain, "Is it similar enough to the other systems to be comparable?". One factor which suggests a negative answer is the substrate difference; the plant enzyme prefers ACP derivatives of the saturated precursor while our system uses the CoA thioester derivative. More importantly, there is no substantial amino acid sequence homology between the plant and animal desaturases. ⁽⁴⁰⁾ Recently a third factor came to light, ⁽⁴⁴⁾ Mossbauer spectroscopy confirmed the presence of two iron atoms per plant desaturase. This apparently adds another difference between the plant and animal systems, although equally definitive evidence for the suggested one iron atom per CoA Δ^9 -desaturase is lacking.

Besides this recent flurry of active site structure-related studies, no detailed mechanistic work has been done on the Δ^9 -stearate desaturase in the past 28 years.

Work from our lab from 1986 to 1988 focussed on producing potential enantiomerically pure compounds from the yeast-mediated Δ^9 -desaturation of various thia- and dithiastearates followed by microbial functionalization and chemical desulfurization. ^(47,48) During the initial step, involving desaturation, it was discovered that the methyl 9-thiastearate substrate was actively sulfoxidized, (Scheme 7a). ⁽⁴⁹⁾ The melting point of the purified biological product (71-72°C) was different than that of the synthetic racemic standard (64-66°C), which suggested that the biological sample might consist of a different enantiomeric composition. In two competition experiments it was suggested that of all thiastearates tested (5 - 13-thia), methyl 10-thiastearate was the only other substrate to actively undergo sulfoxidation. Indeed, the olefin-containing analogue was detected from incubations of thiastearates with sulfur in positions 5,6,7,12 and 13.

Coincidentally, near the same time, Pascal's group noted an inhibition of 9,10-dehydrogenation upon administering 8-,9-,10-, and 11-thiastearates to the protozoan, *Crithidia fasciculata*. ⁽⁵⁰⁾ The biosynthetic route from stearic acid to their cyclopropanated product involved formation of the 9,10-*cis* olefin in a supposedly identical manner to our Δ^9 -desaturation. The results from our lab therefore suggested that the formation of sulfoxide from the 9- and 10-thiastearates, should have occurred in his protozoan system. Unfortunately, production of sulfoxide was not tested for in those experiments, which targeted inhibition of microbial growth and synthesis of cyclopropane-containing fatty acids. In anticipation of sulfoxide production however, the sulfoxides of the 9- and 10-thiastearates were tested and were shown to be non-inhibitory.

Ongoing work by Holland's group, ⁽¹⁹⁾ suggests that highly enantiomer-enriched sulfoxides can often be prepared by exploitation of the high regio- and stereoselectivity inherent in the reactivity of oxidative enzymes. The point is made that sulfoxides of high enantiomeric purity may be useful as chiral relay reagents, but are generally difficult to obtain through chemical synthesis. Keeping this concept in mind then, not only had regioselectivity been demonstrated for our chosen enzyme system, but a possible new route to enantiomer-enriched dialkyl sulfoxides may have been uncovered.

Targeting the stereochemistry of this desaturase-mediated oxidation was then initiated in our group by Skala and coworkers through the use of the methyl *S*-benzyl 8-mercapto-octanoate substrate, (Scheme 7b). ^(51,52) Analysis of the isolated sulfoxide product using the Kagan shift reagent provided ¹H-NMR evidence that the sample was in 35% enantiomeric excess (ee). Confirmation that the observed sulfoxide product was a result of sulfide being processed through the $\Delta 9$ -desaturase complex came from the tlc results of this author's fourth year honours work. ⁽⁵³⁾ The experiments illustrated that a $\Delta 9$ -desaturase-lacking mutant strain of yeast yielded only trace levels of sulfoxide.

Scheme 7 : Desaturase-mediated Sulfoxidations ; a, 9-thiastearate oxidation, b, Benzyl sulfide oxidation

The bulk of this thesis is concerned with the stereochemical and regiochemical analysis of desaturase-mediated oxygenation of sulfide probes. The enzyme regioselectivity, enantioselectivity and product absolute configuration, were all of value towards the validation of our probes as substrates. In aid and in addition to this main target, a more quantitative mutant experiment was performed, a shift reagent discovered, a variety of new substrates was assessed, and a possible new assay method for desaturation was explored.

2.1 Overview

The syntheses of sulfide substrates are discussed first. Secondly, the incubation of these probes in liquid media with a growing yeast culture is described, followed by the procedures used for isolation of the resulting products.

Stereochemical analysis of the thiastearate-type sulfoxides (*22-10,10-d₂*, *23-11,11-d₂*) is introduced by an explanation of how the serendipitous discovery of the MPAA shift reagent stemmed from a mutant study that showed that the desaturase was behaving as a sulfide oxidase. The discovery permitted the enantiomeric excesses (ee's) of the products to be determined, and, along with synthetic standards, enabled the absolute configuration to be deduced.

The stereochemistry of the benzyl-type products (*25,26*) is then discussed in a similar manner, employing a known shift reagent for ee analysis, and an array of techniques to rigorously determine the absolute configuration of the biological sulfoxides.

Next, the regiochemical aspects of the reaction, pertaining to our probes, is reviewed. Yields of isolated sulfoxide are compared for appropriately designed (equal length) substrates with sulfur in positions 9 and 10. Since benzyl-type sulfides (25, 26) have different overall chain lengths, phenyl- and phenethyl-type substrates (27, 28, NVo, 32, 33) are examined. An initially surprising result from the phenyl substrate incubation stemmed an interest in searching for reaction by-products resulting from substrate cleavage, which involved the use of naphthyl-type probes, (30, 31). In all cases the validity of these aromatic probes was tested by ee analysis.

Briefly, the results of a promising new, ^{19}F -NMR-based assay are introduced, along with a those of a less successful study, which employed a chemically-defined media as a means to simplify the original "assay" procedure.

In a final note, the applications of the newly discovered NMR sulfoxide shift reagent, MPAA, are discussed.

Substrates with sulfur in positions 9, 11 and 12 from the carboxylate, were synthesized by starting with the corresponding *w*-bromo acid, (Scheme 8a).

Displacement of bromide with sulfur from thiourea,^(54a) subsequent base hydrolysis of the resulting compound, and acidification yielded crude mercapto acid (approx. 99% yield).

Scheme 8 : The thio-acid / *n*-BuLi route to probe synthesis, a, with commercially available bromocompound or b, synthetic bromocompound

The acidification step required that aqueous acid be stirred vigorously with dichloromethane to avoid urea clathrate formation. ⁽⁵¹⁾ Also, the slow addition of the basic aqueous layer to this mixture demanded use of a blanket of nitrogen to avoid thiol oxidation to disulfide. Common impurities included the corresponding disulfide and ethanol. Analysis by ¹H-NMR (200MHz) clearly showed if any ethanol was present (3.6-3.8 ppm, quartet, CH₃CH₂OH), determined the presence of disulfide (2.60-2.63 ppm, triplet, CH₂CH₂S-S), and generally did not yield a strong signal for the carboxylic acid proton (11-12 ppm, broad), (Figure #5).

The dianion of the thioacid was afforded by dissolving the crude thiol in THF/HMPA (4:1), cooling to 0°C, adding 2 equivalents of *n*-butyllithium in hexane, and stirring for 1 hour. Addition of the bromo-compound dissolved in THF then produced the desired thia-carboxylate. ⁽⁵⁵⁾ Dilution with water, acidification, and extraction with hexane yielded the crude sulfur-containing acid, (ca. 70%). Occasionally, during the extraction procedures, a third layer, positioned between the water and hexane, would appear. This viscous yellow layer was found (tlc, ¹H-NMR) to be composed mainly of HMPA containing ca. 20% desired product. Freely soluble in dichloromethane, this layer was able to yield a small quantity of relatively pure sulfide acid after flash chromatography. In most instances though, the loss in yield to this yellow layer was negligible and the product was left unrecovered.

FIGURE #5 200MHz ^1H -NMR spectra of synthetic thio-acids showing A. common ethanol and disulfide impurities, B. ethanol removed by extended rotary evaporation, C. pure sample without a clear carboxylic acid proton resonance

The preparation of dideuterated species (22-10,10- d_2 and 23-11,11- d_2) required LiAlD_4 reduction of the corresponding straight chain carboxylic acid followed by bromination with phosphorous tribromide, (Scheme 8b). The resulting bromocompound was used without purification to alkylate the prepared thio-acid dianion.

A similar procedure was followed (replacing LiAlD_4 with LiAlH_4) in cases where the bromoalkane was unavailable (eg. preparation of corresponding phenethyl bromide from 2-(*p*-trifluoromethyl)-phenyl ethanoic acid).

When convenient, an alternative scheme was taken to produce the sulfide acid, (Scheme 9a). The alkyl thiolate was linked to the bromoacid by use of ethanolic potassium hydroxide. ⁽⁵⁶⁾ The resulting thia-compound was produced in good yield and with a much better purity than could be afforded from the thioacid-THF/HMPA/*n*-BuLi route.

On occasion where both the bromo alkane and the bromo acid were available, the approach was taken to first thiolate the bromo alkane, then link the resulting alkyl thiolate to the thio acid, (Scheme 9b).

Scheme 9 : The bromo-acid / KOH route to probe synthesis, a, with commercially available thiol, or b, with synthetic thiol.

Methylation of crude acid products with boron trifluoride etherate complex in methanol⁽⁵⁷⁾ gave crude final substrate, which was then purified by flash chromatography (3-6% EtOAc/Hex).

The overall yield from bromooctanoic acid ranged from 50 to 60 % for the original thio-acid/*n*-BuLi procedure and from 80 to 90 % for the bromo-acid/KOH method. The final products were generally characterized by ^1H -NMR, mass spectrometry, and elemental analysis.

Synthesis of substrates with sulfur at the "C-10 position" was more time consuming than the 9-thia substrates. Lack of a good source of 9-bromononanoic acid first led us to another route starting with azelaic acid mono methyl ester, (Scheme 10a).

Scheme 10 : Synthesis of 10-thia substrates ; a, azelaic acid route,

b, nonane diol route

c, alternative final step

The single free acid group of this C₉ dicarboxylate was selectively reduced using BH₃.⁽⁵⁸⁾ The resultant terminal alcohol was then esterified with *p*-toluene sulfonyl chloride.⁽⁵⁹⁾ The product, without purification, was used in place of a bromoacid to produce the thioacid, by a gentler but lengthened thiourea/EtOH reflux.^(54b) Analysis of this crude extract (tlc, ¹H-NMR) showed it to be a mixture of many components, roughly two thirds of which appeared as a single spot on tlc. Flash chromatographic isolation and subsequent analysis (¹H-NMR, GC-MS, Figure #6) of this main component provided evidence for identifying it to be a mixture of ethyl 9-mercaptononanoate and the corresponding disulfide.

The apparent ethylation may have occurred during the work-up of the base/ethanol hydrolysis of the thia-ureayl complex. The disulfide product may have been formed in the acidification step of the workup. During this step urea clathrate formation was avoided by dripping slowly the reaction mixture into a rapidly stirred suspension of acid and organic solvent.⁽⁶¹⁾ As mentioned previously, without the employment of a sufficient blanket of nitrogen the fine stream of basic aqueous material can contact oxygen in the air and thus lead to disulfide formation.

FIGURE #6 Data aiding in the identification of a flash chromatography-isolated unknown, A. GC/MS showing two main components (illustrated) B. 200MHz ¹H-NMR supporting the suggested identities of disulfide and ethyl ester.

In order to isolate the desired thio-acid, the semi-pure mixture was first base hydrolyzed to yield free acid. The second step employed triphenylphosphine in dioxane to break the disulfide linkage ⁽⁶⁰⁾ and yield the desired 9-mercaptononanoic acid (yield from azelaic acid monomethyl ester, ca. 8%). Synthesis to yield the 10-thia substrates then followed those reactions of the 9-thia sequences.

Some time later, a significantly more efficient method of producing the 9-mercaptononanoic acid was utilized, (Scheme 10b). The method produced the 9-bromononanoic acid starting material in good yield by monobromination of nonane diol ⁽⁶¹⁾ and subsequent chromium oxidation of the terminal alcohol to a carboxylic acid. ⁽⁶²⁾

In producing the reference sulfoxides, the oxidation of sulfides employed meta-chloroperoxybenzoic acid (*m*-CPBA) as a reagent. ⁽⁶³⁾ Conditions which minimized sulfone formation included a reaction temperature of 0°C, diluted substrate and reagent (ca. 0.3M concentrations, added dropwise), and a 1:1 molar equivalent of substrate to reagent based on a reagent purity of 70% (Manufacturer suggested purity: ca. 65%). Benzyl sulfoxides produced in this manner yielded a characteristic AB quartet pattern in the ¹H-NMR spectrum, (3.8-4.1 ppm, phenyl-CH₂S(O), eg. Figure #7), while thiastearate-type (dialkyl) products afforded a more complicated characteristic peak, (2.6-2.8 ppm, overlapped ABXY patterns, CH₂S(O)CH₂).

FIGURE #7 200 MHz ^1H -NMR spectrum of synthetic benzyl sulfoxide highlighting the characteristic AB quartet pattern.

2.3 Incubation of Substrates and Isolation of Products

2.3.1 Incubation of Sulfide Substrates

The study of the Δ^9 -stearoyl CoA desaturase enzyme of yeast, using substrate analogues containing sulfur requires a stable biological system which will efficiently take in these proposed precursors. Purification of the yeast desaturase would be very difficult due to its tightly membrane bound nature. Indeed, to date, this enzyme has not been isolated from a yeast source. The options that remained were to use crude cell-free extracts or whole cells as a source of desaturase activity.

Cell-free extracts were not chosen as a source due to the instability and observed low activity of the enzyme in such a preparation, the difficulty in producing the required CoA ester substrates, and the inability to determine ee's on such small scale amounts of processed substrate. The choice of whole cells however, requires certain assumptions to be made when discussing observed results. These include that the various thio-substrates that are compared to one another are not discriminated by the actions of transport into the cells, thio-ester formation with CoA, incorporation into membranes, β -oxidation, random and biosynthetic P-450 oxidation, and excretion.

The use of whole cell cultures of the common Baker's yeast, *Saccharomyces cerevisiae*, grown in liquid YPD medium (yeast extract, peptone, dextrose) was found to be reliable and convenient. This system also proved to be sufficient in yielding the required amounts of product for desired analyses. The previously-used poorly defined

strain ⁽⁶⁴⁾ of this yeast species, was replaced by the strain ATCC 12341, as obtained from the Yeast Genetic Stock Centre, Berkley, California.

Ease in media preparation and aseptic technique was effected by combining all YPD media ingredients before autoclaving. (In future experiments it is suggested that the glucose in dilute solution be autoclaved in separate flasks from the yeast extract and peptone solution to aid in deterring caramelization of the glucose. The resulting solutions while still hot (ca. 50-70°C), can readily be aseptically combined then distributed to individual flasks for later incubation.)

In order to promote uptake (hydrophobic cells attracting hydrophobic substrates), and based on this lab's work ⁽⁵⁵⁾ and the work of others, ⁽⁶⁵⁾ the methyl ester of each fatty acid analogue was the chosen form to administer, instead of the anionic carboxylate form.

All substrates were each fed separately to growing yeast cultures, except in a final competition between the trifluoromethyl substrates. Concentrations were in the range of $6.0\text{-}6.2 \times 10^{-4}\text{M}$ on a 300 mL media per flask scale. The compound was added after inoculation as a 5% (w/v) solution in absolute ethanol. The inoculate consisted of 6 to 8 mL (representing ca. $3\text{-}7 \times 10^8$ cells) of a 17-23 hour old starter culture, as derived from a 55 mL incubation of ca. 10 mg of cells taken from a cold-stored, slant agar culture. Addition of the cells before the substrate was believed to help deter compound "clumping" by making available a dispersion of hydrophobic surfaces to which binding could occur. (Due to its higher efficiency over absolute ethanol to afford a sterile substrate sample, ⁽⁶⁶⁾ the use of 95% ethanol as a substrate solvent is recommended for

future incubations.)

Once added, some of the compound would form a very thin oil or precipitate in the mainly aqueous environment. During the course of cell growth however, (ca. 5×10^8 cells grow to give 3×10^{10} cells), the amount of insoluble material would notably decrease or disappear altogether. This was attributed to both conversion of substrate to product (ie: uptake by the cells), and an increase in dissolution. The rise in apparent solvation was likely due to the increased hydrophobic character of the culture mixture as caused by rising levels of excreted detergents from the cell mass.

An incubation period of 20 to 24 hours took advantage of a period of active cell proliferation, in which time the cells progressed through 4 to 5 generations in a sigmoidal fashion. The desaturase, as a biosynthetic enzyme, displays a desirably high level of activity during this period of cell growth.

2.3.2 Isolation of products

Incubation was halted after ca. 22 hours growth, a time at which corresponded to the pre-senescence stage of a sigmoidal growth curve. After this growth period was over and the cultures were centrifuged, the pelleted cells were washed with distilled water in an attempt to free any cell-bound organics. A dichloromethane extract of such a wash did show the presence of recovered substrate by tlc.

Extraction of the supernatant prior to acidification for the removal of neutral species was not performed due to the emulsions which would result and the likelihood of product loss.

The favoured extraction procedure involved dissolution of a quantity of salt (ca. 20g in 100mL media), followed by acidification with HCl, and dichloromethane extraction. The salt aided in increasing the ionic character of the aqueous solution, and was added prior to acidification so as to have any pH effects factored in immediately. The clear supernatant clouded quickly upon acidification, supposedly due to the insolubility of the resulting, less polar, protonated organic acids. After extraction however, the aqueous layer remained cloudy due to emulsified organic extraction solvent, which separated on standing for a prolonged period of time.

Occasionally, if the extractions seemed excessively emulsified, a more dense solvent such as chloroform or carbon tetrachloride would be added to help "pull down" the emulsion-bound dichloromethane. (Added note: although not attempted, extraction with diethyl ether may help reduce emulsions since it has a density that is further away from that of the media "brine" than the presently used dichloromethane.)

The original lab method for media extraction used hexane to extract the acidified (unsalted) aqueous. The resulting emulsions would then be broken by suction filtration through a pad of celite. The celite pad was often replaced to avoid clogging, and to avoid product loss, each pad required a hexane rinse before disposal.

The dichloromethane/salt procedure was favoured over the hexane/celite procedure due to increased simplicity, decreased working time, and slightly increased yields.

The original work-up procedure ⁽⁵¹⁾ then called for hydrolysis in order to free any esterified (ie: into lipid, etc.) product. This step was omitted after tlc of untreated extracts showed the amount of free acid sulfoxide product to be equivalent to that of a hydrolyzed sample. This suggests that the sulfoxides produced in this manner do not undergo any further biosynthesis (involving ester formation).

Starting material and product sulfoxide, recovered from cell hydrosylate extracts, generally accounted for less than 4% of the recovered net materials. Based on this finding, the cell hydrolysis step was dropped from the routine workup procedure.

Early methylations of crude extracts were carried out using BF_3 -etherate in methanol. After only a few experiments though, the methylation process was greatly shortened and simplified by the use of diazomethane in ether as a reagent. This procedural switch also eliminated the possibility that the BF_3 -etherate reflux was causing sulfoxide racemization.

Flash chromatographic separation of the components, (60 to 80 % EtOAc in Hex) generally yielded a crude substrate-rich fraction and a pure sulfoxide product.

The time savings acquired with the new extraction procedure, the omission of the media hydrolysis reaction, and the use of diazomethane, allowed for the possibility of halting the incubation early in the morning, and having an evaluation of the enantiomeric excess of the product by the evening of the same day.

