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**"The use of fluorinated fatty acids
as a mechanistic probe for the fatty acid desaturation
by the stearoyl-CoA Δ 9-desaturase
of *Saccharomyces cerevisiae*"**

by

Konstantinos A. Alexopoulos, B.Sc.

A thesis

submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements
of the degree of
Master of Science

Department of Chemistry

Carleton University

Ottawa, Canada

December 1994

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"The use of fluorinated fatty acids
as a mechanistic probe for the fatty acid desaturation
by the stearoyl-CoA Δ^9 -desaturase
of *Saccharomyces cerevisiae*"

Submitted by
Konstantinos A. Alexopoulos

in partial fulfillment of the requirements
for the degree of Master of Science

A handwritten signature in black ink, appearing to read "T. Baird", is written over a horizontal line.

Thesis Supervisor

A handwritten signature in black ink, appearing to read "J. Wright", is written over a horizontal line.

Chairman, Department of Chemistry

Carleton University

Abstract

Fluorine substituted fatty acids (*flustrates*) were used as novel probes in order to further investigate the mechanism of fatty acid desaturation by the stearoyl-CoA $\Delta 9$ -desaturase of *Saccharomyces cerevisiae*. The tested flustrates were the methyl (*S*)- and ($\pm$)-12-fluorostearates and the methyl 16-, ($\pm$)-10- and ($\pm$)-9-fluoropalmitates. Incubations of the above flucrofatty acids gave the corresponding olefin as the only product with % conversions 5, 5, 63, 30 and 10 respectively. Analytical procedures included ^1H -decoupled ^{19}F NMR and GC-MS.

The double bond of the biosynthetic product from the incubation of methyl 12-fluorostearate was found to have *cis*-stereochemistry. The two enantiomers of methyl ($\pm$)-12-fluorostearate were not discriminated by the enzyme.

No oxygenated products were formed from the incubations of methyl ($\pm$)-10- and ($\pm$)-9-fluoropalmitates, a result which militates against the obligatory existence of hydroxylated intermediates in desaturation. Finally, the difference between the reactivity of the above flustrates was explained based on studies of hydrogen atom transfer reactions in fluoroalkanes.

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1.0 Introduction

1.1 Fatty acid desaturation

Cis-unsaturated fatty acids are essential molecular components for most organisms^[1]. Their major biological function is to increase the fluidity of cell membranes by introducing a kink in the aliphatic chain so that the m.p. of lipids decreases. *Trans*-unsaturated fatty acids would have little effect on the fluidity of the phospholipid membrane as they resemble saturated fatty acids in terms of conformation^[2]. Some common unsaturated fatty acids are shown below:

Scheme 1. Typical unsaturated fatty acids: palmitoleic ($\Delta^9-16:1$), oleic ($\Delta^9-18:1$), linoleic ($\Delta^9,12-18:2$) and *cis*-vaccenic ($\Delta^{11}-18:1$) acid.

Unsaturated fatty acids can arise *via* two different pathways; anaerobic or aerobic. The anaerobic pathway, present in certain bacterial species, involves an introduction of a *cis*-3 double bond into a medium chain length fatty acid by dehydration of a fatty acid synthetase intermediate^[3]. The aerobic pathway is more unusual from a mechanistic point of view because desaturation occurs in the middle of an essentially homogeneous chain of carbons without any activation by functional groups within the substrate.

The most common desaturase, Δ^9 -desaturase, introduces a *cis*-double bond into the fatty acid chain by regio- and stereoselective removal of two hydrogens from the 9 and 10 carbon atoms. Thus, stearic acid is converted to oleic acid using molecular oxygen as an oxidant and NAD(P)H as an electron source^[4,5] (see Scheme 2). Animal and yeast desaturases are membrane-bound proteins and generally require CoA (coenzyme A) derivatives of fatty acids as substrates, while plant desaturases are soluble proteins and require ACP (acyl carrier protein) derivatives of fatty acids^[6,7].

Scheme 2. Desaturation of stearoyl-CoA to oleoyl-CoA by the stearoyl-CoA Δ^9 -desaturase.

The enzymology of the desaturation has been extensively investigated and the best characterized membrane-bound desaturase, to date, is that isolated from rat liver^[8]. It consists of three enzymes, bound to the endoplasmic reticulum membrane. They are NADH cytochrome b₅ reductase (a flavoprotein), cytochrome b₅ (a heme-iron protein) and stearoyl-CoA desaturase (a non-heme iron protein)^[9]. The electron transfer sequence is shown in the following Scheme:

Scheme 3. Electron flow in the stearoyl-CoA desaturating system.

Cytochrome b₅ reductase and cytochrome b₅ are both amphipathic proteins containing two domains; one hydrophilic and one hydrophobic^[10,11]. The hydrophilic portions are involved with the electron transfer, while the hydrophobic portions are embedded into the phospholipid membrane^[11]. The stearoyl-CoA desaturase is a single polypeptide chain of 458 amino acids (62% non-polar) and it is all embedded into the lipophilic membrane. It contains one atom of tightly bound non-heme iron per molecule, which appears essential for catalysis (catalytic activity destroyed by chelator of Fe²⁺)^[12]. The structure of the non-heme iron oxidant is still not clear, but it is believed that up to five tyrosyl residues are involved in the binding of the iron, possibly *via* the phenolic oxygen^[13]. The yeast desaturase is similar to the rat Δ^9 -desaturase, as was demonstrated after the isolation of the gene (OLE 1) from yeast^[14]. A 257-amino acid internal region of the yeast showed 36% identity and 60% similarity to the rat stearoyl-CoA desaturase protein (see Scheme 4)

Scheme 4. Model for the orientation of the yeast desaturase in the endoplasmic reticulum membrane.

Plant desaturases have also been characterized^[15,16,17]. The most remarkable feature of these desaturases is that they contain two iron atoms as was recently found by Mössbauer spectroscopy for the $\Delta 9$ -desaturase of the plant *Ricinus communis*^[17]. For the soluble enzyme stearoyl-ACP $\Delta 9$ -desaturase, stearoyl-ACP is desaturated to oleoyl-ACP in the presence of O₂, NADPH, NADPH ferredoxin oxidoreductase and ferredoxin^[12].

The conformation of the substrate in the active site of the enzyme is of great importance and it can affect the outcome of the reaction. Previous experiments have suggested that at 9 and 10 positions of the hydrocarbon portion of stearoyl adopt an eclipsed conformation when bound to active site of the enzyme^[18]. A possible model of interaction between enzyme and substrate could involve a binding site which can recognize and hold on the end of the chain (possibly with a hydrogen bond between the CoA thioester and one peptide), a highly reactive group which can remove unactivated hydrogens and which is located at a well-defined distance from the binding site and some

auxiliary hydrophobic binding sites which can immobilize the chain in particular positions (one, for example, could be the end of the fatty acid chain).

Even though the enzymology of fatty acid desaturation has been established, detailed information on the precise mechanism of hydrogen removal by the desaturase is lacking. The failure until now to determine the mechanism is primarily due to the fact that a stable mammalian desaturase system has proved difficult to obtain because of their extraordinary hydrophobic nature and tight association with the membranes. Plant desaturases may be helpful in this case due to their solubility. However, these enzymes might operate by a different mechanism, since they contain two iron atoms at the active site.

Nevertheless, some progress has been made from experiments with the whole cell system of some microorganisms. Thus, Bloch^[5] demonstrated that in the bacterial system *Corynebacterium diphtheriae*, the *pro-R* hydrogens at C-9 and C-10 were the ones removed to form the *cis*-double bond. Studies of isotope effects also indicated that the removal of 9 hydrogen was the initial and rate determining step and the removal of 10 hydrogen followed, suggesting a stepwise hydrogen removal process. However, Morris^[19], working with *Chlorella*, has deduced that synchronous removal of the two hydrogens occurred, since both steps appeared to have similar isotope effects.

All desaturation systems show an obligatory requirement for O₂^[5,19,27]. Molecular oxygen presumably interacts directly with the enzymes to produce a metal-peroxide species; no artificial electron acceptor capable of replacing oxygen has been found to date^[20]. It is thought that an iron oxo species (Fe=O) is formed which is then involved in the desaturation by analogy with hydroxylases^[21] (see Scheme 5).

Scheme 5. Formation of the desaturation-active oxo-derivative.

All the theoretically possible pathways for the formation of $\Delta 9$ *cis*-double bond are shown in Scheme 6.

Scheme 6. Different pathways for the mechanism of Δ^9 -desaturation.

Direct oxygen insertion (pathway A) or direct formation of carbocation (pathway C) are thought to be unlikely on the grounds that they have no well-documented mechanistic equivalent in chemical systems^[22]. The three most probable pathways are the following.

1. Electron transfer/deprotonation ($B \rightarrow E \rightarrow H$): The first step for this model is hydrogen abstraction, which is followed by electron transfer and deprotonation of the resultant carbocation. This model is supported by our group^[23] because of its analogy with the one proposed for the well-studied enzyme P₄₅₀ which is also capable of acting as dehydrogenase (desaturase) with particular substrates^[24]
2. Disproportionation ($B \rightarrow G$): In this case, dehydrogenation is proceeding *via* sequential hydrogen atom removal^[21]. This model involves the use of a free hydroxyl radical by the enzyme, thus, it is less likely to operate.
3. Oxygen rebound/dehydration ($B \rightarrow D \rightarrow F$): This process involves initial hydrogen removal followed by oxygen rebound (hydroxylation) at 9 or 10 position and E₁ dehydration to produce the olefin. The first two steps are similar to hydroxylation by cytochrome P₄₅₀^[25]. The role of Fe^{III} as a Lewis acid, leading to cationic intermediates has recently been proposed for P₄₅₀ oxidation of cyclopropyl hydrocarbons^[26]. Nevertheless, attempts to convert 9- or 10-hydroxystearoyl CoA to oleoyl CoA have been unsuccessful^[27]. These findings do not, however, rule out the possibility of an oxygenated intermediate which is not released from the enzyme^[28]. Furthermore, the existence of the above mechanism is supported by the following findings:

⇒ Non-heme iron proteins are known to be capable of catalyzing the hydroxylation of various aliphatic compounds^[29,30,31,32].

⇒ The plant fatty acyl desaturase of *Ricinus communis* has been demonstrated to have a diiron active site^[17]. Enzymes having a diiron active site, like the methane monooxygenase (MMO), catalyze the hydroxylation of alkanes^[32,33].

There is also another suggested mechanism, based on the “Gif” catalytic reactions which involves formation of an iron-carbon bond as a possible intermediate, followed by oxidative elimination^[32,34] (see Scheme 7). The existence of a similar pathway has been proposed for the mechanism of the desaturative cyclization of the substrate (ACV) by the isopenicillin N synthase^[35].

Scheme 7. Proposed mechanism involving organoiron intermediate.

Our group, working with the Δ^9 -desaturase of baker's yeast (*Saccharomyces cerevisiae*) and using thiafatty acids as fatty acid analogues, has moved one step forward in the understanding of the desaturase mechanism. The main purpose of our experiments was to determine the initial position of oxidation. Incubation of methyl 9-thiastearate and methyl 10-thiastearate in the yeast desaturase gave the corresponding sulfoxides (see Scheme 8). The stereochemistry of sulfoxidation matched that of hydrogen removal in

the desaturation process and furthermore 9-sulfoxidation was always more efficient than 10-sulfoxidation by a factor of at least 2:1^[36].

Scheme 8. Sulfoxidation of methyl 9- and 10-thiastearates by the $\Delta 9$ -desaturase of *Saccharomyces cerevisiae*.

Recent experiments with 9- and 10-dideuterio-7-thiastearic acids showed also that there is a substantial isotope effect at 9 but not at the 10 position^[37]. The above findings confirm a stepwise mechanism in which hydrogen atom removal is the rate limiting step, where the iron oxidant seems to be asymmetrically located between carbons 9 and 10 (see Scheme 6).

For a better understanding of the desaturation, more mechanistic work is required by the use of sterically unobtrusive probes. Replacement of hydrogen atoms by fluorine atoms in certain positions can be very helpful. Thus, fluorine can be used as a possible probe for trapping hydroxyl intermediates, either by blocking hydrogen atom removal, or by raising the redox potential of radical intermediate and preventing carbocation formation.

1.2 "Flustrates" as mechanistic probes for the desaturation of fatty acids by the $\Delta 9$ -desaturase of *Saccharomyces cerevisiae*

1.2.1 General

1.2.1.1 Fluorine in bioorganic chemistry

The attractiveness and utility of fluorine as a substituent in a biologically active molecule is due to the pronounced electronic effects that may result upon fluorination as well the fact that fluorine is not a sterically demanding substituent. Some characteristic features of organofluorine compounds are illustrated below^[38].

Bond energy (kJ mol^{-1})

C-H 416 C-F 485 C-Cl 328

Bond length ($\text{\AA}$)

C-H 1.09 C-F 1.38 C-Cl 1.78

Van der Waals radius ($\text{\AA}$)

CH_3 2.0 CF_3 2.7 CCl_3 3.5 $\text{C}(\text{CH}_3)_3$ 3.5

Van der Waals volume (hemisphere, $\text{\AA}^3$)

16.8

42.6

Electronegativity (according to Pauling)

H 2.1 C 2.5 Cl 3.0 F 4.0

Polarizability (for CH_3X , 10^{-24}cm^3)

H 2.59 F 2.97 Cl 4.72 Br 6.03

Magnetic properties (relative sensitivity)

^1H (spin 1/2) 1.00 ^{19}F (spin 1/2) 0.83 ^{13}C (spin 1/2) 0.016

The C-F bond is particularly strong and of all the bonds involving carbon, the C-F bond is the shortest apart from C-H. The Van der Waals radius of fluorine is very similar to that of hydrogen and despite the fact that a CF_3 group has over twice the Van der Waals volume, it is still capable of replacing the latter statistically in crystals^[39]. The C-F bond is extremely polar, but is nevertheless stable under most conditions. Fluorine has a high electronegativity resulting in a pronounced effect on the electron distribution in a molecule. The magnetic properties of the ^{19}F nucleus facilitate the investigation of receptors and metabolic phenomena without interference from other magnetic nuclei^[40], a property which proved very useful in the work described in the thesis.

1.2.1.2 Effects of fluorine on chemical reactivity

Fluorine has a prominent electron-withdrawing inductive effect by relay of an induced dipole along the chain of σ -bonded atoms. However, the electronic effect of fluorine directly attached to a π system can be more complex, as electrons from fluorine may be donated back to the π system by a π -conjugative effect (resonance or mesomeric effect).