2.4 Stereochemical Analysis of Thiastearate-type Products

2.4.1 Mutant studies and Discovery of a shift reagent reagent for analysis of dialkyl sulfoxides

Along with the use of a whole organism system came the question; "How were we to be sure the sulfoxidation was desaturase-mediated?". The position we had taken was based on the similarity between our thiastearates and the natural stearic acid substrate, plus experimental evidence which showed the production of the 9,10 olefin (in lieu of sulfoxide) when the sulfur was in positions 5,6,7,12 or 13. ⁽⁴⁹⁾

Evidence for answering this question came from an experiment involving a desaturase-deficient strain of yeast. Early results by tlc suggested only trace levels of sulfoxide product from the mutant yeast incubation. ⁽⁵³⁾ This added a degree of validity to our probes by showing that their oxidation did require interaction with a source of active desaturase enzyme.

Quantitation of the results was required however, to link the trace levels of product to the suspected proportion of mutant cells that had reverted to the wild-type phenotype. This quantitation involved determining both the percentage suspected reversion that had occurred in culture and the amount of total sulfoxide yield from an incubation of substrate with a desaturase-lacking mutant.

The key to determining the levels of revertant cells, involved streaking samples of grown-out mutant cultures onto oleic acid-supplemented agar plates to obtain single cell-derived colonies. These colonies were then replica plated onto agar plates with and without the supplement. Those which grew readily on the oleate-lacking media were counted as revertants. We found only 1 out of 132 colonies that grew equally well on both media types, and thus suggested a 0.8% reversion rate. ⁽⁵⁾

Quantification of sulfoxide yield first involved isolation of total sulfoxide by flash chromatography. The resulting residue contained notable levels of other compounds, thus complicating the yield calculation. The simplest and fastest remedy was analysis of the entire sample (ca. 10mg) by ¹H-NMR. The characteristic signal of methylene hydrogens next to a sulfoxide (2.4-2.6ppm) was to be measured (integrated) against a known quantity of added internal standard. The standard was chosen for its solubility, availability, and its having a proton signal at a frequency which was clear in the spectrum. Thus, the use of diphenyl acetic acid allowed the sulfoxide present in the sample to be quantified, by comparative integration between α -sulfinyl protons and the isolated acid standard proton (singlet, 4.9-5.1ppm, Figure 8).

The yield of 9-sulfoxide was determined to be 1.8%, which was similar to the quantity of sulfoxide due to autoxidation obtained in the control experiment (which employed autoclaved cells). Thus, despite the presence of detectable levels of reversion, the determined levels of sulfoxide clearly demonstrated that probe oxidation depended on a functional desaturase system.

FIGURE #8 300MHz ^1H -NMR spectra of flash-isolated total 9-sulfoxide from a culture of the mutant (desaturase-lacking) yeast, *S. cerevisiae*, KD115, A. native isolate B. isolate plus diphenylacetic acid as an internal standard for quantitation. Note: signals for methylene hydrogens next to sulfoxide (2.4-2.6ppm) undergo a splitting upon the addition of the internal standard; sulfoxide quantitation was based on the integral ratio of these protons to that of the unique DPAA proton at ca. 5.0ppm.

FIGURE #9 300MHz ¹H-NMR spectra detailing the effects of adding diphenylacetic acid to a sample of synthetic methyl 9-thiastearate *S*-oxide, A. sample alone, (2 mg) B. with 2.0 mg DPAA added C. with 4.0 mg DPAA D. with 8.0 mg DPAA.

Upon closer inspection of this standard-integrated spectrum (Figure #8) it was noted that the signals for the four AB type hydrogens alpha to the sulfur were shifted downfield and had separated into two groups of peaks. This effect was observed again when diphenyl acetic acid (DPAA) was sequentially added in portions to a purified sample of methyl 9-thiastearate *S*-oxide, (Figure #9). What was unknown however was if the separated groups of peaks were due to hydrogens at C-8 versus those at C-10, or whether the two groups represented the A-type hydrogens versus the B-type hydrogens, (Figure #10).

FIGURE #10 A close-up of the thiastearate-type 9-sulfoxide, illustrating the C-8 versus C-10 hydrogens and the B (chosen here to be "syn" to oxygen) versus A hydrogens.

Unravelling the mystery of this shift effect involved synthesis of a 9-thiastearate probe dideuterated at the C-10 position. Oxidation to the sulfoxide by *m*-CPBA, purification, then analysis by ¹H-NMR with DPAA again yielded the two separated groups of peaks, (Figure #11). This observation suggested that the added acidic compound was increasing the difference in chemical shift between the two diastereotopic hydrogens (A and B), and not those of C-8 versus C-10. After a quick literature search, it was found that similar (non-chiral) shift effects were already reported for diastereotopic methylene hydrogens next to sulfoxides in the presence of certain acids, (trifluoroacetic acid ⁽⁶⁷⁾, see section 2.8).

An idea then emerged for use of a chiral acid complexing agent to discriminate between sulfoxide enantiomers. The concept came by analogy to work by Pirkle, who demonstrated enantiomeric discrimination based on diastereomeric solvates. ⁽⁶⁸⁾ His results suggested to us that similar complexations to acidic chiral reagents may be of use for ee determinations of dialkyl sulfoxides by shift reagent NMR. Thus, a similar compound to diphenylacetic acid, available in optically pure form, (*S*)-methoxy phenyl acetic acid (MPAA), was tested as a shift reagent to discriminate between sulfoxide enantiomers. Employing the dideuterated racemic 9-thiastearate sulfoxide once again, we added the MPAA in 3 mole equivalents. The test yielded positive results; the doubling of peaks was noted and the spectral pattern analyzed as overlapping ABXY systems, (Figure #12).

FIGURE #11 200MHz ^1H -NMR spectrum of methyl 9-thiastearate *S*-oxide $10,10\text{-}d_2$, plus DPAA. Enlargement shows AB-type hydrogens at C-8 to be affected differently.

FIGURE #12 ^1H -NMR expansions of the signals representing AB-type hydrogens to the sulfoxide for racemic methyl 9-thiastearate *S*-oxide $10,10\text{-}d_2$, under the influence of (*S*)-(+)-(α)-methoxyphenylacetic acid (MPAA), A. original 200MHz result, B. 500MHz result to better illustrate the ABXY systems.

A spectral simplification of the ABXY pattern was attempted, for our synthetic dideuterated 9-thiastearate sulfoxide. This involved irradiation of the NMR sample at a frequency corresponding to that of the neighbouring (C-7) hydrogens, eliminating the proton-proton coupling, in hopes of obtaining a simple AB quartet pattern. The resulting spectral pattern however (Figure #13), was a crude AB quartet of poorly resolved triplets. This broadening effect was sufficient to deter observation of the expected double AB quartet, representing the two thiastearate sulfoxide enantiomers. The end result suggested that the better resolved method to determining biological (dialkyl-like) sulfoxide ee's was to remain with the analysis of the more complicated ABXY systems.

FIGURE #13 500MHz ¹H-NMR spectrum of methyl 9-thiastearate *S*-oxide *10,10-d₂*, under the influence of MPAA, A, spectral portion before decoupling, B, same as A, except irradiated at 1.73ppm to effect decoupling (inset; results enlarged).

Thus, in adding more evidence to the claim that we were indeed targeting the desaturase system, we also stumbled across the method by which we were able to determine the enantiomeric excesses and eventually absolute configuration of our nearly symmetrical sulfoxide stearate products.

2.4.2 Stereochemical analysis of sulfoxides derived from thiaustearate-type probes

Comparison of the α -sulfinyl methylene proton signals, under the influence of MPAA, from biological 9-S and 10-S sulfoxides (Figure #14) with that of a dideuterated synthetic standard, clearly showed the expected high ee's. The methyl 9-thiaustearate *S*-oxide (22-10,10-*d*₂) biological product yielded a spectral pattern indicative of a single enantiomer, with no more than 2% of the other enantiomer as based on the available signal to noise ratio of the spectrum. The less efficiently produced 10-sulfoxide however afforded a readily detectable quantity of the other enantiomer, (91% ee). It was suggested that observation of this minor enantiomer be due to a constant amount of autoxidation amongst a now reduced level of biological sulfoxide, thus making it appear as though the sulfoxidation process at the C-10 position was inherently less enantioselective.

FIGURE #14 500MHz ^1H -NMR spectra of dideuterated thiastearate sulfoxides, A. methyl 9-thiastearate *S*-oxide $10,10\text{-}d_2$ plus 3 equivalents of (S)-methoxyphenylacetic acid shift reagent B. methyl 10-thiastearate *S*-oxide $11,11\text{-}d_2$ plus MPAA C. racemic 9-thia sample plus MPAA.

The observed enantiomeric discrimination was illustrated by the formation of a Pirkle-type complexation model, (Figure #15). The model suggests that hydrogen bonding occurs between the sulfoxide oxygen and the MPAA carboxylic proton, and between the sulfoxide lone pair of electrons and the single MPAA hydrogen α to the carboxylate, (also acidic in nature). This complexation has through-space aromatic shielding of the C-8 protons of the major component opposing the through-bond deshielding effect of the sulfoxide-acid complexation. This arrangement would produce the observed spectra with the major sulfoxide enantiomer upfield of the minor one, for both H_A and H_B protons.

FIGURE #15 A Pirkle-style shift reagent complexation model for our dideuterated thiastearate sulfoxides, a typical Pirkle reagent is illustrated for comparison of structure.

2.4.3 Determination of absolute configuration

The proposed complexation model yields an (*R*) absolute configuration for our major biological thiastearate sulfoxide. Comparison to a similar synthetic standard of known absolute configuration was required to substantiate our suggestion. The use of *d*₈-dibutyl sulfoxide was believed to be suitable for this purpose.

Procedures for the synthesis of a dialkyl sulfoxide of mostly *R* configuration (47% ee, Scheme 11) were taken directly from the protocols of Douglass and Poole, ⁽⁶⁹⁾ Mislow *et al*, ⁽⁷⁰⁾ and Drabowicz, Legedz, and Mikolajczyk. ⁽⁷¹⁾

Scheme 11 : Synthesis of a Configurational Standard for Dideuterated Sulfoxides

The synthesis was initiated by saturating a dry, -20°C , dichloromethane solution of dibutyl disulfide with chlorine gas to yield the *n*-butylsulfur trichloride. Slow addition of a carefully measured amount of methanol (2 molar equivalents based on disulfide) produced the corresponding sulfinyl chloride. Removal of all but 10 mL of solvent afforded a concentrate that was used directly in the mentholation reaction. The menthyl ester thus produced was extracted and chromatographed before further reaction, ($[\alpha]_{\text{D}} - 44.5^{\circ}$, c 2.71, acetone; lit. $^{(70)} [\alpha]_{\text{D}} - 50^{\circ}$).

The (-)-*O*-menthyl *n*-butanesulfinate (47% de) was then treated with a perdeuterobutyl Grignard prepared from the commercially available d_5 -1-bromo-*n*-butane. Gentle hydrolysis of the resulting complex with aqueous ammonium chloride afforded the desired product. This dialkyl ("dibutyl") sulfoxide was therefore chiral (predominantly *R*) solely on the basis of deuterium content. Products (synthetic and biological) were analyzed by both ^1H -NMR and ^{13}C -NMR.

^1H -NMR spectral analysis of the α -sulfinyl hydrogens of the synthetic standard of known absolute configuration, under the influence of our novel MPAA shift reagent clearly showed the expected 47% ee. The spectral pattern was readily comparable to the spectra of our biological d_2 thiastearate sulfoxide products, (Figure #16).

FIGURE #16 500MHz ^1H -NMR spectra comparing standard d_7 -dibutyl sulfoxide to biological d_7 -stearate sulfoxide for absolute configuration determination, using MPAA as a shift reagent A. standard 47% ee predominantly (*R*)- d_7 -dibutyl sulfoxide (● *R*-enantiomer signal) B. racemic methyl 9-thiastearate *S*-oxide $10,10-d_7$ (● set of signals now refers to *S* configuration due to priority change) C. biological 9-sulfoxide plus a small amount of racemate to help show nearly absent enantiomer.

It is important to note that the reversal of enantiomer peak intensities between synthetic predominantly (*R*)-*d*₉-dibutyl sulfoxide and our biological *d*₂-thiastearate sulfoxides, does not designate the (*S*) configuration to our product. The dibutyl sulfoxide chirality is based on deuterium, while ours is based on the carboxylate. The predominant enantiomer is *R* in both biological and standard sulfoxides, but the non-deuterated α -sulfinyl positions are opposite one another, creating the observed switching of the peaks of greater intensity. It is this priority change which results in the assigned (*R*) absolute configuration for our biological sulfoxides.

The results thus supported our proposal that the oxidation produced a sulfoxide of the (*R*) configuration, relating perfectly to the pro-*R* hydrogen removal of stearyl CoA as previously reported. ⁽⁴⁾

In addition, the ¹³C-NMR spectra were run and suggested similar results (Figures #17, #18). Although significantly simpler to analyze, the ee determination of sulfoxide products by ¹³C-NMR, was less favoured than that of ¹H-NMR, due to the longer NMR acquisition times and the lower resolution between the peaks representing the two enantiomers.

FIGURE #17 ^{13}C -NMR spectra comparing MPAA effects on carbons near sulfur for standard 47% ee dibutyl sulfoxide and biological sulfoxide, A. predominantly (*R*)-*d*_r-dibutyl sulfoxide detailing clear splitting of enantiomer signals B. biological methyl 9-thiastearate *S*-oxide plus ca. 20% racemate, corresponding carbons (C_8 and C_7) yield signals that clearly illustrate the predominant enantiomer, but show poor separation of peaks for ee analysis. Note: mirror image-style peaks of C_{11} and C_7 signals reflect the mirrored effects of the shift reagent on the sulfoxide, (see Figure #19).

FIGURE #18 ^{13}C -NMR of predominantly (*R*)-*d*₅-dibutyl sulfoxide (47% ee) in the presence of MPAA, detailing enantiomeric discrimination on every carbon signal.

Observation of the mirrored enantiomer peaks for C-7 and C-11 in the ^{13}C -NMR spectrum (Figure #17B), clearly demonstrates that the effect of the shift reagent is opposite for carbon atoms on opposite sides of the sulfur. Again, these effects of enantiomeric discrimination and the observed directions of NMR signal shift can be explained by use of a Pirkle-type complexation model (now including the synthetic standard), for the shift reagent-sulfoxide interaction, (Figure # 19). Note however that the correlation between the standard and biological deuterated sulfoxides, leading to the assignment of configuration, does not depend on the correctness of the model.

FIGURE #19 The Pirkle-style complexation model for MPAA binding to our biological d_2 -sulfoxide (A) compared to that of standard predominantly (*R*)- d_2 -dibutyl sulfoxide (B). Note: major enantiomer in each case has the (*R*)-configuration.

Note added on the assignment of ^{13}C signals for dibutyl sulfoxide:

Due to the unique nature of our d_8 -dibutyl sulfoxide, we were initially able to assign carbon signals in the spectrum by observation of deuterium isotope shift effects as compared to non-deuterated dibutyl sulfoxide (Figure #20). In comparing our assignments with those previously published for dibutyl sulfoxide, ⁽⁷²⁾ we discovered a discrepancy. The C-2 and C-3 assignments were reversed. A quick unambiguous check by COSY and HETCOR (Appendix 1, Figure #40), showed the old report to be in error. In addition, their assignments for the same carbons in dibutyl sulfone were similarly flawed. (See reference 73 for a complete discussion)

FIGURE #20 500MHz ^1H -NMR spectrum of a 2:1 ratio of d_8 -dibutyl sulfoxide to d_0 -dibutyl sulfoxide detailing chemical shift differences caused by deuterium which aided in the assignments shown.

2.5 Stereochemical Analysis of Benzyl-Type Products

Use of benzyl-type probes in our lab commenced after Skala failed to observe splitting of the α -sulfinyl hydrogens of 9-thiastearate sulfoxide using a Europium-based chiral shift reagent. He proposed that if a more dissymmetric substrate could be oxidized by the system, then analysis of the enantiomeric excess of the product would be facilitated. ⁽⁵¹⁾ In a competition incubation of 4 probes (9- to 12-thiabenzyl) he found the 9-S substrate to be processed in 23% isolated yield (35% ee, by Kagan reagent), and only trace levels of the other sulfoxides. This low ee of the 9-thia sulfoxide and the trace yield of the 10-S product were not greatly favourable to validating the benzyl probes as substrates for the desaturase, (believed to be highly stereospecific and able to actively produce a sulfoxide from a 10-thia substrate). It was important therefore to confirm or refute these findings through individual incubations of the 9- and 10-thia benzyl probes.

2.5.1 Stereochemical analysis of sulfoxides derived from benzyl-type probes

The Kagan shift reagent was initially employed as a known chiral shift reagent for determining the ee's of numerous benzyl sulfoxides by ¹H-NMR.

The determined ee's of the benzyl analogues (Figure #21) were substantially higher than that previously found for the one (9-S) analogue by Skala, ⁽⁵¹⁾ (35% ee).

FIGURE #21 ^1H -NMR spectral excerpts (300 and 200MHz) of AB quartet patterns of various benzyl sulfoxides with the Kagan shift reagent, **A**. Previous 9-S results (ca. 70% ee) **B**. Present 9-S results showing higher ee (ca. 98%) **C**. Racemic sulfoxide sample **D**. Present 10-S results (ca. 88% ee).

The best substrate with respect to yield, methyl *S*-benzyl *S*-mercaptooctanoate, afforded sulfoxide bearing >98% ee, (second run results). The 10-*S* produced sulfoxide had an 88% ee. The observed values were similar to those determined previously in the thiastearate-type analogue studies; ie. higher ee's with the 9-sulfoxide than with the 10.

The shortened recovery and work-up procedures were not thought to be the reason for the observed increase in ee's over Skala's results, due to the finding that the ee's remained high even if the procedure was stretched out over the course of a week. The main contributing factor was partial racemization of the existing sulfoxide by the use of BF₃-etherate in the methylation step. In addition, the "second run" results show improvement over earlier ee determinations done by this author, (70%, 85%). These moderately-low ee values may have resulted from autoxidation of substrate during extensive suction filtration through celite, and/or from racemization of product sulfoxide by DCI-contaminated CDCl₃, ¹H-NMR solvent. ⁽⁷⁴⁾

An added note concerning possible bias to some aromatic sulfoxide ee's: A recent report by Kagan ⁽⁷⁵⁾ has suggested that partial enantiomeric separation occurs during flash chromatographic fractionation of certain aromatic sulfoxides. He found that *p*-tolyl methyl and benzyl *t*-butyl sulfoxides (Figure #22) from asymmetric syntheses of modest enantioselectivity, can be enantiomerically enriched by combining only early fractions during common silica gel flash chromatography, (from 86 to 99% ee and from 45 to 53% ee respectively).

Figure #22 Structures of sulfoxides known to exhibit enantiomeric separation upon flash chromatographic fractionation compared to one of our benzyl-type sulfoxides.

In all cases however, our validated probes were not as rigid about the sulfoxide as those tested in the report, and, our purification procedure generally called for combining of all sulfoxide-containing fractions, thus eliminating the fractionation required to observe such effects. Based on this knowledge it is suggested that enantiomeric separation due to flash chromatographic purification of our sulfoxides, did not greatly affect observed ee's.

2.5.2 Determination of absolute configuration

In order to determine the absolute configuration of these sulfoxides, an analogous synthetic standard of known configuration was required. It was believed that benzyl decyl sulfoxides were suitable for this purpose, (Figure #23).

Figure #23 A structural comparison of our 9-thiabenzyl sulfoxide product to the benzyl decyl sulfoxide standard

The crude (-)-*O*-menthyl ($\pm$)-benzylsulfinates were obtained from a summer student (R. Livingstone), and were carefully flash chromatographed to yield the individual diastereomers, (Table #1). The major (more polar) diastereomer, had a melting point and a rotation that compared favourably to literature values, (mp 66-67°C, lit.⁽⁶⁾ 75.7-76.5°C; $[\alpha]_D +99.7^\circ$, c 0.33, CHCl₃, lit.⁽⁷⁾ $[\alpha]_D +105^\circ$). Treatment of the sulfinic acid esters with the *n*-decane Grignard produced benzyl decyl sulfoxides of known absolute configuration, which were taken as comparable equivalents to our biological benzyl sulfoxide fatty acid products.