A α -fluoro substituent can stabilize carbocations by the interactions of a vacant p -orbital of the carbocation with the filled p -orbitals of fluorine, but β , γ , and δ substituents destabilize carbocations due to the inductive effect ($-I$)^[41,42]. The thermodynamic

stability of the fluoro substituted radicals is difficult to assess. Generally some previous experiments^[43] have shown that in *n*-butyl fluorides a β -fluorine retards hydrogen abstraction from carbon and the extent of this retardation depends on the electronegativity of the attacking radical. A γ -fluorine also exerts a slight retarding influence on hydrogen abstraction. α -Fluorine can either activate or deactivate hydrogen abstraction depending again on the attacking group (competition between resonance (+R) and inductive effect (-I)).

1.2.1.3 Effects of fluorine on biological reactivity

All the above features, either individually or in combination, will influence the outcome of a reaction (whether enzyme-catalysed or not) in which a fluoro analogue of the natural substrate is employed. Organofluorine compounds frequently play a role in the synthesis of inhibitors, substrate analogues, so-called enzymatic or metabolic probes, antimetabolites, "transition state analogues" and "suicide substrates or inhibitors" [44,45,46,47,48,49].

An example of the fluorine effect in biological systems is the toxicity of the ω -fluorofatty acids^[50]. The toxicity of this kind of compounds occurs *via* the formation of the toxic fluoroacetic acid, which is the final product from the β -oxidation of the above fatty acids. It can be readily seen that for the $F(CH_2)_nCOOH$ analogues, only when *n* is an odd number does this process of β -oxidation give the toxic fluoroacetic acid. It has been proved that the poisoning by fluoroacetate is brought about by blocking the tricarboxylic acid cycle *in vivo* (see Scheme 9). The fluoroacetate is converted into 2-fluorocitrate (I) which blocks the enzyme aconitase. This enzyme (within the mitochondria) converts *cis*-aconitate to either citric acid or isocitric acid. 2-Fluorocitrate binds irreversibly to the enzyme aconitase and the strong -I effect of fluorine atom ensures that dehydration to form fluoroaconitase is repressed. Accumulation of citric acid is therefore the reason for fluoroacetate poisoning. The cycle is thus completely disrupted by the "lethal synthesis" of an anti-metabolite and the implications for the rational

design of enzyme inhibitors for the use in chemotherapy became immediately apparent^[51].

Scheme 9. Diagram showing the pathway of synthesis of fluoroacetate to fluorocitrate.

Such a mechanism would appear to be dependent upon (a) the stability of the C-F bond in the fluoroacetate; and (b) the small size of the fluorine atom. When the n is even number, then β -fluoropropionic acid is formed, which is non-toxic because in this case the fluorine atom is very labile and is easily removed from the molecule. Difluoroacetic acid and trifluoroacetic acid are non-toxic although the C-F bonds in these compounds are extremely stable. The reasons for the non-toxicity, in this case, is that the CF_2 and CF_3 groups are now significantly larger than the corresponding CH_2 and CH_3 groups and the molecules can no longer mimic acetic acid.

A second classical example of the fluorine effect on biological reactivity is provided by the fluorocorticosteroids^[51]. The parent compound, cortisol (Scheme 10, X = H), is one of the glucocorticoids (these enhance the synthesis and deposition of glycogen

in the liver, but are actually produced in the adrenal cortex) and has anti-inflammatory activity. The 9- α -fluoro analogue (Scheme 9, X = F) has eleven times greater glucocorticoid activity than cortisol. This has been ascribed to the increased acidity of the 11- β -hydroxyl due to the neighboring fluorine atom, with resultant enhanced binding to an hydrogen bond acceptor in liver cells. In addition, oxidation to corresponding cortisone (11-keto), which is probably inactive, is also disfavored due to the difficulty of removal of the hydrogen at C-11. Fluorine was also found to affect microbial hydroxylation of steroids. Thus, hydroxylation did not occur adjacent to the carbon to which a fluorine substituent was attached^[52], while α -fluoro substituents gave ketone as the only product^[53].

Scheme 10. Cortisol

The growing interest in fluorine chemistry will certainly provide a deeper understanding of the effects of fluorine on physical properties and of the frequent unexpected reactivity of this class of compounds. For those reasons a new term has coined for this kind of compounds: *flustrates*^[38] ($\equiv$ fluorine-containing substrates).

1.2.2 Experimental approach

The enzyme which we used in this work is the $\Delta 9$ -desaturase of *Saccharomyces cerevisiae* (baker's yeast) ATCC #12341. The choice of *Saccharomyces cerevisiae* as the selected source of the enzyme for the desaturation was based on the fact that it is a readily available yeast, relatively easy to grow, quite hardy and known to contain a fairly active desaturase which can accept a wide variety of substrates. Furthermore, previous mechanistic studies of desaturation by our group were based on this enzyme.

The propose of this work is to use fluorine to further investigate the mechanism of the $\Delta 9$ -desaturation of fatty acids and also to explore the effects of the fluorine atom on this enzymatic process. In general, this project can be characterized as a feasibility study. This is because fluorine substituted fatty acids could possibly deactivate the enzyme and thus no information might be obtained! The selected flustrates were the (*S*)- and ($\pm$)-12-fluorostearic acids and the 16-, ($\pm$)-10- and ($\pm$)-9-fluoropalmitic acids.

Methyl ($\pm$)-12-fluorostearate was tested first for electronic and steric demand. Its choice was based on the fact that fluorine is close to the active center and also because it can be obtained relatively easily from the readily available (*R*)-12-hydroxystearic acid. Methyl 16-fluoropalmitic acid was chosen as a control substrate to monitor extent of incorporation and desaturation by the yeast cell. It can also be prepared from the readily available 16-hydroxypalmitic acid. Finally, methyl 10- and 9-fluoropalmitic acids were used in order to possibly induce accumulation of any hydroxylated intermediates.

The isolation of products, from the incubation of the above flustrates, was based on their polarity. Therefore, non-polar fatty acids are expected to be in the cells (as membrane components), while polar fatty acids are predicted to be in the medium.

^{19}F NMR and GC-MS were used as analytical techniques to establish the fatty acid profiles. The use of the ^1H -decoupled ^{19}F NMR was of crucial importance in this thesis.

Finally, it should be mentioned that this work is based on a whole cell system. Thus, the substrate is first transported into cells as the methyl ester and then is hydrolyzed and thioesterified with CoA before desaturation can occur. The use of a whole cell system can be considered as more convenient, but the use of pure enzymes can provide more conclusive results.

2.0 Results

2.1 Synthesis and incubation of methyl (*S*)-12-fluorostearate

Methyl (*S*)-12-fluorostearate (**3**) was the first tested substrate for the Δ^9 -desaturase system of *Saccharomyces cerevisiae*, in order to explore the utility of fluorine as a mechanistic probe for the desaturation process. In this way, the degree of rate retardation by a fluorine substituent could be assessed. Furthermore, fluorine is still close to the C-9 and C-10 positions, thus hydroxylated intermediates might be formed.

The synthesis was relatively simple (see Scheme 11) starting from the commercially available (*R*)-12-hydroxystearic acid (**1**)^{ac} and using DAST as fluorinating reagent.

Scheme 11. Synthesis of methyl (*S*)-12-fluorostearate.

^{ac} As it turned out (see page 32), the commercially available sample had been mislabeled and we initially thought we were dealing with the racemic material.