Table # 1 Data for purified (-)-*O*-menthyl ($\pm$)-benzylsulfinic acid diastereomers

Diastereomer (de)	R_f value on tlc (7% EtOAc/Hex)	Collected flash fractions	Yield (mg)	Specific rotations		200MHz ¹ H-NMR proton or menthyl C-1 (Appendix 1)
				EtOH c 0.25	CHCl ₃ c 0.33	
(-)- <i>O</i> -menthyl (+)-benzyl- sulfinic acid (96%)	0.05	68 - 108	241	+ 108	+99.7	3.888 ppm
(-)- <i>O</i> -menthyl (-)-benzyl- sulfinic acid (100%)	0.08	40 - 48	64	- 208	-217	3.898 ppm

Analyses by $^1\text{H-NMR}$ with shift reagents, by polarimetry, and by circular dichroism (CD) were performed for both synthetic benzyl decyl sulfoxides and the biological 9-S benzyl "alkyl" sulfoxide. Both the Kagan and the MPAA shift reagents demonstrated that our major biological product yields a signal which corresponds to the proton signal of the (*S*)-benzyl decyl sulfoxide enantiomer, (Figures #24, #25). Observed CD spectral patterns clearly showed a mirroring of traces for the (*R*)- and (*S*)-benzyl decyl sulfoxide standards, and showed a parallel-to-(*S*) trace for our biological sample, (Figure #26, Table #2). Optical rotation results also strongly correlated our biological sulfoxide with the (*S*)-benzyl decyl sulfoxide, (Table #2). All results therefore, suggested that the absolute configuration of our major biological sulfoxide product was *S*, (Note; due to a priority change from the benzyl group, an *S* configuration in these analogues is equivalent to the determined *R* configuration found in the "dialkyl" or thiastearate cases).

Table #2 Correlation of chiroptical data for (*R*)- and (*S*)- benzyl decyl sulfoxide (bds) with that of (*S*)- S-benzyl-8-mercaptooctanoic acid methyl ester, (biological).

Compound	$[\alpha]$ (CHCl_3 , c 2)	$[\alpha]$ (EtOH, c 2)	CD: $\Delta\epsilon$ (225, 257, 263, 270 nm.) ^a
(<i>R</i>)- bds	+ 77	- 4	- 12.74, - 0.26, - 0.29, - 0.23
(<i>S</i>)- bds	- 73	+ 4	+ 12.68, + 0.22, + 0.27, + 0.21
biological	- 73	+ 6	+ 11.1, + 0.19, + 0.21, + 0.17

^a The $\Delta\epsilon$ values were computed from molar ellipticities $[\Theta]$ which in turn were calculated from the ellipticity data shown in Figure #26 using the appropriate concentrations.

FIGURE #24 300MHz ¹H-NMR of a 2:1 mixture of *R:S* benzyl decyl sulfoxide under the influence of Kagan shift reagent. Inset; biological methyl *S*-benzyl 8-mercaptooctanoate *S*-oxide plus racemate (10%).

FIGURE #25 ¹H-NMR spectra detailing MPAA effects on a variety of benzyl sulfoxides, A. pure biological benzyl 9-sulfoxide B. biological benzyl 9-sulfoxide plus a small amount of racemate C. a 4:1 ratio of *S*:*R* benzyl decyl sulfoxide, suggesting the biological sample to be predominantly *S* D. racemic benzyl 9-sulfoxide.

FIGURE #26 Circular dichroism spectra with intensified inset of various benzyl sulfoxides, A. our biological benzyl 9-sulfoxide B. near pure synthetic (*S*)-benzyl decyl sulfoxide C. pure (*R*)-benzyl decyl sulfoxide.

The observed proton shifting for the two benzyl sulfoxide enantiomers under the influence of the MPAA shift reagent again substantiates our model for a Pirkle-style complexation between shift reagent and sulfoxide. (This is made clear by referring to the model in Figure #19 and by realizing that the diagnostic protons are in this case replacing the positions taken by deuterium, deshielded for the major enantiomer and shielded for the minor.)

Concerning the repeated observation of lower ee for the 10-S substrates over that of the 9-S series, there exists alternative explanations to the "lower yield amongst a constant level of autoxidation" theory previously mentioned.

It may be possible though that sulfoxidation of our 10-thia analogue is indeed less stereocontrolled. Perhaps hydrogen removal at this position in the native substrate is equally stereoselective (ca. 90%), or, perhaps some stereocontrol is lost upon forcing the enzyme to oxidize only the 10 position (and not the nine first). It is also possible that the supposed schemes for biosulfoxidation are different for our 9-S and 10-S substrates.

Oxidation of our analogue in the 9 position may follow the same initial mechanism as suspected for hydrogen removal (Scheme 12a), whereas sulfoxidation at the 10 position could be due to an iron hydroperoxy species, (Scheme 12b). The lower yield of the 10-sulfoxides could then be due to an inherently slower rate of reaction when the iron holds two oxygen atoms. Indeed, it has been reported that the formation of the iron-oxo species only occurs after substrate binding to the active site, ⁽¹⁾ thus allowing for a possible iron hydroperoxy - substrate interaction.

Scheme 12 : Two sulfoxide-yielding scenarios for the reaction of our sulfide probes with the desaturase

a, desaturation by the carbocation route, for comparison

b, formation of sulfoxide with high ee, 9 position illustrated but 10-positioned probe also amenable to this pathway

c, possible route to 10 sulfoxide products of lower ee, involving the hydroperoxy species

Our strongest belief however is that the ee's are reduced in comparison to the 9 position oxidation due to the lower yields, (autooxidation ratio theory). In turn, the lower efficiency of 10 sulfoxidation may reflect relative oxidant positioning between the two positions ie. a regiochemical difference.

In all cases tested so far, the results of our biological sulfoxidation experiments matched the stereochemistry of hydrogen removal suggested by Bloch's work in 1964 with isotopes. ⁽⁴⁾ His group reported that it was the pro-*R* hydrogens at the two positions (C-9 and C-10) being removed by the oxidant, which correlated well with our findings that suggest the oxidant to be stereoselective for reacting with the pro-*R* lone pair of electrons of our sulfide probes.

2.6 Regiochemistry of Desaturation

It is believed that the site of initial hydrogen atom abstraction in desaturase reactions is controlled by the position of the oxidant. Correlation of sulfoxide yields to sulfur position was thought to be indicative therefore of the site of initial attack in the parent reaction.

2.6.1 Regiochemistry based on thiastearate- and benzyl-type sulfoxide yields

Sulfoxides are quickly excreted from the cells and are therefore believed to be waste products. On this basis then, their rate of production by the catalytic cell system is not considered to be under strict metabolic control. This implies that the comparison of sulfoxide yields can be correlated to the relative oxidant position, without concern of interference from metabolic control factors.

Previous experiments comparing relative sulfoxide yields, incubated numerous substrates together in competition. Relative yield determination was deduced from observed relative abundances of characteristic fragment ions in a mass spectrum of the mixed sulfoxide sample. ⁽⁴⁹⁾ Comparison of yields between individual substrate incubations was sought for confirmation of the observed ratio of 9:10 sulfoxide products, (namely 2:1). Indeed, incubation of 9- and 10-thiastearate probes (22,23) individually did support the previous results, (9-sulfoxide, 10% isolated yield; 10-sulfoxide, 5% yield).

A similar experiment which determined the sulfoxide yield ratio from 9- and 10-*S*-benzyl analogues however, did not support the 2:1 result. Surprisingly the 9-*S*-benzyl analogue (25) was a significantly better substrate for sulfoxidation than even the corresponding 9-thiastearate. Incubations typically yielded 50% of substrate converted to recoverable sulfoxide, and only 5% for the 10-*S*-benzyl probe (26).

Our response to this result was to note that the thiastearates had been of equal chain length while the benzyl-type analogue lengths differed by one carbon unit, (see Figure #1 structures 25 and 26). A solution was proposed, suggesting that use of a probe of equal length to our best substrate, (25, 9-*S*-benzyl) but with sulfur in the C-10 position, may restore the expected product ratio. This 10 *S*-phenyl substrate was expected to yield roughly 25% sulfoxide (or better, based on the sulfur having a lower ionization potential than in the 9-*S*-benzyl probe, ca. 7.9 eV for ethyl phenyl sulfide versus 8.4 eV for benzyl methyl sulfide). ^(7b)

2.6.2 Phenyl-type probes

Methyl *S*-phenyl-9-mercaptononanoate (28) was thus expected to be both a good and valid substrate for sulfoxidation by the desaturase enzyme.

Both 9 *S*-phenyl (27) and 10 *S*-phenyl substrates were synthesized and administered, even though it was suspected that the 9 *S*-phenyl substrate would be unable

to bind and react with the active site. This assumption was based on the proximity of the sulfur to the bulky phenyl substituent and the suspected tight conformation of the active site pocket which forces the normal substrate to adopt a "cis" shape, (Figure #27).

FIGURE #27 A crude representation of the desaturase active site pocket, detailing how the *cisoid*-conformation requirement causes rejection of the 9 *S*-phenyl probe through steric interference.

Indeed, negligible levels (3%) of racemic sulfoxide were obtained from such an incubation. Again, our MPAA shift reagent was successful in allowing this determination of racemic character for the sulfoxide product, (Figure #28B).

The incubation of the 10 *S*-phenyl probe was performed alongside the 9-*S* probe experiment. Surprisingly, the results mimicked that obtained for the 9 *S*-phenyl substrate, affording only 3% yield of racemic sulfoxide, (Figure #28C), likely entirely due to autoxidation.

FIGURE #28 400MHz ¹H-NMR spectra of methylene protons adjacent to phenyl sulfoxides under the influence of 3 equivalents of MPAA, A. a racemic sample B. partially pure recovered 9-sulfoxide, C. crude flash-isolated 10-sulfoxide. Note: low yields of sulfoxide helped usually low levels of impurities to be more intrusive in comparison; least obscured peaks (labelled).

The proposal that our probe was autoxidized was based on our findings that desaturase-mediated sulfoxidation is normally a highly enantioselective process, on the similarity of the results to those of the 9 *S*-phenyl experiment, (where steric effects dictated no enzymic involvement), and on the known lower redox potential of thiaphenyls, (compared to methylene-insulated sulfides, eg. 22-26). In addition, the autoxidation theory was supported by a previous report that an aryl sulfide was 5% converted to racemic sulfoxide by a Baker's yeast culture. ⁽⁷⁹⁾

The lower yield of the 10 *S*-phenyl sulfoxide, as well as those previously found for the 10 *S*-benzyl analogue, could be due to a competing reaction involving oxidation/hydroxylation at position 9 and subsequent chain cleavage (Scheme 13b). This reaction would also account for the problems encountered with mass balancing the analogue. It had been generally found that up to 20% of the substrate would be consumed by the culture and not recovered. Previously, this had been explained by extractibility and possible incorporation of the label into cellular components, (including the beta-oxidation of exogenous fatty acids). Now, this new explanation would require consideration. Unfortunately, the product resulting from cleavage with sulfur in position 9, (benzyl alcohol, Scheme 13a), was found to be fully water soluble and undetectable at proposed levels through extraction. Also, the thiophenol cleavage product (sulfur position 10) was somewhat water soluble and difficult to detect at low levels from a culture extract.

Scheme 13 : Mechanisms for chain cleavage of probes a, with sulfur in position 9
b, with sulfur in position 10.

Based on the huge yield difference between the equal length substrates, methyl *S*-benzyl-8-mercaptooctanoate (25) and methyl *S*-phenyl-9-mercaptononanoate (28), considerable amounts of the thiophenol cleavage product, if produced, could accumulate in culture. The toxic nature of this thiol by-product demanded careful analysis of such a possibility. It was determined that ca. 15mg of administered thiophenol did have an inhibitory effect on the growth of the yeast. Inhibition of growth was not observed however for 28-incubated cultures, suggesting that cleavage was not occurring at these high levels.

Investigation of low cleavage levels, as well as the possibility of increasing the assay sensitivity, diverted us to a study involving naphthyl compounds.

2.6.3 Naphthyl-type probes and the search for cleavage products

The probe methyl 10-(2-naphthyl)-9-thiadecanoate (**31**) was tested as a substrate in our system. The results of incubations yielded 12% sulfoxide with 75% ee, as determined by both shift reagent experiments, (Figure #29). Although not nearly as good a substrate as our 9 *S*-benzyl analogue, this very similar but slightly longer probe was undoubtedly a substrate to the same system. Yet again, as with the benzyl results, the observed shifting effects in the NMR spectrum were in complete agreement with that expected from our complexation model for the MPAA reagent. The absolute configuration of the major sulfoxide enantiomer, was considered to be (*S*) on the basis of similarity to the benzyl NMR results. This assignment was also in correlation with the model for shift reagent-sulfoxide binding.

In a similar fashion then, the substrate (**30**) of equal chain length with sulfur in position 10 was tested. Again, as with the 10 *S*-phenyl results, the sulfoxide produced was racemic (2% yield). Although insufficient material was isolated to allow ee determination, the effects of MPAA on a racemic standard was tested, (see Figure #41 in Appendix 1 for spectral result).

FIGURE #29 ¹H-NMR spectra of AB quartet patterns from naphthylmethyl sulfoxide samples with shift reagents for determining ee's, A. (400MHz) biological 9-sulfoxide plus MPAA B. (400MHz) same as A. plus a small amount of racemate C. (200MHz) biological 9-sulfoxide plus Kagan D. (200MHz) racemic 9-sulfoxide plus Kagan. Note: although one enantiomer of the AB pattern collapses into a singlet, ee could still be estimated from integral ratios between the singlet and the quartet.

We looked for the cleavage product, 2-naphthaldehyde, and its suspected reduction product, 2-naphthalene methanol, in the 9-S naphthmethyl (**31**) incubation. Both tlc and ^1H -NMR analysis of extracts (media and cells) suggested levels of these two cleavage products were too low for detection, indeed, the addition of 2-naphthaldehyde to a typical incubation at a level corresponding to 0.2% cleavage of substrate, yielded 2-naphthalene methanol, as a readily extractable and tlc (UV) detectable product. This gave good evidence that cleavage was not occurring at substantial levels for our aromatic 9-thia analogues.

The search for the cleavage product resulting from the corresponding 10-S probe, 2-naphthalenethiol, was equally fruitless. Neither tlc nor ^1H -NMR analysis of the extracts (cell and media) could provide evidence for the existence of this product. The suspected aromatic cleavage product was shown to be both extractable and detectable when fed to a growing yeast culture at a level corresponding to 2% cleavage of substrate.

For both incubations, the cells were hydrolyzed, extracted, and analyzed to again yield no detectable cleavage products.

This work, although not able to fully refute the cleavage hypothesis, did suggest that the comparative yields between corresponding 9-S and 10-S substrates was not affected greatly by losses through cleavage of the probes.

The present hypothesis for the apparent failure of the 10-S-phenyl probe as a substrate, is partly based on the steric environment of the active site, which normally imposes a cisoid conformation on the substrate, (Figure #30). The 10-S substrates which

have sulfur directly bonded to an aromatic substituent would not be able to conform to the *cisoid* structure if, as was found, ⁽⁶⁾ the adjoining bond had at any time sufficient double bond character to hold the ring coplanar with positions 9, 10 and 11. This was especially true if the enzyme were to attempt removal of an electron from a lone pair on the sulfur atom. The resulting sulfur radical cation, would be resonance stabilized by the aromatic ring. The double bond character thus introduced, would cause the steric interference (between the *ortho*-ring hydrogens and C-8 hydrogens) to disrupt the *cisoid* conformation binding requirement.

FIGURE # 30 A crude active site model for the desaturase detailing the role of the *cisoid* requirement in determining probe acceptability A. 9-thiastearate probe B. 9-thiabenzyl, conforms by a tilting of its aromatic ring to avoid *cis*-enforced steric hinderance between C-8 hydrogens and *ortho* ring hydrogens C. 10-thiabenzyl substrate D. the 10-thiaphenyl probe would be rejected due to a steric clash between *ortho* ring and C-8 hydrogens when sulfur radical cation stabilization disallows ring tilting.

In another attempt at producing a better comparison between aromatic substrates, the chain length of the 10 *S*-benzyl analogue was maintained and the position of the sulfur atom moved. Although the 10 *S*-benzyl had not been a very good substrate for producing sulfoxide quantity (4-6%), it was converted with relatively high enantioselectivity (88% ee).

The first trial employed methyl (11-phenyl)-9-thiaundecanoate (NVo) as a substrate (supplied by N. Vo, summer student). Sulfoxide was produced with high enantioselectivity (>98% ee, Figure # 31), but with a lower yield (17%) than in the 9 *S*-benzyl case. In comparison with the equal length 10-*S* substrate however, this 9-*S* aromatic analogue was a better substrate, as was expected. The isolated yields of these isomers (17% versus 5%) suggested an even greater than doubled preference for oxidation of the C-9 position over that of the C-10.

All similar substrates differentiated by sulfur position, showed the 9-*S* oxidation favoured over that of the 10-*S* oxidation. This suggests that the initial site of attack on stearyl-CoA is at carbon position 9. The ability of the enzyme to enantioselectively oxidize sulfur in position 10 with fair yields suggests that the position of the oxidant may be between the C-9 and C-10 positions, (assuming the iron-bound oxo scheme for oxidation).

FIGURE #31 200MHz ^1H -NMR spectra of the C-10 methylene hydrogens of methyl 11-phenyl 9-thiaundecanoate *S*-oxide under the influence of MPAA A. biological sample showing >98% ee B. biological sample plus ca. 40% racemate C. racemic standard sample showing clear peak doubling compared to biological sample A.

2.7 Exploring Novel Assay Methods

2.7.1 Use of ^{19}F -NMR to follow probe fates

In a final comparison between aromatic substrates, a novel approach to yield analysis was attempted. The two substrates would be incubated together in one culture as a competition-style assay, and the production of sulfoxide would be followed by NMR of a suitable nucleus. Fluorine was chosen as a diagnostic nucleus for its high sensitivity to electronic environment, and for its uniqueness in the nuclear composition of the incubations.

As an assurance that fluorinated probes would be valid substrates for the desaturase, a 9 *S*-benzyl analogue was synthesized with a *para*-trifluoromethyl tag on its aromatic ring. Incubation resulted in 26% conversion to a sulfoxide of high ee, (96%, Figure #32). On the basis of this result, combined with those of the ee-validated benzyl and phenethyl probes, future efforts concentrated on demonstrating the usefulness of ^{19}F -NMR as an assay for enzyme activity, (not on ee analysis).

The fluorinated 10-*S* and 9-*S* substrates (32,33) were structurally analogous to their benzyl and phenethyl counterparts, respectively. These substrates, combined with the assay technique, would also be beneficial to supporting or refuting our previous cleavage product experiment findings.

FIGURE #32 200MHz ¹H-NMR spectra of the AB benzylic hydrogens of biological methyl *S*-(*p*-trifluoromethyl)-benzyl-8-mercaptooctanoate *S*-oxide for ee determination, A. nearly collapsed AB quartet of sulfoxide without MPAA shift reagent B. biological sample with MPAA C. same as B with added racemic sulfoxide. (Arrows indicate minor enantiomer, X = impurity)

For the purposes of a full comparison (to present and past results) each substrate was administered both individually and together in competition. Two controls were also run, a blank without substrate, and a competitive feeding with autoclaved (dead) cells from a grown out culture. Aqueous samples were taken at various times of incubation, allowed to settle, stored frozen, then thawed for direct supernatant analysis by NMR.

^{19}F -NMR showed no fluorine signal in the spectral region of concern (-50 to -75ppm) for the blank control, (for signals of sample standards, see Appendix 1, Figure #42). Analysis of the autoclaved cell sample yielded a set of signals (corresponding to the two substrates) that slowly intensified with increasing incubation time. This suggested that the gently shaken cultures were slowly dissolving the dense substrate droplets of the two probes at an equal rate. The substrate-treated growing cultures all displayed the expected results, with only one small twist.