DAST can be used for the direct conversion of an alcohol to a fluoride^[54] (see Scheme 12).

Scheme 12. Fluorination of an alkyl alcohol with DAST.

This reaction generally proceeds cleanly with inversion of configuration at the reacting carbon center (S_N2). DAST was also the reagent of choice for all the other fluorinations, giving high conversion and good yield. Different runs of this reaction showed that better results were obtained for reaction time between three and four hours at low temperatures ($\sim -45^\circ\text{C}$). The only byproduct for all the fluorinations was olefin *via* an elimination side reaction.

For this synthesis the olefin was removed by recrystallization (3 times) from MeOH. The white crystalline product appeared pure by ^1H NMR (disappearance of multiplet at 5.39ppm, which was due to alkene) and also by GC-MS and ^{19}F NMR (see Figures 1, 2, 3, 4 and Table 15).

Incubation of methyl (*S*)-12-fluorostearate (50mg/300mL culture) with growing cultures of *Saccharomyces cerevisiae* was repeated three times. GC-MS of the cell extracts (see Figures 5 and 6) showed only one extra peak which was the suspected desaturation product (from its position on the chromatograph), while the medium extract did not reveal any product.

Figure 1. ^1H NMR of methyl (*S*)-12-fluorostearate.

Figure 2. GC of methyl (*S*)-12-fluorostearate.

Figure 3. MS of methyl (*S*)-12-fluorostearate.

Figure 4. ^{19}F NMR of methyl (*S*)-12-fluorostearate.

Figure 5. GC of cells extract - control.

Figure 6. GC of cells extract from the incubation of methyl (S)-12-fluorostearate.

Figure 7. MS of the product from the incubation of methyl (*S*)-12-fluorostearate.

Figure 8. ¹⁹F NMR of cells extract from the incubation of methyl (*S*)-12-fluorostearate.

The mass spectrum (MS) of the product (see Figure 7) was in agreement with our proposed structure, showing a molecular ion with mass of 294 [loss of HF] and another characteristic peak with mass 262 [loss of CH₃OH].

The % fatty acid composition of the cell extract along with this from the control culture is shown in Table 1, as it was detected by GC.

Table 1. GC fatty acid profiles of cells extract from the incubation of 3 and control incubation.

	% comp. (12F incub.)	% comp. (control)
	30.3	42.2
	8.3	15.6
	24.5	37.6
	1.3	2.6
	2.0	
	33.6	

¹⁹F NMR was used then to confirm the above results (see Figure 8). Indeed the results were quite similar; only one product with low conversion.

Table 2. ¹⁹F NMR analysis of cells extracts from the incubation of methyl (*S*)-12-fluorostearate.

	Me (<i>S</i>)-12F stearate (ppm)	product (ppm)	conversion (%)
1	-180.43	-179.55	5.1 (7.9)*
2	-180.42	-179.57	3.4 (1.2)*
3	-180.43	-179.54	6.3 (5.6)*
average	-180.43	-179.55	4.9 (4.9)*

* Corresponding conversions from the GC-MS analysis.

The average conversion was relatively small (~5%) and no hydroxyl-intermediates were observed. Consequently, the presence of a δ -fluorine seems to drastically affect the rate of desaturation and it might be an inhibitor (for steric or (and) electronic reasons) when placed closer to putative radical intermediate. Indeed, the % conversions of C_{18:0} to C_{18:1} and C_{16:0} to C_{16:1} were lower (5.5 and 1.5 times, respectively) relative to control. The next step was to determine the stereochemistry of the product.

2.1.1 Stereochemistry of the double bond in biosynthetic methyl 12-fluoro-9-octadecenoate.

It is well known^[1,2] that the Δ^9 -desaturase of *Saccharomyces cerevisiae* and generally all the known desaturases introduce a *cis*-double bond during the generation of monounsaturated and polyunsaturated fatty acids. The requirement for the creation of *cis*-unsaturated fatty acids was discussed before (section 1.1). The conformation of the desaturation product could vary, depending on the way which the molecule is complexed to enzyme^[55] (see Scheme 13).

Scheme 13. Conformation model describing the choice of the desaturation product.

It is reasonable that the (J) conformation is favored for the formation of *cis*-double bond, where the two alkyl chains are on the same side because of hydrophobic

interactions. The (K) conformation, where the two alkyl chains are opposite favors the formation of *trans*-double bond. The pronounced electronegativity of fluorine could cause conversion of (J) to (K), by precluding the hydrophobic interaction of the alkyl chains and thus formation of *trans*-olefin. *trans*-Olefins are minor components in yeast fatty acids. It was, therefore, essential to examine the stereochemistry of the formed double bond. ^{13}C NMR, GC-MS and ^{19}F NMR were used as analytical tools for this research.

The plan was to make the pure *trans*- and *cis*-isomers of the (S)-12-fluoro-9-octadecenoic acids (5) and (7) as methyl esters and compare them, as standards, with the desaturation product from the previous incubations. The synthesis of standards was accomplished as follows from the commercially available ricinelaidic acid (4) and ricinoleic (6) (methyl esters) respectively (see Scheme 14).

Scheme 14. Synthesis of *trans*- and *cis*-isomers of methyl (S)-12-fluoro-9-octadecenoate.

The fluorination of **6** was previously performed in our lab^[56] with N-2-chloro-1,1,2-trifluoroethyl-N,N-diethylamine (FAR), another fluorination reagent and gave **7** and **5** in ratio 1:2. **7** and **5** were not the only products from that fluorination, it gave also the *cis*- and *trans*-isomers of the 7-fluoro-9-octadecenoic acids (**8**) and (**9**) as methyl esters, which probably arise from the following rearrangements (see Scheme 15).

Scheme 15. Different products from the fluorination of **6** with DAST.

Taking into account the above results, the fluorination of **6** was performed first using DAST as fluorinated reagent. ^1H NMR and ^{13}C NMR of the product showed many extra peaks, probably due to cyclopropane byproducts and the coexistence of **5**, **7**, **8** and **9**. Shorter times (reaction followed by TLC), less polar solvent and partial purification of the product (flash chromatography) were performed on a second run of that reaction. ^{13}C NMR showed purer product and furthermore no *trans*-isomers were detected (see Figure 9). That observation was derived from the fact that the above *trans*- and *cis*-isomers give

characteristic peaks at the ^{13}C NMR (in a *cis*-olefin the allylic carbons have strong γ steric interaction with each other^[56,57], see also Tables 3, 17 and 19). It can be readily seen from these Tables that there is a noticeable difference for the chemical shifts of the two isomers at C-8 and C-11 positions. Thus, no peaks around 32.6 and 38.4 were found in the ^{13}C NMR from the fluorination of 6.

Table 3. ^{13}C NMR data of 5 and 7 isomers.

carbons	C ₈	C ₉	C ₁₀	C ₁₁	C ₁₂	C ₁₃
<i>cis</i> isomer (<u>7</u>)	27.4	132.7	123.8	33.1	94.0	34.7
<i>trans</i> isomer (<u>5</u>)	32.6	133.8	124.6	38.4	94.0	34.5

Applying the same method for the fluorination of 4, the ^{13}C NMR of the product did not reveal any noticeable amount of *cis*-isomer (no peaks around 27.4 and 33.1 were found, see also Figure 10). The next step was to compare the GC-MS and ^{19}F NMR data from the above fluorinations with those from the incubation of 3. For the GC-MS of 5 and 7 it was necessary to do a simulation, by adding the proper amounts of methyl stearate and 3 so as to have more accurate retention times (see Figures 11 and 12). Nevertheless, the R.T's were quite close to each other (15:50 *versus* 15:45), so any comparison with the retention time from the incubation product (R.T: 15:48) would not lead to a valid conclusion.