Firstly, and as may have been expected, the sulfoxide from the 9-S substrate was quickly formed, readily detectable even after only 2 hours of incubation, (Figure #33A). The 10-S product was relatively very slow to build up to detectable amounts, and was only readily visible after about 20 hours of incubation, (Figure #33B). No cleavage products were detected in any of these experiments, supporting our conclusions from the naphthyl analogue studies. The presence of an impurity (X) in the 9-S probe sample (possibly the corresponding phenethyl bromide) was found to be occasionally useful as an internal "standard", since the absolute chemical shifts of the various compounds often fluctuated while their relative positioning to one another remained nearly constant.

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FIGURE #33 ^{19}F -NMR spectra comparing rates of sulfoxidation for 9-thia and 10-thia methyl 11-(*p*-trifluoromethyl)-phenyl undecanoate substrates, A. 9-sulfoxide appeared to be readily detectable after only 2 hours of incubation B. 10-sulfoxide required about 20 hours of incubation to reach observable levels. (? = unknown, X = constant impurity to 9-S substrate sample)

The surprising discovery was that the substrates seemed to be hydrolyzing to the free acid at an alarming rate. After only 10 hours the amount of 9-S substrate which was left (not sulfoxidized) was divided almost equally (ca. 45:55) into two peaks. 0.04ppm apart, (Figure #34b). The peaks closely coincided to those of the methyl ester and free acid forms of the sulfide, (see reference spectra in Appendix 1, Figure #42). Similar standards for the 10-S substrate's ester and acid forms however, did not show a resolvable chemical shift difference when dissolved in a sample of the blank medium. The lack of a substantial free acid peak in the dead cell control even after 31 hours of incubation (Figure # 34a) suggests the observed apparent hydrolysis to be actively yeast-mediated. Assuming this involvement then, it is likely that the 10-S substrate is also undergoing the same rate of hydrolysis, (an assumption unfortunately not verifiable by ^{19}F -NMR).

Since the samples are relatively cell-free upon analysis, the finding suggests that the yeast cells import the methyl ester and export the free acid and the sulfoxide. What remains unclear is the ability of the cells to retrieve the sulfide free acid and perform the sulfoxidation. A simple experiment wherein only the free acid form of the substrate was fed would readily determine the answer to this question. If significant levels of sulfoxide product resulted from such an experiment, then it would show that the yeast can accept the free acid as a substrate.

FIGURE #34 ^{19}F -NMR spectra showing evidence for yeast-mediated hydrolysis of starting material to the free acid, A. a dead cell control showed only the two major peaks for starting ester substrates B. after 10 hours of live-culture incubation it appeared as though the 9-thia substrate had undergone hydrolysis to a considerable extent; the 10-thia substrate had an unsuitable standard for differentiation between its ester and acid forms.

2.7.2 Wickerman's chemically defined media

An attempt at observing product sulfoxide, including ee analysis, involved use of a culture media, sufficiently defined in content, so as to perhaps allow direct ^1H -NMR observation of product in the crude extract. Growth of cells in the chosen, dextrose-enriched, Wickerman's medium, ⁽⁸⁰⁾ was slower than that in the standard YPD culture, but was considered sufficient for observing desaturase activity.

The use of this chemically defined media was occasionally beneficial in the following ways. It often lessened the severity of the emulsions typically encountered in the media extraction procedure. It sometimes allowed for the omission of the column chromatography step by providing a sufficiently clear ^1H -NMR spectrum that the product's ee (for the benzyl case) could be directly determined using the crude methylated extract, (Figure #35).

FIGURE #35 200MHz ^1H -NMR spectra of crude methylated extracts from incubations of our best substrate (25) A. Wickerman's chemically-defined media, note readily detectable AB quartet of benzyl sulfoxide, 3.9 - 4.1 ppm, B. YPD media, yields a significantly dirtier spectrum.

Disadvantages included slower cell growth and a resulting lower yield of sulfoxide product. It also introduced increased complexity in the making up of the growth media. One great concern was small background spectral peaks being mistaken for a detectable amount of the other sulfoxide enantiomer during ee determinations. Observation of such a noise signal before the addition of shift reagent was suggestive of it not being the other enantiomer, but was then in the way of allowing any of the lesser enantiomer to be detected, (eg. 9-thiabenzyl (25) incubation product, Figure #36).

The given example illustrates a result of incubation of our best substrate with respect to yield. Other probes afford less sulfoxide and would therefore yield spectra with a heightened relative level of interfering background signals.

There were sufficient drawbacks to the method to outweigh the advantages, and thus it was decided to abandon further research using this media.

FIGURE #36 200MHz ¹H-NMR spectra of AB benzylic hydrogens of biological 9-S-benzyl sulfoxide as extracted from Wickerman's chemically-defined medium without isolation, A. sample without shift reagent B. under the influence of the Kagan shift reagent C. with excessive racemic sulfoxide added. (Notes: most impurities (1-4) remain constant; others (X) seem less predictable)

The discovery of the MPAA shift reagent and formation of the Pirkle-type complexation model, depended on the specific binding between acidic protons of the reagent and the one oxygen and lone pair of electrons of the sulfoxide. This specificity was supported when attempted complexation to sulfide and sulfone substrates, failed to show any significant ^1H -NMR shifting effect, (eg. sulfone, see Appendix 1, Figure #43).

2.8.1 Comparison to other acids and reagents

Besides the aforementioned diphenyl acetic acid and (S)-methoxy phenyl acetic acid, other organic acids were used to help expand the knowledge of the sulfoxide binding affinity. Acetic acid (a), monochloroacetic acid (b), phenylacetic acid (c), benzoic acid (d), and 2-trifluoromethyl-2-methoxy-2-phenylacetic acid (e) all caused at least partial separation (increased differentiation) of the methylene protons next to the sulfoxide, (examples in Figure #37). It was suggested that the amount of shift observed was proportional to the pKa of the acid. Those with relatively high pKa's (4.0 and higher, a, c, d) showed little effect, while those of lower pKa values (b, e) yielded significantly stronger effects. Our diphenyl- acetic acid and methoxyphenylacetic acid fit into this range of low (below 4) pKa values.

FIGURE #37 200MHz ^1H -NMR expansions illustrating the effects of certain organic acids on the chemical shifts of the α -sulfinyl (AB-type) hydrogens of a thiastearate *S*-oxide A. the signal without acid reagent B. with acids of $\text{pK}_a > 4.0$; acetic acid (a, pK_a 4.7), benzoic acid (d, pK_a 4.2) C. with acids of $\text{pK}_a < 4.0$; 2-trifluoro-2-methoxyphenylacetic acid (e), monochloroacetic acid (b, pK_a 2.8).

During a literature search on the subject of shift reagents for sulfoxides, it was found ⁽⁶⁷⁾ that use of trifluoroacetic acid caused an increased differentiation in the diastereotopic proton signals of *para*-substituted benzyl phenyl sulfoxides. The proton that was noted to be more deshielded by the complexation effect was not given an unambiguous assignment by the author of that work. This question went unanswered for acyclic sulfoxides until interest from our group spawned an experiment designed to afford a concrete solution. Enantiomerically enriched methyl 10-*d*, 9-thiastearate was synthetically prepared by B. Behrouzian from our lab, then incubated in yeast culture in the normal fashion. Isolation of the sulfoxide product and analysis by ¹H- and ²H-NMR with and without acid shift reagent, clearly showed a decrease in intensity for one of the two peaks representing the A- and B-type hydrogens. The result demonstrated that the proton *syn* to the oxygen of the sulfoxide yields the more deshielded signal in the ¹H-NMR spectrum. Thus, concerning the complexation to MPAA, the observed greater enantiomeric shift effect for the downfield set of signals (refer to Figure #14, $\Delta\delta$ 0.012ppm vs $\Delta\delta$ 0.007ppm) can now be attributed to MPAA's effects on that hydrogen *syn* to the sulfoxide oxygen.

Non-acidic reagents such as the Pirkle shift reagent, the Kagan shift reagent, methyl diphenylacetate, and methyl methoxyphenylacetate, were shown to have no detectable effect on the methylene proton signals concerned.

2.8.2 Other sulfoxides analyzed by MPAA

The methoxyphenylacetic acid reagent was first invented for determination of the ee of the dideuterated thiastearate sulfoxide product, (22-10,10- d_2). However, there are a host of other sulfoxides for which MPAA has allowed enantiomeric discrimination. Some of which include naphthyl analogues where the Kagan reagent worked but only with unclear results, the S-phenyl sulfoxide products, and the S-phenethyl sulfoxide.

A variety of synthetic sulfoxides were also tested, (courtesy of professors T. Durst and H. Holland). Those which were readily analyzed by the system included benzyl methyl sulfoxide, benzyl *n*-propyl sulfoxide, benzyl *n*-butyl sulfoxide, (*p*-carboxymethyl)-benzyl methyl sulfoxide, (*p*-nitro)-benzyl methyl sulfoxide, (*p*-trifluoromethyl)-benzyl methyl sulfoxide, phenyl methyl sulfoxide, phenyl *n*-butyl sulfoxide, and (1,1-dimethyl)-phenethyl phenyl sulfoxide, (for structures see Figure #38). Observed enantiomeric splitting was noted for lone methyl groups on sulfur, either one or both of the benzylic (AB) methylene hydrogens, and/or the AB-style hydrogens of the dimethyl-phenethyl sulfoxide lone methylene, (example , Figure #39).

In general, the MPAA reagent was found to have a much broader range of acceptable sulfoxide substrates than the Kagan reagent, which is usually limited to sulfoxides with simple ^1H -NMR signals, (for example see Appendix 1, Figure #44). MPAA has allowed the determination of absolute configuration for more than 70 tested sulfoxides to date, by use of the Pirkle-type complexation model.

FIGURE #38 Structures for sulfoxides that showed enantiomeric ^1H -NMR signal splitting upon complexation with (*S*)-(+)-methoxyphenylacetic acid, (MPAA).

FIGURE #39 200MHz ^1H -NMR showing application of MPAA to use in determining ee's for sulfoxides; example: optically-active (*p*-trifluoromethyl)-benzyl methyl sulfoxide A. sample without shift reagent (tight, unresolved AB quartet signal enlarged) B. with 0.8:1 mole ratio of MPAA:sulfoxide (note methyl peak doubled) C. with 3 equivalents MPAA (arrow = minor enantiomer). (Compound courtesy of H. Holland)

3.1 Synthesis

3.1.1 General

The ^1H NMR (200, 400, and 500 MHz), ^{13}C NMR (50.29, 100.6, and 125.8 MHz) and ^{19}F -NMR (376.5 MHz) spectra were recorded on Varian XL 200, Bruker AMX 400, and Bruker AM 500 spectrometers. Abbreviations used in assigning peaks were the following: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; b, broad; i, imperfect or resembling. Chemical shifts are expressed in ppm with reference to tetramethylsilane (TMS) using deuteriochloroform (CDCl_3) as solvent. Chiral shift experiments on biological and optically active sulfoxides were carried out using CDCl_3 treated with basic alumina and anhydrous magnesium sulfate.

The elemental analyses were obtained on a Perkin-Elmer Elemental Analyzer 240C operated by Ann Webb at the Biological and life Sciences Department at N.R.C. Mass spectra were obtained by direct probe for sulfoxides or by GC/MS for most other final products using a VG 7070E mass spectrometer at 70eV, interfaced with a Varian 3300 GC equipped with a 30m megabore DB-5 column, (temperature program 100°C to 300°C at 10°C/min, carrier gas helium at 20mL/min, operator Clem Kazakoff, Ottawa U., with DEC PD P8 data collection system).

All polarimetric measurements were carried out at 20°C. Optical rotations were carried out on a Perkin-Elmer model 141 polarimeter at 589nm, equipped with a 1ml sample cell of length 10cm. Circular dichroism (CD) spectra were obtained on a JASCO J-600 using a cuvette pathlength of 1cm, operated by Dr. Gordon Willick at M51, at N.R.C. Montreal Road.

Melting point determinations (uncorrected) were performed using a Buchi SMP-20 melting range apparatus, or by employing a thermometer in a stirred cold water bath engulfing a sample in a vial, (for compounds melting between 2 and 20°C).

Analytical tlc was performed using Merck glass plates precoated with silica G/UV 254. Visualization of UV-active components was performed by use of a hand-held UV lamp at 254nm and/or by a UVP inc. Chromato-vue UV cabinet (model C-70 G) at 365nm. UV-inactive materials were visualized by using a combination of iodine vapour followed by a water spray; regions of sufficient hydrophobicity showed up as white spots on a grey background.

Most samples were purified by flash chromatography using Merck Silica Gel 60 (230-400 mesh) as a stationary phase and a hexane/ethyl acetate solution as a mobile phase, in one of three available column systems (11mm inner diameter, 25cm length; 22mm i.d., 35cm l.; 32mm i.d., 50cm l.) with ca. 50 kPa air pressure. Some small sized samples were further purified by bulb to bulb distillation at reduced pressure using a Buchi brand Kugel-Rhor boiling point/distillation oven and either a water aspirator or an Edwards 8 two stage high vacuum pump (model E2M8, operated without a gas ballast, system pressure measured on an Edwards Pirani 501 needle gauge linked to a

model PRE10K gauge head).

Unless otherwise stated, all reagents and starting materials were purchased from Aldrich Chemical Company and used without purification. Chiral compounds were used without attempting to improve their optical purity. All air- and moisture-sensitive reactions and distillations were performed under nitrogen (Liquid Carbonic Inc., lab grade) that had been passed through a cell containing Drie-Rite brand absorbant chips to remove water.

All organic solvents for reactions, extractions, cleaning, and chromatography were distilled in house prior to use, or were 'absolute' or 'spectral grade' quality. Dry ether was freshly distilled as required from LiAlH_4 and collected in a septum-sealed 3-neck RB containing molecular sieves. Tetrahydrofuran (THF) was distilled in a similar fashion but on a larger scale and stored in a stopcock- and septum-sealed receiver vessel. Dimethyl sulfoxide (DMSO) was distilled *in vacuo* from CaH_2 and stored over molecular sieves under nitrogen. Transfers of these anhydrous solvents and other moisture-sensitive liquids was aided by use of oven-dried ground glass syringes with metal needles.

Solvents were removed *in vacuo* on a Buchi ROTOVAPOR R, rotary evaporator, (water bath temperatures not exceeding 40°C for biological extracts or chiral materials, pressure ca. 10mmHg by water aspirator). Solvents from small samples in vials were removed with gentle heating under a nitrogen stream via a Pierce Reacti-Therm heating module.

All pH determinations were done using pH indicator papers having both wide and narrow pH ranges.

'Oven-dried' refers to clean glassware or equipment that had been stored in a Fisher Isotemp oven (model 501) at 130°C for at least 12 hours.

3.1.2 Synthesis of probes

A. Synthesis of Thiols

Example; 8-thiooctanoic acid (1)

An oven-dried 2 litre rb flask was fitted with a condensor and cooled under a gentle flow of nitrogen. To this was added 25.4g (114mmol) 8-bromooctanoic acid, 200mL absolute ethanol, 9.1g (120mmol) thiourea, and some boiling chips. The mixture solvated with added heat, and this solution was refluxed for 2.5 hours. The now pale yellow solution was taken off the heat source and allowed to cool for 20 minutes. 400mL of a freshly prepared 2M sodium hydroxide solution (ethanol:water 1:1) was added, resulting in a white cloudy mixture which clarified upon reheating. Reflux for 1.5 hours effected the hydrolysis, and was followed by cooling to room temperature and dilution with 350mL water. This mother liquor was placed in a large separatory funnel and was allowed to stream slowly (5 min.) into an efficiently stirred mixture of sulfuric

acid in dichloromethane (180mL 6M H_2SO_4 , 400mL CH_2Cl_2) in a 2 litre Erlenmeyer flask. The resultant aqueous layer was further extracted (3 X 70mL CH_2Cl_2) and the organics combined. This organic was washed with acidified saturated sodium chloride solution (2 X 50mL; 5 drops 6M H_2SO_4 in 100mL NaCl solution), dried over anhydrous sodium sulfate (ca. 30g, 1 hr.) and then evaporated *in vacuo* (4-5 hrs., $T = 47^\circ\text{C}$) to yield 20.18g of a white lard-like solid.

a. 8-thiooctanoic acid (1): R_f 0.34-0.37 (60% EtOAc/Hex); ^1H NMR (200 MHz) 2.1-2.5 (1H, s, HSCH_2), 2.4-2.6 (2H, t/m, HSCH_2CH_2), 2.20-2.35 (2H, t, $\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.5-1.7 (4H, bm, $\text{SCH}_2\text{CH}_2\text{CH}_2$ and $\text{CH}_2\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.0-1.4 (6H, b, $\text{SCH}_2\text{CH}_2(\text{CH}_2)_3\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$).

b. 4-thiobutanoic acid (2): R_f 0.19-0.25 (60% EtOAc/Hex); ^1H NMR (200 MHz) 2.1-2.5 (1H, s, HSCH_2), 2.4-2.6 (2H, t/m, HSCH_2CH_2), 2.20-2.35 (2H, t, $\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.7-1.9 (2H, m, $\text{SCH}_2\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$).

c. (para-trifluoromethyl) phenyl methanethiol (3): ^1H NMR (200 MHz) 7.35-7.65 (4H, 2d, phenyl), 3.75-3.85 (2H, d, $\text{phenylCH}_2\text{SH}$), 1.7-1.9 (1H, t, CH_2SH).

d. 10-thiodecanoic acid (4): R_f 0.39-0.41 (60% EtOAc/Hex); ^1H NMR (200 MHz) 2.1-2.5 (1H, s, HSCH_2), 2.4-2.6 (2H, t/m, HSCH_2CH_2), 2.20-2.35 (2H, t, $\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.5-1.7 (4H, bm, $\text{SCH}_2\text{CH}_2\text{CH}_2$ and $\text{CH}_2\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.0-1.4 (10H, b, $\text{SCH}_2\text{CH}_2(\text{CH}_2)_5\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$).

e. 11-thioundecanoic acid (5): R_f 0.44-0.47 (60% EtOAc/Hex); ^1H NMR (200 MHz) 2.1-2.5 (1H, s, HSCH_2), 2.4-2.6 (2H, t/m, HSCH_2CH_2), 2.20-2.35 (2H, t, $\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.5-1.7 (4H, bm, $\text{SCH}_2\text{CH}_2\text{CH}_2$ and $\text{CH}_2\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.0-1.4 (12H, b, $\text{SCH}_2\text{CH}_2(\text{CH}_2)_6\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$).

B. Synthesis of sulfide acids (Method 1; thio-acid + bromo-compound)

Example; 9-thiastearic acid (6)

A dry, 3-neck, 250mL rb flask was equipped with a stirring bar, a rubber septum, a condensor, a glass stopper, and a nitrogen atmosphere. To this was added 4.17g (approx. 22mmol) cold 8-thiooctanoate, followed by 28mL dry THF and 8mL

hexamethylphosphoramide (HMPA). The mixture was stirred, (warmed by the still hot glass), for 5 minutes to dissolve the thiol, before being cooled by stirring on an ice-water bath. Approximately 2 equivalents of n-butyllithium in hexanes (30mL, 1.5 M, 45mmol) was then added by syringe as a slow stream over a two minute period, and allowed to stir 40 minutes further. 1-Bromononane (4.4g, 21mmol) was then injected in dropwise to the stirring, ice-cold yellow mixture. (Note: a solid brominated compound would be added as a concentrated solution in THF) The mixture was stirred for 22 hrs. at room temperature before being quenched by the injection of 10mL of distilled water. Transferred to a separatory funnel, (effected by rinses in order with 10mL dH₂O, 5mL 3 M HCl, 5mL hexane), the mixture was acidified to below pH 2 by addition of approx. 7mL of 6 M HCl. 10mL more hexane was added, the mixture gently shaken, and the hexanes removed. The aqueous was extracted further with four 15mL portions of hexane, and finally with two 15mL portions of CH₂Cl₂. The CH₂Cl₂ extracts were rotary evaporated to yield a yellow oil (mostly composed of HMPA) which was rinsed/extracted three times with 15mL portions of hexane. All hexanes were combined, washed twice with 20mL 0.1 M HCl, dried over anhydrous sodium sulfate, and rotary evaporated to yield 4.44g crude 9-thiastearic acid.

a. 9-thiastearic acid (6): mp 61-62°C; R_f 0.30-0.37 (47% EtOAc/Hex); ¹H NMR (200MHz) 2.35-2.50 (4H, t, CH₂SCH₂), 2.20-2.35 (2H, t, CH₂CH₂C(O)OH), 1.5-1.7 (6H, bm, CH₂CH₂SCH₂CH₂ and CH₂CH₂CH₂C(O)OH), 1.2-1.4 (18H, b, CH₃(CH₂)₆CH₂CH₂SCH₂CH₂(CH₂)₃CH₂CH₂C(O)OH), 0.8-0.9 (3H, t, CH₃(CH₂)₆).

b. 9-thiastearic acid-10,10-*d*₂ (6-10,10-*d*₂): ¹H NMR (200MHz) similar to that of 6 except for 2.35-2.50 (2H, t, CD₂SCH₂).

c. 10-thiastearic acid (7): mp 61-62°C; R_f 0.32-0.38 (47% EtOAc/Hex), R_f 0.40-0.45 (65% EtOAc/Hex); ¹H NMR (200MHz) 2.35-2.50 (4H, t, CH₂SCH₂), 2.20-2.35 (2H, t, CH₂CH₂C(O)OH), 1.5-1.7 (6H, bm, CH₂CH₂SCH₂CH₂ and CH₂CH₂CH₂C(O)OH), 1.2-1.4 (18H, b, CH₃(CH₂)₅CH₂CH₂SCH₂CH₂(CH₂)₄CH₂CH₂C(O)OH), 0.8-0.9 (3H, t, CH₃(CH₂)₅).

d. 10-thiastearic acid-11,11-*d*₂ (7-11,11-*d*₂): ¹H NMR (200MHz) similar to that of 7 except for 2.35-2.50 (2H, t, CD₂SCH₂).

e. 5-thiastearic acid (8): R_f 0.34-0.38 (65% EtOAc/Hex); ¹H NMR (200 MHz) 2.4-2.6 (4H, t, CH₂SCH₂), 2.2-2.3 (2H, t, CH₂CH₂C(O)OH), 1.7-1.9 (2H, m, SCH₂CH₂CH₂C(O)OH), 1.5-1.7 (2H, m, CH₂CH₂CH₂S), 1.2-1.4 (20H, b, CH₃(CH₂)₁₀CH₂CH₂S), 0.8-0.9 (3H, t, CH₃(CH₂)₁₀).

f. *S*-benzyl-8-mercaptooctanoic acid (9): ¹H NMR (200MHz) 7.2-7.4 (5H, m, phenyl), 3.68-3.72 (2H, s, phenylCH₂S), 2.35-2.55 (2H, t, SCH₂CH₂), 2.20-2.35 (2H, t, CH₂CH₂C(O)OH), 1.5-1.7 (4H, bm, SCH₂CH₂ and CH₂CH₂CH₂C(O)OH), 1.2-1.4 (6H, b, SCH₂CH₂(CH₂)₃CH₂CH₂C(O)OH).

g. *S*-benzyl-9-mercaptononanoic acid (10): R_f 0.24 (60% EtOAc/Hex); ^1H NMR (200MHz) similar to that of 9 except for 1.2-1.4 (8H, b, $\text{SCH}_2\text{CH}_2(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$). Note; 10 also made by Method 2.

h. 10-(2-naphthyl)-9-thiadecanoic acid (11): ^1H NMR (200MHz) 7.4-7.9 (7H, 2m, naphthyl), 3.84-3.85 (2H, s, naphthyl CH_2S), 2.35-2.55 (2H, t, SCH_2CH_2), 2.20-2.35 (2H, t, $\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.5-1.7 (4H, bm, SCH_2CH_2 and $\text{CH}_2\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.2-1.4 (6H, b, $\text{SCH}_2\text{CH}_2(\text{CH}_2)_3\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$).

i. 12-thiamyristic acid (12): mp 43-44°C; R_f 0.30-0.38 (80% EtOAc/Hex); ^1H NMR (200MHz) 2.35-2.55 (4H, t, CH_2SCH_2), 2.20-2.35 (2H, t, $\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.5-1.7 (4H, bm, SCH_2CH_2 and $\text{CH}_2\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.2-1.4 (15H, t and b, $\text{CH}_3\text{CH}_2\text{S}$ and $(\text{CH}_2)_6\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$).

j. 5-thiamyristic acid (13): R_f 0.20-0.25 (80% EtOAc/Hex); ^1H NMR (200MHz) similar to that of 8 except for 1.2-1.4 (12H, b, $\text{CH}_3(\text{CH}_2)_6\text{CH}_2\text{CH}_2\text{S}$).

k. *S*-benzyl-10-mercaptodecanoic acid (14): R_f 0.36-0.38 (60% EtOAc/Hex); ^1H NMR similar to that of 9 except for 1.2-1.4 (10H, b, $\text{SCH}_2\text{CH}_2(\text{CH}_2)_5\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$); 10mg purified on preparatory tlc. (Product visualized by UV only. Glass plate carefully broken to yield section with pure acid. Product removed by soaking in stirring solvent, 2 X 30ml MeOH: CHCl_3 2:1.)

1. *S*-benzyl-11-mercaptoundecanoic acid (15): R_f 0.42-0.44 (60% EtOAc/Hex); ^1H NMR similar to that of 9 except for 1.2-1.4 (12H, b, $\text{SCH}_2\text{CH}_2(\text{CH}_2)_6\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$); 4mg purified on preparatory tlc. (As per 14)

C. Synthesis of sulfide acids (Method 2; thiol + bromo-acid)

Example; *S*-phenyl-8-mercaptooctanoic acid (16)

To a 2-neck 100mL rb flask and stir bar (oven-dried) was added 0.87g KOH (16mmol) and 36mL absolute ethanol. Under nitrogen and with a condensor and septum completing the system, the mixture was stirred for 3 minutes to effect the solvation of the pellets. 541mg (4.9mmol) of thiol was dissolved in 2mL of abs. EtOH and added by syringe, along with a 1mL abs. EtOH rinse of the holding vessel. The solution was stirred for 5 minutes before the bromo-acid was introduced (1.105g, 4.95mmol, in 6mL abs. EtOH, followed by 2mL rinse). Precipitate started forming within 20 minutes of stirring at room temperature. The mixture was very slowly heated (40 setting on a "Powerstat" type L116B variable autotransformer with cloth heating mantle) to a final temperature of $70 \pm 5^\circ\text{C}$ over a 16 hr. period. While still hot, the mixture was transferred to a 250mL RB and rotary evaporated down to a volume of approximately

15mL, then cooled under a flow of nitrogen for 20 minutes. Transferred to a separatory funnel with water rinses (3 X 10mL), the mixture was acidified (3mL 6 M HCl, diluted to 10mL with dH₂O) and extracted with CH₂Cl₂ (3 X 10mL). The combined organics were dried (Na₂SO₄) and rotary evaporated to yield 1.25g (4.96mmol) of the 8-thiophenyl acid. (Note: 100% yield reflects nearly pure material containing small amounts of EtOH which evaporated with time)

a. *S*-phenyl-8-mercaptooctanoic acid (16): ¹H NMR (200MHz) 7.1-7.4 (5H, m, phenyl), 2.80-2.95 (2H, t, SCH₂CH₂), 2.20-2.35 (2H, t, CH₂CH₂C(O)OH), 1.5-1.7 (4H, bm, SCH₂CH₂ and CH₂CH₂CH₂C(O)OH), 1.2-1.5 (6H, b, SCH₂CH₂(CH₂)₃CH₂CH₂C(O)OH).

b. *S*-phenyl-9-mercaptononanoic acid (17): ¹H NMR (200MHz) similar to that of 16 except for 1.2-1.5 (8H, b, SCH₂CH₂(CH₂)₄CH₂CH₂C(O)OH).

c. *S*-(2-naphthyl)-8-mercaptooctanoic acid (18): ¹H NMR (200MHz) 7.4-7.9 (7H, 2m, naphthyl), 2.95-3.10 (2H, t, SCH₂CH₂), 2.25-2.40 (2H, t, CH₂CH₂C(O)OH), 1.5-1.7 (4H, bm, SCH₂CH₂ and CH₂CH₂CH₂C(O)OH), 1.2-1.5 (6H, b, SCH₂CH₂(CH₂)₃CH₂CH₂C(O)OH).

d. *S*-(2-naphthyl)-9-mercaptononanioic acid (19): ^1H NMR (200MHz) similar to that of 18 except for 1.2-1.5 (8H, b, $\text{SCH}_2\text{CH}_2(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$).

e. *S*-(*para*-trifluoromethyl)benzyl-9-mercaptononanoic acid (20): ^1H NMR (200MHz) 7.2-7.6 (4H, 2d, phenyl), 3.68-3.72 (2H, s, $\text{phenylCH}_2\text{S}$), 2.35-2.55 (2H, t, SCH_2CH_2), 2.20-2.35 (2H, t, $\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.5-1.7 (4H, bm, SCH_2CH_2 and $\text{CH}_2\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.2-1.4 (8H, b, $\text{SCH}_2\text{CH}_2(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$); ^{19}F NMR (376.5 MHz) (-62.7)-(-62.9) (CF_3).

f. 11-thiamyristic acid (21): R_f 0.27-0.32 (80% EtOAc/Hex); ^1H NMR (200MHz) 2.40-2.55 (4H, 2t, CH_2SCH_2), 2.30-2.40 (2H, t, $\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.5-1.7 (6H, bm, $\text{CH}_2\text{CH}_2\text{SCH}_2\text{CH}_2$ and $\text{CH}_2\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.2-1.4 (10H, b, $(\text{CH}_2)_5\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 0.94-1.03 (3H, t, $\text{CH}_3\text{CH}_2\text{CH}_2\text{S}$).

D. Synthesis of methyl esters

Example; methyl 9-thiastearate (22)

To a one-neck 250mL rb flask with boiling chips, reflux condensor, and nitrogen flow was added 1.24g (4.9mmoles) of the thia acid and 10mL absolute methanol for partial solvation. 7.7mL cold boron trifluoride etherate complex was added to 40mL absolute methanol, and this solution plus 17mL of abs. MeOH rinses were added to the reaction flask, yielding a final solution of approximately 10% (v/v) $\text{BF}_3\text{Et}_2\text{O}$ in MeOH. The clear colourless or slightly yellow solution, was refluxed for ca. 1.3 hrs and became a pale pink colour. Partially cooled (ca. 45°C) the solution was directly rotary evaporated down to a volume of about 20mL, then transferred to a separatory funnel with rinses (alternately, 2 X 20mL dH_2O , 2 X 10mL CH_2Cl_2), and extracted. The aqueous was extracted twice more (20mL portions of CH_2Cl_2) and the combined organics dried (Na_2SO_4) and rotary evaporated to yield 1.30g of a pale brown/yellow oil representing the crude methyl ester.

^1H NMR (200MHz) each similar to the corresponding carboxylic acid except for 3.60-3.68 (3H, s, $\text{C}(\text{O})\text{OCH}_3$) added and 2.20-2.35 (2H, t, $\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OCH}_3$) shifted to 2.15-2.30. Gas chromatography retention time (GC-RT) is based on a 20M DB5-A column ramped from 100°C to 280°C at 15°C per minute.

a. Methyl 9-thiastearate (22): mp 20-22°C; R_f 0.27 (5% EtOAc/Hex); GC-RT 10min. 53sec.; MS (EI, 70eV) m/e 316 (M^+), 285 ($(\text{M}-\text{OCH}_3)^+$), 157 ($(\text{M}-\text{CH}_3(\text{CH}_2)_8\text{S})^+$), 159 ($(\text{CH}_3(\text{CH}_2)_8\text{S})^+$); Anal. calcd. for $\text{C}_{18}\text{H}_{36}\text{O}_2\text{S}$: C, 68.30; H, 11.46; S, 10.13. Found: C, 68.41; H, 11.88; S, 9.94.

b. Methyl 9-thiastearate-10,10- d_2 (22-10,10- d_2): The spectral data was similar to that of 20 except for MS (EI, 70eV) m/e 318 (M^+), 287 ((M-OCH₃)⁺), 157 ((M-CH₃(CH₂)₇CD₂S)⁺), 161 ((CH₃(CH₂)₇CD₂S)⁺).

c. Methyl 10-thiastearate (23): mp 19-20°C; R_f 0.28 (5% EtOAc/Hex); GC-RT 10min. 47sec.; MS (EI, 70eV) m/e 316 (M^+), 285 ((M-OCH₃)⁺), 171 ((M-CH₃(CH₂)₇S)⁺), 145 ((CH₃(CH₂)₇S)⁺); Anal. calcd. for C₁₈H₃₆O₂S: C, 68.30; H, 11.46; S, 10.13. Found: C, 68.00; H, 11.76; S, 10.04.

d. Methyl 10-thiastearate-11,11- d_2 (23-11,11- d_2): The spectral data was similar to that of 21 except for MS (EI, 70eV) m/e 318 (M^+), 287 ((M-OCH₃)⁺), 171 ((M-CH₃(CH₂)₆CD₂S)⁺), 147 ((CH₃(CH₂)₆CD₂S)⁺).

e. Methyl 5-thiastearate (24): R_f 0.16 (5% EtOAc/Hex), R_f 0.25 (6% EtOAc/Hex).

f. Methyl S-benzyl-8-mercaptooctanoate (25): bp 106-112°C (10mmHg); R_f 0.65 (60% EtOAc/Hex), R_f 0.17 (6% EtOAc/Hex); GC-RT 10min. 11sec.; MS (EI, 70eV) m/e 280 (M^+), 189 ((M-C₇H₇)⁺), 157 ((M-C₇H₇S)⁺), 123 ((C₇H₇S)⁺), 91 ((C₇H₇)⁺); Anal. calcd. for C₁₆H₂₄O₂S: C, 68.52; H, 8.63; S, 11.43. Found: C, 68.41; H, 8.67; S, 11.05.

g. Methyl S-benzyl-9-mercaptononanoate (26): bp 118-124°C (10mmHg); R_f 0.16 (5% EtOAc/Hex), R_f 0.18 (6% EtOAc/Hex); GC-RT 10min. 50sec.; MS (EI, 70eV) m/e 294 (M^+), 203 ($(M-C_7H_7)^+$), 171 ($(M-C_7H_7S)^+$), 123 ($(C_7H_7S)^+$), 91 ($(C_7H_7)^+$); Anal. calcd. for $C_{17}H_{26}O_2S$: C, 69.34; H, 8.90; S, 10.89. Found: C, 68.86; H, 9.07; S, 10.56.

h. Methyl S-phenyl-8-mercaptooctanoate (27): R_f 0.19 (6% EtOAc/Hex); MS (CI iso, 70eV) m/e 267 ($(M+H)^+$), 235 ($(M-OCH_3)^+$), 110 ($((phenylS)+H)^+$); Anal. calcd. for $C_{15}H_{22}O_2S$: C, 67.63; H, 8.32; S, 12.03. Found: C, 67.47; H, 8.69; S, 11.98.

i. Methyl S-phenyl-9-mercaptononanoate (28): R_f 0.22 (6% EtOAc/Hex); MS (CI iso, 70eV) m/e 281 ($(M+H)^+$), 249 ($(M-OCH_3)^+$), 110 ($((phenylS)+H)^+$); Anal. calcd. for $C_{16}H_{24}O_2S$: C, 68.53; H, 8.63; S, 11.43. Found: C, 68.27; H, 9.03; S, 11.03.

j. Methyl S-(2-naphthyl)-8-mercaptooctanoate (29): R_f 0.15 (6% EtOAc/Hex).

k. Methyl S-(2-naphthyl)-9-mercaptononanoate (30): mp 36-37°C; R_f 0.67 (40% EtOAc/Hex), R_f 0.29 (8% EtOAc/Hex); MS (CI iso, 70eV) m/e 331 ($(M+H)^+$), 299 ($(M-OCH_3)^+$), 160 ($((naphthylS)+H)^+$), 128 ($((naphthyl)+H)^+$); Anal. calcd. for $C_{20}H_{26}O_2S$: C, 72.69; H, 7.93; S, 9.70. Found: C, 72.82; H, 7.96; S, 9.71.

- l. Methyl 10-(2-naphthyl)-9-thiadecanoate (31):** mp 23-26°C; R_f 0.50 (25% EtOAc/Hex), R_f 0.25 (8% EtOAc/Hex); MS (CI iso, 70eV) m/e 331 ((M+H)⁺), 299 ((M-OCH₃)⁺), 173 ((naphthylCH₂S)⁺), 141 ((naphthylCH₂)⁺); Anal. calcd. for C₂₀H₂₆O₂S: C, 72.69; H, 7.93; S, 9.70. Found: C, 72.54; H, 7.82; S, 9.74.
- m. Methyl 11-((para-trifluoromethyl)-phenyl)-9-thiaundecanoate (32):** oil; R_f 0.16 (6% EtOAc/Hex); ¹⁹F NMR (376.5 MHz) (-62.6)-(-62.8) (CF₃); supplied by Nataly Vo. Anal. calcd. for C₁₈H₂₅O₂SF₃: C, 59.65; H, 6.95; S, 8.85; F, 15.72. Found: C, 59.66; H, 7.05; S, 8.56.
- n. Methyl S-(para-trifluoromethyl)-benzyl-9-mercaptononanoate (33):** oil; R_f 0.17 (6% EtOAc/Hex); ¹⁹F NMR (376.5 MHz) (-62.7)-(-62.9) (CF₃); Anal. calcd. for C₁₈H₂₅O₂SF₃: C, 59.65; H, 6.95; S, 8.85; F, 15.72. Found: C, 59.17; H, 6.95; S, 8.98.
- o. Methyl 11-thiamyristate (34):** R_f 0.13 (4% EtOAc/Hex); MS (CI ether, 70eV) 261 ((M+H)⁺), 229 ((M-OCH₃)⁺), 185 ((M-CH₃CH₂CH₂S)⁺).
- p. Methyl 12-thiamyristate (35):** R_f 0.14 (4% EtOAc/Hex); MS (CI ether, 70eV) 261 ((M+H)⁺), 229 ((M-OCH₃)⁺), 199 ((M-CH₃CH₂S)⁺).
- q. Methyl 5-thiamyristate (36):** R_f 0.11 (4% EtOAc/Hex), R_f 0.23 (6% EtOAc/Hex).

Example; methyl 9-thiastearate *S*-oxide (37)

Sulfide (9-thiastearate, 202mg, 0.63mmoles) was dissolved in dichloromethane (3mL) with stirring in a 50mL rb flask on ice. Meta-chloroperoxybenzoic acid (*m*-CPBA, 157mg, 0.63mmoles, ca. 70% pure) dissolved in 2mL CH₂Cl₂ was then added dropwise, followed by a 0.5mL rinse. The reaction solution was stirred 30 minutes on ice, and a further 30 minutes warming to room temperature. Transferred to a separatory funnel, the organics were washed with aqueous base (4 X 20mL; 1.0g NaOH + 8.0g NaHCO₃ in 80mL dH₂O; combined washes back-extracted with 10mL CH₂Cl₂), combined, dried over anhydrous Na₂SO₄ (ca. 0.3g), and rotary evaporated to yield 220mg (greater than 100% yield) crude sulfoxide product as white crystals.