Having already an idea about the purity of the products (5 and 7), ^{19}F NMR was tested to compare their spectra with the one from the incubation product ($\delta = -179.55\text{ppm}$). Both spectra, of the *cis*- and *trans*-isomers (see Figures 13 and 14), contained many peaks at the region between -168.5 and -175.5ppm (probably due to many cyclopropane byproducts), but they also showed one intense peak at -179.86ppm (for the *trans*-isomer) and at -179.57ppm (for the *cis*-isomer). The spectra showed also that very small amounts of "5-contaminated" samples of 7 and *vice-versa*. It was clear from these data that the desaturated product had the *cis*-configuration, corresponding to methyl (*S*)-12-fluorooleate.

Figure 9. ^{13}C NMR of the product from the fluorination of methyl ricinoleate.

Figure 10. ^{13}C NMR of the product from the fluorination of methyl ricinelaideate.

Figure 11. GC of methyl (*S*)-12-fluorooleate (after simulation).

Figure 12. GC of methyl (*S*)-12-fluoroeleaidate (after simulation).

Figure 13. ^{19}F NMR of methyl (S)-12-fluorooleate.

Figure 14. ^{19}F NMR of methyl (S)-12-fluorolaidate.

2.1.2 Stereochemistry at C-12 of methyl *cis*-12-fluoro-9-octadecenoate.

Since the starting material (**1**) was sold as racemic, we initially thought it would be interesting to see if the desaturase discriminated between the two enantiomers of the methyl 12-fluorostearate. The stereochemistry of CHF center in the starting material was determined by the following method: Aqueous solutions of KMnO_4 were used to cleave the double bond of the desaturation product (**10** for $n = 5$ and $\text{X} = \text{F}$), along with the other unsaturated fatty acids (**10** for $n = 5, 7$ and $\text{X} = \text{H}$). The product then was complexed with a chiral shift reagent and the formed diastereomers were determined by ^{19}F NMR. As standards for that experiment were used the ($\pm$)-, (*R*)-, and (*S*)-3-fluorononanoic acids, which were previously prepared in our lab^[58]. The oxidative cleavage of double bond for the cell extract is depicted at the following Scheme:

Scheme 16. Oxidative cleavage of double bond of cells extract.

The first difficulty of that reaction was that we had to work with limited amounts of the extract (the total amount of fatty acids was never more than 20mg). Following the modified original procedure^[59] by P. M. Seigel^[60], GC-MS did not show any product. Furthermore, GC showed that the peaks due to methyl palmitoleate and methyl oleate were still present along with many alcohol peaks (oxidative intermediates). The above

findings indicate that the reaction was incomplete. Thus, the same reaction was repeated for different reaction times and keeping the amount of oxidant constant. Finally, it was found that the reaction time had to be increased to 48 hours so as to detect product. Under these conditions, GC showed completion of the reaction which was obvious from the disappearance of the methyl palmitoleate and methyl oleate (see Figure 15). The MS of the new peaks on the chromatograph were in agreement those expected for the products of Scheme 16 (see Table 4). Especially important was that the MS of the peak with retention time 5:15 was identical with that of 3-fluorononanoate (standard, see also Figure 15).

Table 4. Some characteristic peaks of the oxidative cleavage of cells extracts according to their GC-MS analysis.

II for n = 5, X = F	5:15	170 (M⁺ - HF)	159 [(M⁺ - HF) - CH₃O]
II for n = 5, X = H	5:00	172	141 (M⁺ - CH₃O)
II	8:55	185 (M⁺ - CH₃O)	

¹⁹F NMR of the oxidatively cleaved cell extract showed clearly disappearance of the peak at -179.55ppm and appearance of a new peak at -180.27ppm (see Figure 17). The identity of that peak was confirmed from the ¹⁹F NMR of the readily available 3-fluorononanoic acid (δ = -180.25ppm, see also Figure 18). The chiral shift reagent which was used for the complexation was the (*S*)-(-)-1-(naphthyl)ethylamine (NEA). The complexation was efficient only for the free acids and not for the methyl esters (because of the hydrogen bonding interaction). Only a few mg (~5) of shift reagent were needed to form the complexed product, despite the coexistence of the other acids which also interact with the shift agent.

Figure 15. GC of the oxidative cleavage of cells extract from the incubation of methyl (S)-12-fluorostearate.

Figure 16. MS of methyl 3-fluorononanoate.

Figure 17. ^{19}F MNR of the oxidative cleavage of cells extract from the incubation of methyl (S)-12-fluorostearate.

Figure 18. ^{19}F NMR of 3-fluorononanoic acid.

Figure 19. ^{19}F MNR of the oxidative cleavage product ((*S*)-isomer) plus NEA.

Figure 20. ^{19}F MNR of (*R*)-3-fluorononanoic acid plus NEA.

Figure 21. ^{19}F MNR of (S)-3-fluorononanoic acid plus NEA.

Figure 22. ^{19}F MNR of the oxidative cleavage product plus NEA and a spike of (R)-3-fluorononanoic acid.

Scheme 17. Complexation of 3-fluorononanoic acid with NEA.

After complexation, ^{19}F NMR showed only one peak at -179.52ppm (Figure 19). This peak was compared with the peaks which arised from the complexation of the standards with NEA (-179.26ppm for the (*R*)-isomer and -179.43ppm for the (*S*)-isomer, see also Figures 20, 21) and showed clearly that the product was the enantiomerically pure (*S*)-12-fluorooleic acid. That was more obvious after the addition of some of the (*R*)-isomer into the NMR tube (see Figure 22). Thus, the commercial sample of 12-hydroxystearate was most probably the pure (*R*)-enentiomer, which yielded the (*S*)-enantiomer upon fluorination, and not racemic as labeled.

2.2 Synthesis and incubation of methyl ($\pm$)-12-fluorostearate

It was, therefore, necessary to prepare racemic 12-fluorostearate (16) in order to check the “behavior” of the enzyme towards the two isomers. The synthesis was performed by carrying out an oxidation-reduction sequence on (*S*)-12-hydroxystearic acid (see Scheme 18).

Scheme 18. Synthesis of methyl (±)-12-fluorostearate.

The oxidation of 13 to 14 was accomplished by using chromic acid solution as an oxidant which was prepared from the appropriate amount of sodium dichromate and sulfuric acid as indicated by the following equation^[61].

For the reduction of 14 to 15, the widely employed $NaBH_4$ was used as reagent. The product after the fluorination (16) was contaminated with the elimination byproduct

(85% pure by ^1H NMR) and purified as before (section 2.1) by recrystallization from MeOH. Before the incubation, 16 was analyzed by ^1H NMR and GC-MS and it was found to be pure. Both ^1H NMR and GC-MS of 16 (racemic) and 3 ((*S*)-isomer) were identical.

The incubation was carried out once and only one product was detected by GC-MS and ^{19}F NMR analysis of the cell extract (see Figures 22, 23 and 24). The product had the same characteristics with this from the incubation of 3 as is shown from the following Table.