¹H NMR similar to those of methyl esters of sulfides except for protons near sulfur.

a. Methyl 9-thiastearate *S*-oxide (37): mp 66.0-66.5°C; R_f 0.16 (75% EtOAc/Hex);

¹H NMR (300MHz) 3.62-3.69 (3H, s, C(O)OCH₃), 2.60-2.68 (4H, m, CH₂S(O)CH₂), 2.15-2.30 (2H, t, CH₂CH₂C(O)OCH₃), 1.6-1.8 (6H, bm, CH₂CH₂S(O)CH₂CH₂ and

CH₂CH₂CH₂C(O)OCH₃), 1.2-1.4 (18H, b,
 CH₃(CH₂)₆CH₂CH₂S(O)CH₂CH₂(CH₂)₃CH₂CH₂C(O)OCH₃), 0.8-0.9 (3H, t,
 CH₃(CH₂)₆); MS (EI, 70eV) 315 ((M-OH)⁺), 301 ((M-OCH₃)⁺), 174
 ((OS(CH₂)₇CO)⁺), 175 ((CH₃(CH₂)₈SO)⁺), 157 (((CH₂)₇C(O)OCH₃)⁺); ¹³C-NMR
 (100.6 MHz) shifts in ppm 174.15, 52.56, 52.42, 51.47, 34.0, 31.83, 29.34, 29.23,
 29.21, 28.92, 28.87, 28.86, 28.71, 24.81, 22.66, 22.63, 22.57, 14.09; IR (CHCl₃)
 1025 cm⁻¹ (S=O stretch). Anal. calcd. for C₁₈H₃₆O₃S: C, 64.24; H, 10.78; S, 9.53.
 Found C, 64.31; H, 11.09; S, 9.58.

b. Methyl 9-thiastearate S-oxide-10,10-d₂ (37-10,10-d₂): The spectral data was
 similar to that of 37 except for the following ¹H NMR (300MHz) 2.60-2.68 (2H, t,
 CD₂S(O)CH₂); MS (EI, 70eV) 317 ((M-OH)⁺), 303 ((M-OCH₃)⁺), 174
 ((OS(CH₂)₇CO)⁺), 177 ((CH₃(CH₂)₇(CD₂)SO)⁺), 157 (((CH₂)₇C(O)OCH₃)⁺).

c. Methyl 10-thiastearate S-oxide (38): mp 65.5-66.0°C; R_f 0.19 (75% EtOAc/Hex);
¹H NMR (300MHz) similar to that of 37; MS (EI, 70eV) 315 ((M-OH)⁺), 301 ((M-
 OCH₃)⁺), 188 ((OS(CH₂)₈CO)⁺), 161 ((CH₃(CH₂)₇SO)⁺), 171 (((CH₂)₈C(O)OCH₃)⁺);
 IR (CHCl₃) 1025 cm⁻¹ (S=O stretch). Anal. calcd. for C₁₈H₃₆O₃S: C, 64.24; H,
 10.78; S, 9.53. Found C, 64.93; H, 11.23; S, 9.73.

d. Methyl 10-thiastearate *S*-oxide-11,11- d_2 (38-11,11- d_2): The spectral data was similar to that of 37-10,10- d_2 except for the following MS 317 ((M-OH)⁺), 303 ((M-OCH₃)⁺), 188 ((OS(CH₂)₈CO)⁺), 163 ((CH₃(CH₂)₆(CD₂)SO)⁺), 171 (((CH₂)₈C(O)OCH₃)⁺).

e. Methyl 5-thiastearate *S*-oxide (39): ¹H NMR (200 MHz) 3.60-3.65 (3H, s, C(O)OCH₃), 2.6-2.8 (4H, m, CH₂S(O)CH₂), 2.2-2.3 (2H, t, CH₂CH₂C(O)OCH₃), 2.0-2.1 (2H, m, S(O)CH₂CH₂CH₂C(O)), 1.6-1.8 (2H, m, CH₂CH₂CH₂S(O)), 1.2-1.4 (20H, b, CH₃(CH₂)₁₀CH₂CH₂S), 0.8-0.9 (3H, t, CH₃(CH₂)₁₀); MS (EI, 70eV) 315 ((M-OH)⁺), 301 ((M-OCH₃)⁺), 118 ((OS(CH₂)₄CO)⁺), 215 ((CH₃(CH₂)₁₁SO)⁺), 101 (((CH₂)₄C(O)OCH₃)⁺).

f. Methyl *S*-benzyl-8-mercaptooctanoate *S*-oxide (40): mp 73-74°C; R_f 0.098 (60% EtOAc/Hex); ¹H NMR (300MHz) 7.2-7.4 (5H, m, phenyl), 3.87-4.07 (2H, ABq, J_{AB}=12.9 Hz, phenylCH₂S(O)), 3.65-3.69 (3H, s, C(O)OCH₃), 2.49-2.59 (2H, t, S(O)CH₂CH₂), 2.20-2.35 (2H, t, CH₂CH₂C(O)CH₃), 1.5-1.8 (4H, bm, S(O)CH₂CH₂ and CH₂CH₂CH₂C(O)), 1.2-1.4 (6H, b, S(O)CH₂CH₂(CH₂)₃CH₂CH₂C(O)); MS (EI, 70eV) 296 (M⁺), 91 ((C₇H₇)⁺); IR (CHCl₃) 1031 cm⁻¹ (S=O stretch). Anal. calcd. for C₁₆H₂₄O₃S: C, 64.83; H, 8.16; S, 10.82. Found: C, 64.75; H, 8.44; S, 10.39.

g. Methyl S-benzyl-9-mercaptononanoate S-oxide (41): R_f 0.12 (60% EtOAc/Hex);

^1H NMR (300MHz) similar to that of 40 except for 1.2-1.4 (8H, b,

$\text{SCH}_2\text{CH}_2(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$); MS (CI ether, 70eV) 311 ((M+H) $^+$),

295(((M+H)-OH) $^+$), 171 ((M-phenylCH $_2$ S(O)) $^+$).

h. Methyl S-phenyl-8-mercaptooctanoate S-oxide (42): oil; R_f 0.21 (60%

EtOAc/Hex); ^1H NMR (400MHz) 7.4-7.7 (5H, m, phenyl), 3.65-3.69 (3H, s,

$\text{C}(\text{O})\text{OCH}_3$), 2.71-2.85 (2H, it, $\text{S}(\text{O})\text{CH}_2\text{CH}_2$), 2.20-2.35 (2H, t, $\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{CH}_3$),

1.5-1.8 (4H, bm. $\text{S}(\text{O})\text{CH}_2\text{CH}_2$ and $\text{CH}_2\text{CH}_2\text{CH}_2\text{C}(\text{O})$), 1.2-1.5 (6H, b,

$\text{S}(\text{O})\text{CH}_2\text{CH}_2(\text{CH}_2)_3\text{CH}_2\text{CH}_2\text{C}(\text{O})$); MS (CI iso, 70eV) m/e 283 ((M+H) $^+$), 251 ((M-

OCH_3) $^+$), 125 ((phenylS(O)) $^+$); Anal. calcd. for $\text{C}_{15}\text{H}_{22}\text{O}_3\text{S}$: C, 63.80; H, 7.85.

Found: C, 63.37; H, 7.85.

i. Methyl S-phenyl-9-mercaptononanoate S-oxide (43): oil; R_f 0.25 (60%

EtOAc/Hex); ^1H NMR (400MHz) similar to that of 42 except for 1.2-1.5 (8H, b,

$\text{S}(\text{O})\text{CH}_2\text{CH}_2(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{CO}$); MS (CI iso, 70eV) 297 ((M+H) $^+$), 265 ((M-

OCH_3) $^+$), 125 ((phenylS(O)) $^+$); Anal. calcd. for $\text{C}_{16}\text{H}_{24}\text{O}_3\text{S}$: C, 64.83; H, 7.82.

Found: C, 64.59; H, 8.16.

j. Methyl *S*-(2-naphthyl)-8-mercaptooctanoate *S*-oxide (44): R_f 0.24 (60%

EtOAc/Hex); ^1H NMR (200MHz) 7.5-8.0 (7H, 2m, naphthyl), 3.65-3.69 (3H, s, $\text{C}(\text{O})\text{OCH}_3$), 2.80-2.92 (2H, it, $\text{S}(\text{O})\text{CH}_2\text{CH}_2$), 2.22-2.32 (2H, t, $\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{CH}_3$), 1.5-1.8 (4H, bm, $\text{S}(\text{O})\text{CH}_2\text{CH}_2$ and $\text{CH}_2\text{CH}_2\text{CH}_2\text{C}(\text{O})$), 1.2-1.5 (6H, b, $\text{S}(\text{O})\text{CH}_2\text{CH}_2(\text{CH}_2)_3\text{CH}_2\text{CH}_2\text{C}(\text{O})$).

k. Methyl *S*-(2-naphthyl)-9-mercaptononanoate *S*-oxide (45): mp 36-37°C; R_f 0.27

(60% EtOAc/Hex); ^1H NMR (200MHz) similar to that of 44 except for 1.2-1.5 (8H, b, $\text{S}(\text{O})\text{CH}_2\text{CH}_2(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{C}(\text{O})$); MS (CI iso, 70eV) m/e 347 (($\text{M}+\text{H}$) $^+$), 315 (($\text{M}-\text{OCH}_3$) $^+$), 176 ((naphthyl $\text{S}(\text{O})+\text{H}$) $^+$), 128 ((naphthyl $+\text{H}$) $^+$); Anal. calcd. for $\text{C}_{20}\text{H}_{26}\text{O}_3\text{S}$: C, 69.33; H, 7.56; S, 9.25. Found: C, 69.37; H, 7.58; S, 9.03.

l. Methyl 10-(2-naphthyl)-9-thiadecanoate *S*-oxide (46): mp 87-88°C; R_f 0.085 (60%

EtOAc/Hex); ^1H NMR (400MHz) 7.4-7.9 (7H, 2m, naphthyl), 4.02-4.25 (2H, ABq, $J_{\text{AB}}=12.8$ Hz, naphthyl $\text{CH}_2\text{S}(\text{O})$), 3.62-3.65 (3H, s, $\text{C}(\text{O})\text{OCH}_3$), 2.50-2.65 (2H, t, $\text{S}(\text{O})\text{CH}_2\text{CH}_2$), 2.20-2.35 (2H, t, $\text{CH}_2\text{CH}_2\text{C}(\text{O})$), 1.5-1.8 (4H, bm, $\text{S}(\text{O})\text{CH}_2\text{CH}_2$ and $\text{CH}_2\text{CH}_2\text{CH}_2\text{C}(\text{O})$), 1.2-1.4 (6H, b, $\text{S}(\text{O})\text{CH}_2\text{CH}_2(\text{CH}_2)_3\text{CH}_2\text{CH}_2\text{C}(\text{O})$); MS (CI iso, 70eV) m/e 347 (($\text{M}+\text{H}$) $^+$), 315 (($\text{M}-\text{OCH}_3$) $^+$), 189 ((naphthyl $\text{CH}_2\text{S}(\text{O})$) $^+$), 141 ((naphthyl CH_2) $^+$); Anal. calcd. for $\text{C}_{20}\text{H}_{26}\text{O}_3\text{S}$: C, 69.33; H, 7.56; S, 9.25. Found: C, 69.36; H, 7.68; S, 9.09.

m. Methyl 11-((*para*-triflouromethyl)-phenyl)-9-thiaundecanoate *S*-oxide (47):

crude mp 20-22°C; R_f 0.073 (60% EtOAc/Hex).

n. Methyl *S*-(*para*-triflouromethyl)-benzyl-9-mercaptononanoate *S*-oxide (48): mp

90-91°C; R_f 0.11 (60% EtOAc/Hex); ^1H NMR (200MHz) 7.4-7.7 (4H, 2d, phenyl), 3.91-4.07 (2H, ABq, $J_{AB}=13.0$ Hz, phenylCH₂S(O)), 3.65-3.69 (3H, s, C(O)OCH₃), 2.52-2.65 (2H, it, S(O)CH₂CH₂), 2.24-2.35 (2H, t, CH₂CH₂C(O)CH₃), 1.5-1.8 (4H, bm, S(O)CH₂CH₂ and CH₂CH₂CH₂C(O)), 1.2-1.4 (8H, b, S(O)CH₂CH₂(CH₂)₄CH₂CH₂C(O)). Anal. calcd. for C₁₈H₂₅O₃SF₃: C, 57.13; H, 6.66; S, 8.47. Found: C, 57.31; H, 6.57; S, 8.60.

o. Methyl 11-thiamyristate *S*-oxide (49): mp 48-49°C; R_f 0.13 (75% EtOAc/Hex);

^1H NMR (200MHz) 3.55-3.60 (3H, s, C(O)OCH₃), 2.45-2.62 (4H, m, CH₂S(O)CH₂), 2.35-2.45 (2H, t, CH₂CH₂C(O)CH₃), 1.4-1.8 (6H, bm, CH₂CH₂S(O)CH₂CH₂ and CH₂CH₂CH₂C(O)), 1.2-1.4 (10H, b, (CH₂)₅CH₂CH₂C(O)), 0.94-1.03 (3H, t, CH₃CH₂CH₂S(O)); MS (CI ether, 70eV) 277 ((M+H)⁺), 261 ((M-CH₃)⁺), 245 ((M-OCH₃)⁺), 185 ((M-CH₃CH₂CH₂S(O))⁺). Anal. calcd. for C₁₄H₂₈O₃S: C, 60.82; H, 10.21; S, 11.60. Found: C, 60.42; H, 10.47; S, 11.37.

p. Methyl 12-thiamyristate *S*-oxide (50): mp 53-54°C; R_f 0.099 (75% EtOAc/Hex); ^1H NMR (200MHz) 3.55-3.60 (3H, s, $\text{C}(\text{O})\text{OCH}_3$), 2.50-2.65 (4H, t, $\text{CH}_2\text{S}(\text{O})\text{CH}_2$), 2.16-2.27 (2H, t, $\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OCH}_3$), 1.45-1.75 (4H, bm, $\text{S}(\text{O})\text{CH}_2\text{CH}_2$ and $\text{CH}_2\text{CH}_2\text{CH}_2\text{C}(\text{O})$), 1.15-1.45 (15H, t and b, $\text{CH}_3\text{CH}_2\text{S}(\text{O})$ and $(\text{CH}_2)_6\text{CH}_2\text{CH}_2\text{C}(\text{O})$).
Anal. calcd. for $\text{C}_{14}\text{H}_{28}\text{O}_3\text{S}$: C, 60.82; H, 10.21; S, 11.60. Found: C, 60.67; H, 10.53; S, 11.93.

q. Methyl 5-thiamyristate *S*-oxide (51): ^1H NMR (200MHz) similar to that of 39 except for 1.2-1.4 (12H, b, $\text{CH}_3(\text{CH}_2)_6\text{CH}_2\text{CH}_2\text{S}(\text{O})$).

Example: 11-thiamyristic acid *S*-oxide (52).

Purified methyl ester (0.95g, 3.44mmol) was dissolved in absolute ethanol (4mL) in a RB flask with stirring bar, boiling chip, and nitrogen atmosphere. 4mL of 2M KOH_(aq) was added, and the resulting cloudy mixture was heated to reflux, (10min.). Cooled slowly to room temperature and acidified (pH 1, ca 6mL 3M HCl), this aqueous was extracted with CH₂Cl₂ (3 X 10mL). The combined organics were washed once (10mL 0.1M HCl), dried (Na₂SO₄), and rotary evaporated to yield 11-thiamyristic acid *S*-oxide, (0.72g, 2.75mmol).

a. 11-thiamyristic acid *S*-oxide (52): *R_f* 0.038 (80% EtOAc/Hex); ¹H NMR similar to that of 49 except for 3.55-3.60 (3H, s, C(O)OCH₃) missing.

b. 12-thiamyristic acid *S*-oxide (53): *R_f* 0.032 (80% EtOAc/Hex); ¹H NMR similar to that of 50 except for 3.55-3.60 (3H, s, C(O)OCH₃) missing.

To a dry 2-neck, 100mL rb flask with stirring bar, septum, and N₂ flow was added 4.56g (22.5mmoles) azelaic acid, monomethyl ester and 10mL dry THF. Cooled on ice, BH₃ (28mL of a 1M solution in THF) was slowly syringed in to the stirring solution over a 15min. period, so that the temperature did not exceed ca. 20°C, (a white soapy froth appeared on the solution surface with each addition). The solution was stirred 20 min. on ice, then 1.5hrs at room temperature before being cooled on ice and hydrolyzed by the slow injection of 8mL chilled dH₂O (producing more white froth and a cloudy mixture). The mixture was transferred to a single neck rb flask with rinses (4mL dH₂O, 4mL CH₂Cl₂) then rotary evaporated (bath temperature not greater than 45°C) for ca. 1 hr to yield a white creamy paste (ketchup consistency). Transferred to a separatory funnel (alternating rinses; 3 X 12mL dH₂O, 2 X 8mL CH₂Cl₂) the aqueous was extracted this time and three times further (10mL CH₂Cl₂ portions). Combined organics were dried (Na₂SO₄) and rotary evaporated to yield 4.2g crude 9-hydroxy nonanoic acid methyl ester (oil, 99% yield).

Methyl 9-hydroxynonanoate (54): R_f 0.37-0.39 (60% EtOAc/Hex); ^1H NMR (200 MHz) 3.66-3.68 (3H, s, $\text{C}(\text{O})\text{OCH}_3$), 3.55-3.70 (2H, t, HOCH_2CH_2), 2.2-2.4 (2H, t, $\text{HOCH}_2(\text{CH}_2)_6\text{CH}_2\text{C}(\text{O})\text{OCH}_3$), 1.5-1.7 (5H, bm, $\text{HOCH}_2\text{CH}_2(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OCH}_3$), 1.2-1.4 (8H, bs, $\text{HOCH}_2\text{CH}_2(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OCH}_3$).

H. Synthesis of methyl 9-(*p*-toluene sulfonyl ether)-oxynonanoate (55)

Alcohol, (13.5g, 72mmol) was transferred from a dry vessel by syringe to the oven dried reaction RB (500mL) which was N_2 purged and contained a dry stirring bar. 15mL dry pyridine was used to quantitate the transfer before the solution was stirred on ice for 10 minutes. Purified *p*-tosyl-Cl (16g, 84mmol) was then dropped in while the stirred mixture was maintained under a blanket of nitrogen, a fine white precipitate formed immediately. The mixture was stirred, starting on ice (which was allowed to melt) for 20 hours (temperature not exceeding 20°C). Returned to an ice bath, the stirring mixture was acidified (15mL 50% H_2SO_4 + 30mL dH_2O), transferred to a

separatory funnel (rinses in order; 8mL -10°C CH₂Cl₂, 10mL 20°C dH₂O, 8mL -10°C CH₂Cl₂) and gently extracted. The aqueous was extracted three more times with near 0°C CH₂Cl₂ (15mL portions). The cold combined organics were washed with an ice-chilled 1M copper sulfate solution (2 X 25mL, turned organic layer pale green), then once with cold saturated sodium chloride solution (25mL), and finally dried over anhydrous Na₂SO₄ for 30 minutes (flask kept chilled on ice). Careful rotary evaporation (bath temperatures 10 to 20°C) for ca. 8hrs yielded a pale green oil (23.2g, 94% crude yield). Storage under N₂ at -10°C for ca. 60hrs yielded a pale yellow oil containing fine white precipitate.

Methyl 9-(*para*-toluene sulfonyl ether) oxy nonanoate (55): ¹H NMR (200 MHz) 7.2-7.8 (4H, 2d, phenyl), 3.85-4.00 (2H, t, (tosyl)OCH₂CH₂), 3.55-3.58 (3H, s, C(O)OCH₃), 2.34-2.35 (3H, s, CH₃C₆H₄S(O₂)), 2.1-2.3 (2H, t, OCH₂(CH₂)₆CH₂C(O)OCH₃), 1.4-1.6 (4H, bm, OCH₂CH₂(CH₂)₄CH₂CH₂C(O)OCH₃), 1.1-1.3 (8H, bs, OCH₂CH₂(CH₂)₄CH₂CH₂C(O)OCH₃).

I. Synthesis of 9-thiononanoic acid (attempt, from tosylate, 55) (56)

In a cool one-neck 250mL rb flask (oven dried) equipped with condensor, boiling chips, stir bar and nitrogen flow, was placed 10.10g (30mmol) crude tosylated alcohol and 40mL absolute ethanol. Thiourea, (4.9g, 64mmol) was added to the mixture as it stirred on ice. After 5 minutes stirring, the bath was removed and the mixture was given 1 hour to slowly warm to room temperature. The continually stirred mixture was then very gently heated in a controlled water bath (starting at ca. 25°C, ending at ca. 55°C over 30 min., gradually dissolving the thiourea), and finally brought to a reflux in a cloth heating mantle. Refluxed for 1 hour, the solution was cooled for 10 minutes before addition of the aqueous base for hydrolysis (solution containing 5.0g NaOH, 47mL dH₂O, 18mL 95% EtOH). The now pale yellow-brown cloudy mixture was returned to a reflux for 50 minutes, cooled, diluted with 50mL dH₂O, then poured slowly over a 2 minute period into an efficiently stirred acid-dichloromethane mixture in a 1 litre Florence flask, (40mL 6M H₂SO₄, 100mL CH₂Cl₂, magnetically stirred). The resultant mixture (pH well below 2) was cooled in ice water, placed in a separatory funnel and the organics removed. The aqueous was extracted with four 30mL portions of CH₂Cl₂, and

the combined organics were washed once (30mL) with an acidified saturated NaCl solution, dried (Na_2SO_4 , overnight, 4°C), then rotary evaporated to yield ca. 8g of crude product, (desired: 9-thiononanoate ; suspected: ethyl ester and disulfide). Crude yield was greater than 100% for both this trial and second trial (13g tosylated alcohol gave 9.7g crude product). Two fast large-scale flashes (65% EtOAc in hexane, some components insoluble) yielded major component as a mixture (12.8g combined) suspected by ^1H -NMR and GC-MS to be ethyl esters of free thiol (56est) and disulfide (56di-est), (a white opaque solid at -20°C ; approx. yield on this basis: 86%).

Ethyl 9-thiononanoate (56est) and Disulfide (56di-est): ^1H NMR (200 MHz) 4.0-4.2 (2H, q, $\text{CH}_3\text{CH}_2\text{OC(O)}$), 2.63-2.75 (4H, t, $\text{CH}_2\text{CH}_2\text{SSCH}_2\text{CH}_2$), 2.40-2.55 (2H, bq(d of t overlapped), $\text{CH}_2\text{CH}_2\text{SH}$), 2.20-2.35 (2H, t, $\text{OC(O)CH}_2\text{CH}_2$), 1.5-1.7 (5H, bm, SH (1.6-1.7ppm) and $\text{SCH}_2\text{CH}_2(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{C(O)OCH}_2$), 1.20-1.45 (8H, bs, $\text{SCH}_2\text{CH}_2(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{C(O)OCH}_2$), 1.15-1.30 (3H, t(aside bs), $\text{CH}_3\text{CH}_2\text{OC(O)}$). MS (EI, 70eV) m/e (GC second most prominent component, 9min. 21sec.) 219 (MH^+), 273 ($(\text{M}-\text{CH}_3\text{CH}_2\text{O})^+$), thought to be free thiol; (GC most prominent component, 18min. 57sec.) 435 (MH^+), 390 ($(\text{MH}-\text{CH}_3\text{CH}_2\text{O})^+$), thought to be disulfide.

I. (i) hydrolysis of ethyl esters (yielding thioacid **56** and disulfide diacid **56di**)

To 12.8g of ester mixture in a one-neck rb flask (N₂ flow, boiling chips, condensor) was added 100mL of a 2M NaOH solution (H₂O:EtOH = 1:1). Refluxed for 2.5 hrs., the pale yellow solution was then cooled on ice, acidified (pH less than 1.0, ca. 30mL 25% H₂SO₄), diluted with 150mL water, and extracted (4 X 20mL CH₂Cl₂). The organics were dried over ca. 2g anhydrous Na₂SO₄ (2hrs) and rotary evaporated (twice with added CH₂Cl₂ to rid sample of EtOH) to yield a creamy white solid at room temperature (11.1g, 99% yield).

9-thiononanioic acid (56) and corresponding disulfide (56di): ¹H NMR (200 MHz) 9.7-9.8 (1H, bs, HOC(O)), 2.62-2.72 (4H, t, CH₂CH₂SSCH₂CH₂), 2.44-2.58 (2H, bq(d of t overlapped), CH₂CH₂SH), 2.30-2.40 (2H, t, HOC(O)CH₂CH₂), 2.24-2.34 (1H, bt, CH₂SH), 1.5-1.7 (4H, bm, HOC(O)CH₂CH₂(CH₂)₄CH₂CH₂S), 1.20-1.45 (8H, bs, HOC(O)CH₂CH₂(CH₂)₄CH₂CH₂S).

I. (ii) disulfide reduction to free thiol (yielding desired thioacid 56)

In a 1 litre rb flask with stirring bar and strong nitrogen flow was placed 200mL dioxane, 200mL dH₂O (both N₂ bubbled for 20 min.), 1.8mL concentrated HCl, 60mg EDTA (0.2mmol) and 2.99g KCl (40mmol). After dissolution, 5.03g of the disulfide mixture (maximum 13 mmoles disulfide) was added, followed by 7.00g triphenylphosphine (TPP, 27mmol). The system was closed except for the now gentle nitrogen flow, and warmed by a water bath to 30°C for ca. 1.8 hrs, (by which time most of the solid had dissolved). The bath temperature was raised to 40 ± 2°C and maintained there for 18 hrs stirring. With the bath removed, 72g NaCl was added followed by 50mL diethyl ether. The mixture was given 20 minutes to stir under N₂ in order to continue cooling and help dissolve the salt. The ethereal layer was noted to nearly triple in volume during this stirring period. The liquid was decanted off the leftover salt (washed with ether 2 X 15mL), placed in a separatory funnel, and the organics removed. The aqueous was extracted (2 X 40mL Et₂O), the organics combined, dried (Na₂SO₄), and rotary evaporated to yield 19.5g of a slightly yellow liquid (very crude thio-acid; max. yield 26mmol, 5g).

Alkylation was performed as before with *n*-BuLi base and bromo compound except that the moles of thiol used was calculated by the ratio of maximum moles of pure thiol expected (26mmol) with mass of crude product obtained (19.5g). Thus if 6g crude material was used for alkylation, this was taken to represent $6\text{g} \times 26\text{mmol} / 19.5\text{g} = 8\text{mmoles}$ (maximum).

9-thiononanoic acid (56): ^1H NMR (200 MHz) 3.4-3.7 (1H, s, HSCH_2), 2.3-2.5 (2H, t/m, HSCH_2CH_2), 2.2-2.3 (2H, t, $\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.4-1.6 (4H, bm, $\text{SCH}_2\text{CH}_2\text{CH}_2$ and $\text{CH}_2\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.0-1.4 (8H, b, $\text{SCH}_2\text{CH}_2(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), the carboxylic proton was not observed.

J. Reduction of carboxylic acids to alcohols

Example: 1- D_2 octanol (57)

To an oven-dried 250mL rb flask and stir bar, cooled under a nitrogen flow, was added LiAlD_4 (4.83g, 115mmol), freshly distilled anhydrous diethyl ether (27mL), and then dropwise, with the mixture stirring on ice, octanoic acid dissolved in dry ether,

(10.9g, 75mmol in 5mL). The mixture was allowed to stir for 2hrs (5 - 15°C), then placed on ice during the slow addition of 10mL 6M HCl, which caused the evaporation of most of the ether. Off ice and without nitrogen, the mixture was diluted with 5mL dH₂O then 30mL 3M HCl, transferred to a separatory funnel (alternating rinses; 4 X 20mL dH₂O, 3 X 15mL CH₂Cl₂), and shaken. The initial emulsion was partially clarified by the addition of 100mL 3M HCl. The CH₂Cl₂ was removed and the aqueous was further extracted (4 X 20mL CH₂Cl₂), the combined organics were washed once (60mL 0.2M HCl), dried (Na₂SO₄), and rotary evaporated to yield 11.8g crude dideutero-alcohol (10g = 75mmol = 100% yield).

a. 1-D₂ octanol (57): ¹H NMR (200 MHz) 1.5-1.6 (2H, bt, HOCD₂CH₂CH₂), 1.2-1.4 (10H, bs, HOCD₂CH₂(CH₂)₅CH₃), 0.8-0.9 (3H, t, CH₂CH₃).

b. 1-D₂ nonanol (58): ¹H NMR (200 MHz) similar to that of 57.

Example: 1-D₂ bromooctane (59)

11.8g crude alcohol (75mmol maximum) was dissolved in 25mL fresh distilled dry ether in a dry rb flask (stir bar, N₂ flow, on ice). PBr₃ (4.8mL, 50mmol) was syringed in, and the clear pale yellow solution was allowed to warm to room temperature over a 3hr period. Following the connection of a water-cooled condensor, the solution was refluxed for 1.5 hrs., cooled on ice, and finally quenched with the dropwise addition of 5mL dH₂O. The solution was diluted further, (10mL dH₂O), transferred to a separatory funnel, and once the initial ethereal layer was removed, extracted (2 X 20mL Et₂O). The combined ethereals were washed once with 20ml of a 5% sodium thiosulfate solution (which was back-extracted with 2 X 10mL ether), dried over Na₂SO₄, and rotary evaporated to yield 14.4g of the 1-D₂ bromooctane (98% overall yield from the octanoic acid).

a. 1-D₂ bromooctane (59): ¹H NMR (200 MHz) 1.8-1.9 (2H, bt, BrCD₂CH₂CH₂), 1.2-1.5 (10H, bs, BrCD₂CH₂(CH₂)₅CH₃), 0.8-0.9 (3H, t, CH₂CH₃). GC-MS (EI, 70eV) (retention time: 5min. 32sec.) *m/e* 137/139 ((M-C₄H₉)⁺), low intensity (10%) peaks 151/153 ((M-C₃H₇)⁺).

b. 1-D₂ bromononane (60): ¹H NMR (200 MHz) similar to 59 except for 1.2-1.5 (12H, bs, BrCD₂CH₂(CH₂)₆CH₃).

L. Synthesis of 9-bromononanol (61)

Glass apparatus was assembled in a manner to allow the continual extraction of product in solvent via atmospheric reflux. The reaction flask was charged with 48% aqueous HBr (150mL), 1,9-nonanediol (22.8g, 143mmol), and *n*-heptane (1.20L). The resulting solution was heated to reflux (75°C) and extracted over a period of 24 hrs. The cooled distillate was neutralized by stirring with sodium bicarbonate (solid, ca. 3g), then dried over anhydrous magnesium sulfate. Rotary evaporation of the filtered product yielded a rust-yellow oil.

9-bromononanol (61): ¹H-NMR (200MHz) 3.8-4.0 (1H, s, CH₂OH), 3.5-3.6 (2H, t, CH₂CH₂Br), 3.4 (2H, t, CH₂CH₂OH), 1.8-1.9 (2H, m, BrCH₂CH₂CH₂), 1.5-1.6 (2H, m, HOCH₂CH₂CH₂), 1.2-1.4 (10H, bs, HOCH₂CH₂(CH₂)₅CH₂CH₂Br).

A three-necked rb flask was equipped with a thermometer, a gas inlet, and a stir bar. 9-bromononanol (3.34g, 15mmol) was added, dissolved in 40mL acetone, and cooled slightly on an ice bath. Freshly prepared Jones's reagent (11mL; from : 16g CrO_3 , 10mL H_2O , and 60ml 3M H_2SO_4), was mixed with 240mL acetone, and slowly added to the dissolved alcohol. The temperature of the mixture was maintained below 22°C by use of an ice bath, until the addition was complete. Stirred for 2.5 hrs. at room temperature, the mixture was then quenched by bubbling in SO_2 gas. This reduction of excess oxidant yielded a green solution and precipitate. The solution was quantitatively transferred to a separatory funnel using ether (120ml, then 2 X 40mL rinses). The ether was washed with water (ca. 3 X 50mL) and saturated NaCl solution (ca. 2 X 30mL) until the final aqueous layer was clear. The combined aqueous were back-extracted with ether (40mL), which was then washed once with sat. NaCl and combined with the initial ethereals. Rotary evaporation yielded an oil, which, upon dissolving in hexane and drying (Na_2SO_4) afforded the crude crystalline acid. The product was partially purified through recrystallization in hexane.

9-bromononanoic acid (62): $^1\text{H-NMR}$ (200MHz) 3.5-3.6 (2H, t, $\text{CH}_2\text{CH}_2\text{Br}$), 2.2-2.3 (2H, t, $\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OH}$), 1.8-1.9 (2H, m, $\text{BrCH}_2\text{CH}_2\text{CH}_2$), 1.4-1.5 (2H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH}$), 1.2-1.4 (10H, bs, $\text{BrCH}_2\text{CH}_2(\text{CH}_2)_5\text{CH}_2\text{CH}_2\text{COOH}$).

3.1.3 Syntheses (and purification) for obtaining configurational standards

N. Synthesis of *n*-butylsulfonyl chloride (63)

The apparatus consisted of a 3-neck 250mL rb flask, fitted with a mechanical stirrer, a gas inlet tube designed to come to within 1cm of the reaction mixture surface, and a gas outlet port connected first to an empty trap and second to a trap containing dilute acetic acid, (through which was bubbled all exiting gasses). The RB was oven-dried, cooled first by N_2 flow and room temperature then cooled secondly on an acetone / dry ice bath. With the nitrogen flow on, 75mL spectrograde CH_2Cl_2 and 9.50ml (50mmol) dibutyl disulfide were introduced to the flask through the gas outlet neck. With the N_2 flow stopped and the stirrer started, Cl gas was slowly introduced, turning the liquid a deep orange-yellow. With all gas flow stopped, a fine precipitate formed and the mixture became a very pale yellow colour. Nitrogen was then purged into the system to clearly see the yellow colour of the mixture, which increased in intensity when the Cl gas once again briefly replaced the N_2 flow. The intermediate (trichloride, a thin paste after 10 minutes stirring), was apparently saturated with chlorine due to no observable decrease in the intensity of the yellow colour once the Cl gas flow had been stopped. Absolute methanol (4.05mL, 100mmol) was then added, and the mixture allowed to

slowly warm (dry ice chunks removed from bath) under nitrogen for 1 hr, yielding a clear yellow solution. Most of the CH_2Cl_2 was evaporated off (ca. 63mL) *in situ* to yield a readily handled yellow solution, assumed to be a dry source of the sulfinyl chloride product.

O. Synthesis of butyl sulfinate (-) menthyl ester (optically active) (64)

To a dry, one-neck 100mL rb flask (N_2 flow and stir bar) was added 2mL crude sulfinyl chloride (14mmol max.) and 40mL dry ether. The solution was stirred and cooled on an acetone / dry ice bath before introduction of the stock menthol solution by slow injection (1.4mL of a solution containing 4.04g (-) menthol, 4.00mL dry pyridine, and ca. 0.2mL dry ether; 1.4mL = 9mmol menthol). A white precipitate fomed immediately and persisted throughout the four minute injection period. The mixture was stirred in the dry ice bath for 1 hr, then in the bath with the ice chunks removed for 30 minutes, and finally out of the bath for 5 minutes. Transferred to a separatory funnel with two ethereal rinses (10mL each), this organic was washed with ice-cold aqueous solutions (25mL each in order; 5% Na_2CO_3 , dH_2O , 1% HCl , dH_2O), dried over Na_2SO_4

(14g, 30 min.), then rotary evaporated to yield 1.91g crude ester (82% yield). Flash chromatography (10% EtOAc in hexane) afforded a white crystalline solid product (1.16g, 50% purified yield).

P. Synthesis of perdeutero butyl magnesium bromide (Grignard reagent) (65)

Magnesium metal (144mg, 5.8mmol) was crushed with a mortar and pestle, quickly washed with spectrograde acetone, then washed with fresh dry ether, and left to dry (5min.) under a gentle nitrogen stream in a dry rb flask (25mL, with stir bar and small condensor). A small (ca. 5mg) crystal of iodine was added, followed by dry ether (1mL), and dropwise, 0.6mL (5.4mmol) perdeutero-1-bromobutane. After the addition of the compound and the syringe rinse (2mL dry ether), the mixture was stirred and gently refluxed for 15 minutes. Once cooled, the resultant solution was used as a source of active Grignard reagent.

Q. Synthesis of 'optically active' 1,2,3,4-nonadeutero-dibutyl sulfoxide (66)

To a dry 10mL rb flask with stir bar under N_2 was added half the volume of a freshly prepared Grignard reagent (65) solution in ether (perdeutero-butyl magnesium bromide, 1.75mL, ca. 2.7mmol). Optically active menthyl ester (64) dissolved in dry ether was then carefully added dropwise (0.71g, 2.8mmoles in 3mL ether). Excessive initial reaction (partially due to poor stirring) caused bubbling and possible loss of ca. 0.1g of material. After the addition, the mixture was stirred under N_2 at room temperature for 1.5 hrs, before being quenched by the injection of ca. 2mL 10% aqueous ammonium chloride. Diluted with 2mL dH_2O , the mixture was transferred to a separatory funnel, extracted, and the aqueous layer removed. The ethereal layer was washed with water (2 X 4mL; combined with initial aqueous) then set aside. The combined aqueous layers were extracted (3 X 5mL CH_2Cl_2), and the resultant organics combined with the ether, dried (Na_2SO_4), and rotary evaporated to yield 248mg crude D_9 -dibutyl sulfoxide. Flash chromatography (EtOAc) provided 169mg of pure crystalline product (36% yield).

1,2,3,4-nonadeutero-dibutyl sulfoxide (66): ^1H NMR (400MHz) 2.55-2.72 (2H, m, $\text{CH}_2\text{CH}_2\text{S}(\text{O})$), 1.65-1.80 (2H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{S}(\text{O})$), 1.36-1.58 (2H, m, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{S}(\text{O})$), 0.90-1.00 (3H, t, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{S}(\text{O})$); ^{13}C NMR (400MHz) 52.1 (1C, $\text{CH}_2\text{CH}_2\text{S}(\text{O})$), 24.6 (1C, $\text{CH}_2\text{CH}_2\text{CH}_2\text{S}(\text{O})$), 22.1 (1C, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{S}(\text{O})$), 13.7 (1C, CH_3CH_2).

Benzyl sulfonate menthyl esters;

Obtained as a partially purified mixture of diastereomers from the synthetic work of a 498 student, R. Livingstone.⁽⁸¹⁾ Careful flash chromatography (7% EtOAc / hexane, 3-5mL fraction volume) effected the separation of the two diastereomers, yielding 241mg of the major component from the reaction (specific rotation in EtOH, c 0.25 : + 108), and 64mg of the minor component (specific rotation in EtOH, c 0.25 : - 208).

3.2.1 General

The strain of yeast used in this study was wild type *Saccharomyces cerevisiae*, ATTC #12341, obtained from the Yeast Genetic Stock Centre at the University of California-Berkeley. A Δ 9-desaturase deficient mutant (strain KD115) was used to demonstrate the dependency of sulfoxidation on this enzyme.

The wild type organism was grown at 30°C in liquid YEPD culture medium in a New Brunswick Scientific incubator shaker (model G25) at 150 rpm. The YEPD liquid growth media contained by weight 1.0% yeast extract, 2.0% bacto-peptone, and 2.0% D-glucose dissolved in distilled deionized (Millipore) water. Cultures were maintained as slants at 4°C on a solid YEPD-agar medium for up to 3 months. The slant medium was prepared as above with the addition of 3.0% bacto-agar.

The mutant was grown under similar conditions in liquid culture with a media supplement of 1.0% Tween 80 (by volume) as a source of essential fatty acids. The mutant was actively maintained (stored at 4°C for no longer than 1 month) by streaking for single-cell promoted cultures and replica plating on petri-agar with and without the Tween supplement. Cultures observed to grow only on the Tween supplemented media were considered to be true mutants (non-revertants) and were immediately used for inoculations and incubations with substrate. The petri-plate agar medium was similar to the YEPD, except that only 2% bacto-agar was used.