Table 5. ^{19}F NMR analysis of cells extract from the incubation of methyl ($\pm$)-12- fluorostearate.

run	Me ($\pm$)-12F stearate (ppm)	product (ppm)	conversion (%)
1	-180.43	-179.55	5.0 (2.0)*

* % Conversion calculated from the GC-MS analysis.

2.2.1 Stereochemistry of the incubation product

The enantioselectivity of the enzyme could be determined by measuring the enantiomeric excess of the (*S*)- and (*R*)-isomers of the desaturation product using the methodology already applied for the (*S*)-isomer (section 2.1.2).

As before, the ^{19}F NMR of the cleaved product gave a single resonance at -180.27ppm and after complexation with NEA showed two peaks at -179.34 and -179.50ppm (see Figure 26). The (*R*)- and (*S*)-3-fluorononanoic acids were shifted after complexation with NEA at -179.26ppm and -179.43ppm respectively (see Figures 20 and 21). From the above ppm values it was clear that the high field peak in Figure 26 was responded to (*R*)-isomer and the low field one to (*S*)-isomer. The two peaks had almost the same intensity (10:9) indicating no enantiomeric discrimination from the enzyme.

Figure 23. GC of cells extract from the incubation of methyl ($\pm$)-12-fluorostearate.

Figure 24. MS of the product from the incubation of methyl ($\pm$)-12-fluorostearate.

Figure 25. ^{19}F NMR of cells extract from the incubation of methyl ($\pm$)-12-fluorostearate.

Figure 26. ^{19}F NMR of the cleaved (racemic) product plus NEA.

2.3 Synthesis and incubation of methyl 16-fluoropalmitate

The low conversion of 3 to 7 indicates that fluorine is retarding the desaturation and possibly the degree of retardation could be larger when fluorine is at C-9 or C-10 position. To examine how potent the fluorine substituent effect was, a second analogue was tested as substrate, having the fluorine atom far away from C-9 and C-10 positions. This substrate was the methyl 16-fluoropalmitate (19) and was synthesized from the commercially available juniperic acid (17) as shown in Scheme 19. A small amount of alkene was formed from the fluorination reaction and it was removed along with some other impurities by flash chromatography to give the pure 19 by ^1H NMR and GC-MS (see Figures 27, 28, 29 and Table 24).

Scheme 19. Synthesis of methyl 16-fluoropalmitate.

The incubation proceeded normally (ω -fluorofatty acids with even number of carbons are toxic compounds) and GC-MS analysis of the cell extract showed excellent incorporation and desaturation of the substrate (see Figure 30 and Tables 6 and 7). Some

Figure 27. ¹H NMR of methyl 16-fluoropalmitate.

Figure 28. GC of methyl 16-fluoropalmitate.

Figure 29. MS of methyl 16-fluoropalmitate.

Figure 30. ^{19}F NMR of methyl 16-fluoropalmitate.

Figure 31. GC of cells extract from the incubation of methyl 16-fluoropalmitate.

Figure 32. MS of the product from the incubation of methyl 16-fluoropalmitate.

Figure 33. ^{19}F NMR of cells extract from the incubation of methyl 16-fluoropalmitate.

other small peaks were also observed on the chromatograph which are due to chain elongation of 16 and its product (the enzyme usually elongates substrates by adding two CH₂ groups, starting from the C-1^[62]).

The position of that large, new peak on the chromatograph and its mass spectra peaks (M⁺ 286, M⁺-HF 266, M⁺-CH₃O⁺ 257 and (M⁺-HF)-CH₃OH 234, see also Figure 30) implied that the product was the methyl 16-fluoropalmitooleate (20). Applying ¹⁹F NMR as second analytical tool to confirm the above results we were gratified to see separate peaks for the 19 and 20, even though fluorine was seven bonds away from the double bond (see Figure 32)! Indeed, only one extra peak appeared in the ¹⁹F NMR spectrum of the extract which corresponds to 20.

Table 6. GC fatty acid profiles of cells extract from the incubation of 19.

% composition (16F incubation)
	21.4
	10.7
	21.6
	13.3
	19.2
	5.5
	8.3

Table 7. GC and ¹⁹F NMR analysis of cells extract from the incubation of 19.

	R.T (GC)		ppm ¹⁹ F NMR
	14:36		-218.47
	14:24		-218.55

The high conversion of 16 to 20 prompted us to use palmitate analogues as substrates for the desaturase enzyme in future experiments, as we would have a convenient control substrate with a remote fluorine substituent.

2.4 Synthesis and incubation of methyl ($\pm$)-10-fluoropalmitate

Our approach was to determine the fluorine substituent effect (at C-9 and C-10) on desaturation, in order to trap possible hydroxylated intermediates. The first substrate to be tested was the methyl ($\pm$)-10-fluoropalmitate (25). The total synthesis of (25) was accomplished as follows:

Scheme 20. Synthesis of methyl ($\pm$)-10-fluoropalmitate.

The terminal double bond of (21) was chosen, instead of any other functional group, because it “survives” the Grignard reaction. The cleavage of the terminal double bond of (23) to give the carboxylic acid (24) was accomplished with aqueous KMnO_4 solution^[63]. Oxidations by permanganate, although quite effective, are usually plagued by the insolubility of the organic substrate in water. The solubility was enhanced by rapid stirring to facilitate interfacial contact between the reactants, addition of a cosolvent such as acetic acid and use of a phase-transfer agent^[64]. The stoichiometry of the cleavage of terminal olefins is represented by the equation:

Under those conditions, the one-carbon cleavage products were contaminated with the overoxidation product (methyl ($\pm$)-9-fluoropentadecanoate), resulting from a loss of two carbons^[63]. After methylation of 24 to give 25, the product was purified by flash chromatography (2% EtOAc/Hexanes). GC-MS analysis (see Figures 34, 35 and 36) showed that the overoxidation impurity was about 7%. It was amazing to detect that impurity by ^{19}F NMR ($\delta = -180.54\text{ppm}$, see Figure 36) even though the fluorine was 10 bonds away from the carboxyl group! Once again the high chemical shift range of the ^{19}F NMR was demonstrated.

Since the overoxidation product could not be easily removed, the incubation of 25 took place without further purification of substrate. Two different runs from the incubation of 25 gave a large peak at -105.57ppm as a product in the ^{19}F NMR of the cell extract (see Figure 38, and Table 9). A literature search (^{19}F NMR data for a similar CF=CH group)^[65] revealed that this peak could probably be assigned to methyl 10-fluoropalmitooleate (26). Furthermore, ^{13}C NMR of the cell extract gave clear peaks for the C-9 and C-10 of 26 (C-10: $\delta = 160.3\text{ ppm}$, $J_{\text{CF}} = 245.9\text{ Hz}$; C-9: $\delta = 105.5\text{ ppm}$, $J_{\text{CF}} = 21.5\text{ Hz}$, see also Figure 39).

Figure 34. GC of methyl ($\pm$)-10-fluoropalmitate.

Figure 35. MS of methyl ($\pm$)-10-fluoropalmitate.

Figure 36. MS of methyl (±)-9-fluoropentadecanoate (overoxidation by-product from the synthesis of methyl (±)-10-fluoropalmitate).

Figure 37. ^{19}F NMR of methyl (±)-10-fluoropalmitate.

Figure 38. ^{19}F NMR of cells extract from the incubation of methyl ($\pm$)-10-fluoropalmitate.

Figure 39. ^{13}C NMR of cells extract from the incubation of methyl ($\pm$)-10-fluoropalmitate.