All media was sterilized in volumes no greater than 300mL each for 30 minutes at 1.3kg / cm² and 121°C in an Amsco Eagle Series (model 2021) Gravity Autoclave. Aseptic techniques were carried out in a freshly 75% EtOH-swabbed Canadian Cabinets Flowhood (model HE-3-97-T) in close proximity to a Bunsen burner flame. Cell counts were made on water-diluted culture samples, employing a glass haemocytometer counting chamber (slide), visualized through a microscope at 400 X magnification. A minimum number of ten sets of ten squares were counted then averaged per sample. Autoclaved media and flowhood sterility was routinely checked by opening an autoclaved media sample in the operating flowhood for 2 to 5 minutes, then incubation of the sample for at least two days. In every case the clarity of the solution was maintained, providing good evidence for sterility. After the typical incubation period (20-24hrs), the cells were separated from the medium by centrifugation for 10 minutes at 5,000rpm (1,600 X g) at ca. 5°C, using a GSA type rotor in a Sorvall (Beckman) model RC-5B refrigerated centrifuge. Like cells were resuspended, combined, washed with water (20-30mL), and repelleted.

A. Example of YPD media preparation and a typical incubation

To a 3L beaker was added 32.0g D-glucose, 32.0g bactopectone, and 16.0g yeast extract, followed by sufficient distilled deionized water (ca. 1585mL) to yield a final volume of 1600mL. Heated and stirred to aid dissolution, the warm solution was divided among five 1L Erlenmeyer flasks (ca. 304mL each) with about 60mL reserved for a starter culture (placed in a 250mL flask). Plugged with a small 7cm diameter ball of cotton wool and capped with a double layer of tinfoil, the flasks were autoclaved (30 min.) along with two 10mL graduated cylinders and six cotton-plugged glass pipettes, (foil wrapped). All was then removed from the autoclave and cooled in a non-operating flowhood for a minimum of 3 hours and no longer than 22 hours.

The starter culture flask was inoculated with a small (ca. 10mg) portion of yeast, taken aseptically with a flamed wire loop from a cold agar slant. Growth for 17 to 23 hours, yielded a stock culture of ca. 5×10^8 cells per mL. Aseptically, using the autoclaved graduated cylinder, a constant volume (± 0.2 mL, generally between 6 and 8 mL) of starter culture was then distributed to each flask (unless a sterile control was used, which required only the addition of 1mL ethanol). This was followed by 50mg of a chosen substrate, dissolved in 1mL absolute ethanol, transferred to each flask by use of the autoclaved and plugged pipettes. The spare autoclaved graduated cylinder and pipettes were used any time contamination of the old item was suspected (brushed against with a wrist, etc.), more than 3 transfers had been performed, or whenever the contents

to be transferred were changed.

All flasks (except the remaining starter culture) were then incubated with agitation for 20 - 24 hours, yielding a final cell concentration of 2×10^8 cells/mL, representing between 4 and 5 generations of growth. Common cultures were combined at this end point without worry of contamination. Samples were immediately centrifuged and cell pellets combined with water washes to yield one cell pellet for each type of experiment culture. Clear, tea-coloured supernatant, held in refrigeration, was then extracted using one of two methods.

The first method involved acidification to pH 1 (ca. 10mL conc. H_2SO_4 per 600mL media, indicator paper - red), followed by hexane extraction (1 X 80mL, 4 X 50mL, per 600mL media). Emulsions were broken by suction filtration through a large Celite pad (8 - 15mm thick, 15 - 20cm diameter).

The second method required acidification of the medium to approximately pH 2 (ca. 15mL conc. HCl per 600mL media), dissolution of solid NaCl (20g per 100mL), and extraction with dichloromethane (1 X 70mL, 4 X 50mL per 600mL). Emulsions, less severe than those of the previous method, were dissipated with a combination of time (10 - 40min.) and 'spinning' of the separatory funnel by hand, (both centrifuge-style and spinning top-style). Occasionally, severe or stubborn emulsions were partially resolved by the addition of CHCl_3 and / or CCl_4 followed by very gentle swirling.

Combined organics were dried (ca. 15g, 2 - 20hrs), rotary evaporated, and either stored under nitrogen (4°C) or methylated then stored. Methylation was performed by the addition of diazomethane in ether to the crude extract in ether, holding both solutions on ice. The addition of the diazomethane solution was continued until a yellow colour persisted at room temperature or (if the extract solution started out as yellow or green) until no obvious effervescence resulted from a gentle addition. The resulting solution was brought to room temperature for 1 - 2 minutes then rotary evaporated in a 25 - 30°C water bath to yield the methylated extract.

TLC analysis was performed against standard synthetic sulfoxide, to determine the presence or absence of product, and the correct ratio of hexane to ethyl acetate to use in the flash chromatography which followed. The solvent system of choice resulted in an R_f value between 0.17 and 0.20 for the compound most interested in purifying. Purified sulfoxide would then be weighed and analyzed by NMR.

B. List of compounds administered

Methyl 9-thiastearate (22); Methyl 9-thiastearate-10,10- d_2 (22-10,10- d_2); Methyl 10-thiastearate (23); Methyl 10-thiastearate-11,11- d_2 (23-11,11- d_2); Methyl 5-thiastearate (24); Methyl *S*-benzyl-8-mercaptooctanoate (25); Methyl *S*-benzyl-9-mercaptononanoate (26); Methyl *S*-phenyl-8-mercaptooctanoate (27); Methyl *S*-phenyl-9-mercaptononanoate (28); Methyl *S*-(2-naphthyl)-8-mercaptooctanoate (29); Methyl *S*-(2-naphthyl)-9-mercaptononanoate (30); Thiophenol; 2-Naphthalenethiol; Methyl 10-(2-naphthyl)-9-thiadecanoate (31); Methyl 11-((*para*-trifluoromethyl)-phenyl)-9-thiaundecanoate (32); Methyl *S*-((*para*-trifluoromethyl)-benzyl)-9-mercaptononanoate (33).

C. Wickerman's media preparation ⁽⁸⁰⁾

The following stock solutions were prepared.

(i) Trace Elements ; The following was dissolved in 200mL ddH₂O;

H₃BO₃, 49.8mg; MnSO₄(4H₂O), 40.1mg; ZnSO₄(7H₂O), 40.0mg; FeCl₃(6H₂O), 20.0mg; Na₂MoO₄(2H₂O), 20.1mg; KI, 10.5mg; and CuSO₄(5H₂O), 4.3mg.

(ii) Salts Mixture ; The following was dissolved in 400mL ddH₂O;

KH₂PO₄, 10.00g; MgSO₄(7H₂O), 5.00g; NaCl, 3.00g; CaCl₂, 1.50g.

(iii) Vitamin Mixture ; The following was added to 100mL ddH₂O;

inositol, 406.6mg; pantothenic acid (calcium salt), 100.8mg; nicotinic acid, 40.0mg; pyridoxine-HCl, 40.2mg; thiamine-HCl, 40.8mg; p-amino benzoic acid, 20.0mg; riboflavin, 20.5mg; biotin, 1.5mg; folic acid, 0.2mg (*0.1mg).

The resulting mixture was warmed to 50 - 60°C and suction filtered. The fine orange insoluble material on the filter paper was washed with water (3 X 30mL, 60°C), dilute salt solution (4 X 20mL, 70°C, 0.03M), more water (4 X 30mL, 80°C) and finally dilute sodium bicarbonate solution (2 X 10mL, 80°C, 1% w/v). The solution was cooled and filter sterilized by suction through a Millipore glass frit filter fitted with a Gelman 0.05 μ m sterile filter paper. The bright yellow solution was stored refrigerated and kept sterile.

1 litre of complete media was prepared in the following manner;

20.0g D-glucose was dissolved in 600mL ddH₂O in a 2 litre Erlenmeyer flask, cotton-plugged and foil-capped.

40mL of salts solution, 2.0mL of trace elements, and 354mL of ddH₂O was used to dissolve the following in a 1 litre Erlenmeyer; ammonium sulphate, 4.0g; L-aspartate, 1.5g; L-histidine, 10mg; L-methionine, 10mg; D,L-tryptophan, 20mg.

Prepared for sterilization, these solutions were autoclaved (35min.) along with three 1-litre Erlenmeyer flasks, a cotton-plugged 10mL graduated glass pipette, and the usual 10mL graduated cylinders and plugged glass pipettes. Cooled to approximately 50 - 60°C, the salt-containing solution was added aseptically to the glucose solution. 4.1ml of the sterile cold vitamin solution was then transferred in by use of the graduated pipette. After complete mixing, this net solution was divided out among the empty sterile flasks, (ca. 50mL/250mL flask and 300mL/1-litre flask). The second 250mL flask produced by this method was used as a sterility control, important to each set of defined-media experiments due to the number of transfers performed. This control was touched with a flame-sterilized wire loop (air-cooled), and treated with 0.5mL abs. EtOH (via plugged pipette).

D. Special incubations

(i) thiophenol, 2-naphthylene thiol

Incubations were performed in the typical manner in YPD media except that the substrate was replaced with thiol (ca. 15mg). The experiment was concluded by the observation of growth after 22 hours.

(ii) fluorine assay

An experiment involving the trifluoromethyl substituted phenyl ring was firstly analyzed in the above typical (YPD media) manner, to ensure highly enantio-selective sulfoxidation had occurred. The second experiment competed 9-thia versus 10-thia substrate in the same flask, and product formation was followed by aseptic removal of 2mL aliquots from the growing cultures at a variety of sampling times, (2, 10, 22, and 30 hrs). The aliquots were allowed to settle, stored frozen, and the thawed supernatants (plus ca. 10% D₂O) directly analyzed for product by ¹⁹F-NMR.

E. Hydrolysis of pelleted cells

During initial experiments, including the search for products resulting from cleavage, it was necessary to look at the total fatty acid content that was harboured inside the cells. The following procedure was used for cellular fatty acid content analysis.

Frozen pelleted cells (wet, 10g) were transferred to a one-neck 250mL round bottom flask with boiling chips (30 - 40). The storage bottle and transfer device (spatula or glass pipette) were rinsed with 10mL distilled water, which was added to the cells. Potassium hydroxide pellets (3.5g, 63mmol) were dissolved in 18mL dH₂O, then added to the cell mixture, followed by 30mL of 95% EtOH. Under nitrogen and with a water-cooled condensor, the mixture was heated to a gentle reflux for 2 hours. Cooled 1 hour to room temperature, solid particulate was removed by mild suction filtration and washed with water (2 X 5mL). The filtrate was acidified (11mL 6M HCl + 29mL dH₂O), transferred to a separatory funnel and extracted with dichloromethane (3 X 25mL). Combined organics were dried (Na₂SO₄, overnight) then rotary evaporated to yield a crude pale green oil (246mg).

NOTE: Methylation with diazomethane in ether at this point, occasionally produced ether insoluble material, which when removed accounted for approx. 5 - 20% of the yield.

4.1 Appendix 1 : Supplementary Spectra

- Contents;**   **Figure #40 COSY and HETCOR spectra for dibutyl sulfoxide and dibutyl sulfone**
- Figure #41 ¹H-NMR of Methyl *S*-(2-naphthyl)-9-mercaptononanoate *S*-oxide (45) in the presence of MPAA**
- Figure #42 ¹⁹F-NMR spectra of synthetic standards in blank YPD media**
- Figure #43 ¹H-NMR of Methyl 9-thiastearate sulfone in the presence of MPAA**
- Figure #44 ¹H-NMR spectra of (*p*-carboxymethyl)-benzyl methyl sulfoxide in the presence of the Kagan reagent and MPAA**
- Figure #45 ¹H-NMR spectra of the proton on menthyl C-1 for the two pure (-)-*O*-menthyl (±)-benzylsulfinate diastereomers (re: Table #1)**

FIGURE #40 COSY and HETCOR spectra (Bruker AMX 400) for the purposes of unambiguous assignment of the ^{13}C -NMR signals for A. dibutyl sulfoxide
B. dibutyl sulfone

FIGURE #41 400MHz ¹H-NMR spectral segment for the α -sulfinyl hydrogens of synthetic methyl S-(2-naphthyl)-9-mercaptononanoate **A.** without shift reagent **B.** with 3 equivalents MPAA added

FIGURE #42 ^{19}F -NMR (376.5MHz) spectra of CF_3 -probe and product standards in spent blank media with ca. 10% D_2O A. Methyl (*p*-trifluoromethyl)-benzyl-9-mercaptanonanoate, (33) (10-S(Me)) B. sulfoxide of 10-S(Me), (48) (10-S(O)Me) C. crude 10-S acid (ester also visible by tlc) (10-S(OH)) D. sulfoxide of 10-S acid (10-S(O)OH) E. methyl 11-((*p*-trifluoromethyl)-phenyl)-9-thiaundecanoate *S*-oxide, (47) (9-S(O)Me, with sulfide (9-S(Me)) impurity) F. 2:1 ratio of 9-S(Me):9-S acid (9-S(OH)).

FIGURE #43 200MHz ^1H -NMR of α -sulfinyl hydrogens of methyl 9-thiastearate sulfone A. without shift reagent B. in the presence of 4 equivalents of MPAA

FIGURE #44 200MHz ^1H -NMR spectra comparing the effectiveness of shift reagents on the AB-type benzylic protons of (p-carboxymethyl)-benzyl methyl sulfoxide
 A. without shift reagent B. with Kagan reagent C. with MPAA

FIGURE #45 200MHz ^1H -NMR spectra of the menthyl C-1 proton (doublet of triplets) for purified benzylsulfinate esters A. (-)-O-menthyl (+)-benzylsulfinate centered near 3.888ppm B. (-)-O-menthyl (-)-benzylsulfinate centered near 3.898 ppm.

4.2.1 Synthesis of *N*-nitroso-methyl urea (67) ⁽⁸²⁾

To a stirring solution of methylamine (ca. 1.58moles, 153g of a 30-35% aqueous solution in a 2L RB) on ice was slowly added ca. 150mL of concentrated HCl to yield a pH of about 0.0. Water (ca. 165mL) was added to bring the total solution mass near 500g. Urea (300.2g, 5.00moles) and a few boiling chips were added, the mixture was removed from the ice, connected to a condensor, and brought to a steady reflux for 2.5 hours. The resulting solution was cooled in ice to room temperature, then 111g (1.61moles) sodium nitrite was added and dissolved. Ice-cold, this solution was slowly dripped (ca. 35 minutes) into a magnetically stirred mixture of 602g ice and 101g concentrated sulfuric acid, held in a 4L Erlenmeyer on ice. The resulting precipitate was suction filtered to near dryness, yielding ca. 100g of damp pale yellow needle-like crystals.

4.2.2 Purification of *p*-toluene sulfonyl chloride (p-Tosyl-Cl)

Ca. 55g crude material was partly dissolved in hot benzene (200mL) and gravity filtered hot into a separatory funnel (with 2 X 10ml rinses). This organic was washed with sodium hydroxide solution (5 X 20mL, made fresh 5.6g / 100mL dH₂O), dried over anhydrous magnesium sulfate, rotary evaporated down to ca. 40mL, then allowed to crystallize on ice, (to ca. 5°C). The excess benzene was decanted off, the crystals were washed with ice cold benzene (2 X 10mL, removed by pipette), then freed of benzene by rotary evaporation at room temperature (5hrs). Yield ca. 22g white crystals.

4.3.1 Introduction

Personal interest in the insect pheromone system, coupled with the recent confirmation of Δ 11-desaturase activity in the spruce budworm moth sex pheromone gland, prompted a short study into the use of our sulfide probes with this system. The question that was posed was, "could this insect desaturase be diverted to oxygenase activity with use of sulfide substrates as demonstrated already for the yeast system?"

4.3.2 Results and discussion

Results by tlc of both the 11-S and 12-S substrates (thiamyrystate and thiabenzyl) showed the absence of detectable levels of sulfoxide. Suspected reasons for reduction of product yield were estimated sequentially at; 10% loss for administering each substrate, 25% loss to non-gland tissues and missed during gland removal, a 66% extraction efficiency, and only 33% sulfoxidation. It was based on these values in conjunction with the determined tlc detection limits, that a 10 μ L sample from the 400 μ L extract should contain detectable sulfoxide. In searching for the sulfoxide product however, this value was doubled and occasionally quadrupled during subsequent attempts.

(i) General

Initial synthesis of 11- and 12-thiamyristic acids and the 11-thiabenzyl compounds, was performed by linking the dianion of the thio acid to the corresponding bromo compound (Method 1 of main procedure). A second set of myristate syntheses were similar except that the 11-thiamyristic acid substrate was afforded by linkage of propane thiol to the corresponding 10-bromodecanoic acid (Method 2 of main procedure).

All three substrates were purified and chemically oxidized as their methyl esters, then returned to free acid form by base hydrolysis. In most cases a final purification of the sulfide acid was performed by preparatory tlc (95 % EtOAc/Hexane) to rid the sample of any possible sulfoxide contaminant.

Determining product detection limits involved a comparison between ultraviolet light-aided visualization and water-aided visualization. The UV system required that the crude acid product be ester-linked to a p-nitrobenzyl group (2hrs., 80°C, 0.1M reagent in CH_2Cl_2), then visualized on the tlc plate with the aid of a UV lamp. The water-based visual detection system made use of the strongly hydrophobic nature of the sulfoxide product, especially when the acid group was methylated. The crude extracts would be subjected to diazomethane in ether, solvent and excess reagent evaporated, product dissolved, run on tlc, then sprayed with a water mister and placed on a black background to aid in the visual detection of white regions. These white spots were characteristic of hydrophobic products which "pushed" water away from themselves,

causing the plate to dry faster in that region.

A decision to administer 80 nanomoles of substrate to each insect was based on determined detection limits, sources of substrate and product loss, and previous publications of similar material.^(83,84,85) The starting materials were dissolved in DMSO which contained approximately 0.005% (w/v) butylated hydroxy toluene (BHT) as an antioxidant, guarding against sulfide autoxidation by exposure to air.

Sexed pupae of the Eastern Spruce Budworm, *Choristoneura fumiferana*, were obtained from Dr. G. Grant's laboratory at the Great Lakes Forestry Research Laboratory in Sault Ste. Marie, Ontario. Insects were hatched in a 35cm cubic wire screen cage at approximately 70% humidity (maintained by household humidifier), at 22 - 24°C, with a 15 hour light : 9 hour dark, cycle.

(ii) Detailed example of an experiment

Female budworm pupae (70-80) were carefully counted and spaced out onto a paper towel on the bottom of the cage. They were misted directly with a water spray then maintained in a humid environment until the start of the feeding experiment. A fluorescent lamp was used to maintain the light cycle (approx. 5:30 pm - 8:30 am) while a heavy cloth was employed for the dark. Emergence of a few moths occurred after two days of this cycle, and within three more days the majority of the moths had hatched (68/78). Unhatched pupae were removed and destroyed at this point by freezing. The

moths were allowed to age through two light cycles before being used for experimentation near the end of the second.

Myristate substrate (2.0mg, 8.1 μ mol) was freshly dissolved in 50 μ l DMSO (0.005% BHT) in a 100 μ l point-bottomed Reacti-vial. One hour before the dark phase was to begin, moths were collected into groups of 4 to 6, kept in the light, and anaesthetized with carbon dioxide between a porous pad and an inverted funnel. Individually, each moth was squeezed near the end of the abdomen with a modified smooth-jawed alligator clip so that the sex pheromone gland was exposed. A 0.5 μ l drop of dissolved myristic acid analogue (81nmol) was applied to the exposed gland by use of a 10 μ l glass microsyringe. The moth was hung on a wire by the alligator clip for 1 - 2 hours to allow adsorption of the substrate solution. The moth was then released for 5 hours before being anaesthetized and clipped again. Finally the exposed gland was removed with the aid of a pair of needle-point tweezers and crushed in a Reacti-vial containing 200 μ l CHCl_3 and 200 μ l MeOH.

In this experiment, 20 moths were administered the 11-thiamyristic acid, another 20 the 12-thiamyristic acid, 10 moths the 5-thiamyristic acid (control), and three moths were treated with just the DMSO. Dead moth controls were created by subjecting 15 freshly clipped moths to a hot water bath (80 - 85°C, 15min.). This was followed by the administering of substrate (one type per 5 moths), incubation, gland abstraction, and crude product recovery as above.

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