Figure 40. GC of cells extract from the incubation of methyl ($\pm$)-10-fluoropalmitate.

Figure 41. MS of the product from the incubation of methyl ($\pm$)-10-fluoropalmitate.

Table 8. ^{19}F NMR analysis of cells extracts from the incubation of methyl ($\pm$)-10- fluoro-palmitooleate.

	Me ($\pm$)-10-fluoropalmitate (ppm)	product (ppm)	conversion (%)
1	-180.49	-105.56	28.6
2	-180.46	-105.58	31.6
average	-180.48	-105.57	30.1

Some small peaks in both the cell and medium extract were also found by ^{19}F NMR and they were identified by GC-MS (see Figure 40). The large product peak, detected by ^{19}F NMR, was not visible on the GC chromatograph because it overlapped with the peak of the methyl palmitate. Nevertheless, the mass spectrum was clear showing peaks with mass 286 [M^+], 266 [$\text{M}^+ - \text{HF}$] and 234 [$(\text{M}^+ - \text{HF}) - \text{CH}_3\text{OH}$] which correspond to unsaturated product (see also Figure 41). Mass spectra of the other GC peaks did not show any characteristic fragmentation for hydroxyfatty acids^[66]; therefore their *in vivo* production was excluded. The two extra peaks on the GC graph of the cell extract (R.T's: 15:00, 15:58) belong to elongated fluorofatty acids, according to their MS. The fatty acids which underwent elongation were the methyl ($\pm$)-9-fluoropentadecanoate, and the 25. The % fatty acid composition of the cell extract, as was detected by GC, is shown in Table 9 (methyl palmitate and 26 overlapped).

Table 9. GC fatty acid profiles of cells extract from the incubation of 25.

	% composition (10F incubation)
	5.0
	28.1
	5.2
	11.2
	26.1
	22.5
	1.3
	0.6

2.5 Synthesis and incubation of methyl (±)-9-fluoropalmitate

Encouraged by the excellent % conversion of the methyl (±)-10-fluoropalmitate, the synthesis and incubation of methyl (±)-9-fluoropalmitate (32) was carried out. The synthesis of 32 was somewhat similar to that for 25, starting with the 9-decen-1-ol (27) as a precursor for the Grignard reaction. The oxidation of 27 to 28 was performed under mild conditions, with the use of dimethyl sulfoxide "activated" by oxalyl chloride^[67]. The total synthesis of 32 is shown in Scheme 21.

Scheme 21. Synthesis of methyl (±)-9-fluoropalmitate.

The final product (32) was again contaminated (~8%) with the overoxidation product, (methyl ($\pm$)-8-fluoropentadecanoate), as was found by GC-MS and ^{19}F NMR. Further purification of the substrate was not attempted. Incubation of 32 took place twice again and both cells and medium extracts were analyzed by GC-MS and ^{19}F NMR. ^{19}F NMR of the cell extract showed one major product which was in all likelihood methyl 9-fluoropalmitoleate (33) (see also Figure 42 and Table 10). ^{13}C NMR of the cell extract confirmed the biosynthesis of 33: C-10: $\delta = 105.6$ ppm, $J_{\text{CF}} = 21.5$ Hz; C-9: $\delta = 159.8$ ppm, $J_{\text{CF}} = 246.5$ Hz (tentative assignments, see also Figure 43). Medium extracts did not reveal any oxygenated product as shown by ^{19}F NMR and GC-MS.

Table 10. ^{19}F NMR analysis of cells extracts from the incubation of methyl ($\pm$)-9-fluoropalmitoleate.

	Me ($\pm$)-9-fluoropalmitate (ppm)	product (ppm)	conversion (%)
1	-180.50	-105.77 (-105.56) ^a	9.6 (11.1)*
2	-180.48	-105.78 (-105.58) ^a	15.4
average	-180.49	-105.78 (-105.57) ^a	12.5

^a ppm of the desaturation product from the incubation of 25.

* In one of the two GC-MS analysis, the column was able to separate the product (see Figure 44) which was confirmed by its mass spectra to be 33 (286 [M^+], 266 [$\text{M}^+ - \text{HF}$], see also Figure 45).

The mass spectra of some other peaks on the graph (R.T's: 15:00, 15:58) showed that they arised from chain elongation of methyl ($\pm$)-8-fluoropentadecanoate (which was also found in the ^{19}F NMR at $\delta = -105.58$ ppm) and 32. The % composition of fatty acids in the cell extract is shown in Table 11.

Figure 42. ^{19}F NMR of cells extract from the incubation of methyl ($\pm$)-9-fluoropalmitate.

Figure 43. ^{13}C NMR of cells extract from the incubation of methyl ($\pm$)-9-fluoropalmitate.

Figure 44. GC of cells extract from the incubation of methyl (±)-9-fluoropalmitate.

Figure 45. MS of the product from the incubation of methyl (±)-9-fluoropalmitate.

Table 11. GC fatty acid profiles of cells extract from the incubation of 32.

<i>% composition (9F incubation)</i>
	2.0
	34.1
	6.4
	3.6
	28.5
	23.9
	1.0
	0.5

Therefore, the only biosynthetic product from the incubation of 32 was its unsaturated analogue. The % conversion to 33 was lower by a factor of ~3, relative to the conversion of 25 to 26.

2.6 Competition incubation of methyl ($\pm$)-9- and 10-fluoropalmitate

Since the relatively low % conversion of 32 to olefin could be due to enzyme inhibition, we were motivated to carry out a competition incubation between those two substrates. Equal amounts of 25 and 32 (25.0mg each) were combined and incubated to the yeast (300mL culture). The cell extract was analyzed by ^{19}F NMR (Figure 46) and the results are listed in Table 12.

Figure 43. ^{19}F NMR of cells extract from the competition incubation between methyl $(\pm)$ -10-fluoropalmitate and $(\pm)$ -9-fluoropalmitate.

Table 12. ^{19}F NMR analysis of cells extract from the incubation of methyl ($\pm$)-9- and 10-fluoropalmitoleate.

Fluoro fatty acids	ppm	conversion
	-180.50	
	-105.56	33.8
	-180.53	
	-105.76	10.2

The results of the competition incubation are in agreement with the previous separate incubations, showing that the two fluistrates (25 and 32) give product with ratio roughly 3:1. This result clearly shows that 32 is processed at a slower rate than 25. Figure 43 shows also that 25 and 32 have different intensities suggesting that the starting material was not in a large excess into cells.

3.0 Discussion

From the introduction (section 1.1), it seems possible that desaturases could operate as mixed-function oxygenases. Therefore, desaturation and hydroxylation may lie on the same reaction pathway^[24,68,69]. The choice of the oxidative pathway might be controlled by the redox potential of the putative radical or the redox potential of the iron.

Introduction of fluorine at C-10 might be expected to increase the redox potential of the carbon radical (destabilization of corresponding carbocation due to inductive withdrawal) and thus induce formation of hydroxylated intermediates. (*R*)-10-Fluoro substrates cannot give olefin and thus might be forced to give alcohol. In contrast (*S*)-9-fluoro substrates could give a stabilized carbocation, while (*R*)-9-fluoro substrates can give no reaction. Thus, the expected products from the incubation of 9- and 10-fluorofatty acids would be the following (Scheme 22):

10-Fluorofatty acid

9-Fluorofatty acid

Scheme 22. Possible products from the incubation of 9- and 10-fluorofatty acids.

Nevertheless, only olefinic products were observed for all the incubations, indicating that presence of monofluorine substrates is not sufficient to raise redox potential of putative radical and divert the pathway to hydroxylation. Furthermore, the obligatory existence of hydroxylated intermediates in desaturation cannot be correct. This result can also be regarded as relatively strong evidence against direct oxygen insertion as the only means of oxidizing hydrocarbons.

The difference between the reactivity of methyl ($\pm$)-10-fluoropalmitate (25) and methyl ($\pm$)-9-fluoropalmitate (32) and particularly the high conversion of 25 to olefin were the most surprising and interesting results of the thesis. Since we know from our previous work, using thia-analogues and deuterium-labeled substrates, that hydrogen removal from substrate is initiated at C-9 and is rate-limiting, our fluorine substituents are probably probing this initial event. Thus, it is critical to evaluate literature on the effect of fluorine and other electronegative substituents in hydrogen atom transfer reactions.

The only literature data for hydrogen atom abstraction in radical reactions using fluoroalkanes as substrates are from Tedder's work^[43,70]. Free-radical substitution of *n*-butyl fluorides was performed in the gas phase, using a variety of atoms and radicals as reactants. The results from the above halogenations are shown in Table 13.

Table 13. Radical substitution in *n*-butyl fluorides. In brackets the $RS_p^\ddagger$ for the *n*-butane.

	T (°C)		ΔH (kJ mol ⁻¹)
	25		-150
	25		-20
	25		-17
	150		-29
	150		+33
	150		+46

These results clearly show that there is a marked difference in the nature of selectivity of the above atoms and radicals as hydrogen abstractors. Thus, the substituent fluorine atom has no appreciable effect on hydrogen abstraction at the γ -carbon atom and further down the chain. The β -position is deactivated to attack by all the atoms and radicals shown, but the α -position is activated to attack in comparison to the other terminal position (δ -position), except to attack by fluorine and chlorine atoms.

The lower degree of radical substitution at the β -position can be explained in terms of polarity. If the attacking radical is very electronegative then the H-X bond being formed can be highly polar, in the direction $H^{\delta+} \rightarrow X^{\delta-}$. The more polar the H-X, the more

+----->

polar the transition state $-C \cdots H \cdots X$ is expected. Now, if substrate, (R-H), contains electronegative substituents close to the hydrogen atom to be abstracted by the X radical (like in our case with the fluorine atom), the R-H bond will be polarized and the formation of the H-X will be opposed^[70,71,72,73]. All the radicals and atoms in Table 13 are electrophilic, so the deactivation of the β -position can be attributed to the reaction of a dipole in the transition state which is in opposition to the dipole of the rest of the molecule.

Scheme 23. Reversal of the polarity effect.

When we come to compare the attack of different atoms and radicals at the α -position we find a substituent which is activating to one atom or radical may be deactivating to another. This is due to opposing inductive (-I) and resonance (+R) effects. A substituent with filled *p*-atomic orbitals can in many instances interact with an adjacent radical center, especially if the radical is planar (see Scheme 24). At the α -position in 1-

fluorobutane the incipient radical is stabilized by interaction with the non-bonded $2p$ electrons of the fluorine and, in comparison with the other terminal position (δ), attack is facilitated except by highly electronegative atoms such as chlorine and fluorine where the opposing polar effect will be maximized.

Scheme 24. Resonance stabilization of radical intermediate by α -halogen.

Considering that $\text{Fe}^{+3}=\text{O}$ is a very electrophilic group, then we should expect that the controlling factor regarding the outcome of desaturation is the polar effect. Thus, the rate of conversion of 9- and 10-fluorofatty acids to product would be different; the substrate with fluorine at 10-position (β -position relative to the incipient radical) should retard the formation of the putative radical, while the one with fluorine at 9-position (α -position relative to the incipient radical) might retard its desaturation in a degree depending on the competition between the inductive ($-I$) and the resonance ($+R$) effect. This becomes better understood from the relative stabilities of α and β cations relative to fluorine (see section 1.2.1.2).

Considering that the conversions of 25 and 32 to product were about 30% and 10% respectively. one only enantiomer for both substrates can give desaturation product and equal incorporation of both enantiomers in each case, then the corrected conversions involving both active isomers would be about 60% for the 25 and 20% for the 32. The conversion of methyl palmitate to methyl palmitooleate varies between 70% and 80%.

Thus, 25 is deactivated only slightly, as a substrate, while the conversion of 32 is retarded to a higher degree. Indeed, competition between 25 and 36 confirmed that the low conversion of 36 to olefin was not due to enzyme inhibition, showing that the ratio between the conversions of 25 and 32 to the corresponding olefin remained the same (3:1).

The above findings were somehow unexpected since fluorine retards desaturation at α -position much more than at β -position. However, considering the effect of the polarity in hydrogen atom transfer reactions, a reasonable explanation can be given. When there is polarity at the transition state, then the outcome of the reaction depends greatly on the nature of the attacking radical. Therefore, the α -position could be strongly deactivated if the inductive effect ($-I$) overcomes the resonance effect ($+R$).

The only similarity between our findings and Tedder's experiments is when fluorine atoms were used as hydrogen abstractors ($RS^\bullet$, between the α - and β -position had roughly ratio 1:3). Nevertheless, this model is still very different from the one involved on desaturation because:

1. $Fe-O^\bullet$ as an attacking group is different from the $F^\bullet$ and probably even more polar.
2. Reaction with fluorine radicals ($F^\bullet$) are very exothermic, comparing to thermoneutral enzymatic reactions.

For the above reasons a better model than Tedder's is currently being tested^[74], involving 3-fluorohexane as substrate and various oxy radicals as attacking group (see Scheme 25). Measurement of the % attack of oxy radicals can be obtained by trapping the formed radicals with TEMPO and analyze them by HPLC-MS and ^{19}F NMR.

Scheme 25. Investigating model for hydrogen atom transfer reactions.

The lower conversion of 32 relative to 25 could also be explained by fortuitous steric reasons. The oxidant attaches asymmetrically the 9-10 carbon bond and is located closer to 9-C, therefore, steric hindrance due to fluorine atom is expected to be higher for the 32.

A better knowledge of the fluorine effect for the proposed radical-mediated mechanism of desaturation can be provided by synthesizing the 7-, 8-, 11- and 12-fluoropalmitic acid analogues and comparing their conversions to olefin. Thus, for example, (*S*)-8- and (*S*)-10-fluorostearates should give similar conversions, if H^+ removal is not rate limiting step, since both would exert a β -effect on the initial attack by oxidant.

The lower degree of desaturation of methyl 12-fluorostearate, relative to 25 and 32 cannot be commented on because the substrates do not have the same chain length. In fact, the difference in chain length can cause this small conversion, as we have seen from our work with some thia-analogues^[60].

3.1 Conclusions

- ◆ Incubations of all fluistrates gave the corresponding olefin as the only product. No oxygenated products were formed. This constitutes good evidence against obligatory hydroxylated intermediates in desaturation.
- ◆ Using methyl 12-fluorooleate and methyl 12-fluoroelaidate as standards, the geometry of double bond for the desaturation product of methyl 12-fluorostearate was assigned. The configuration of double bond was the *cis*-, showing that the stereochemistry of the reaction was not affected by the fluorine atom.
- ◆ The geometry of fluorine at C-12 was determined for the products from the incubations of methyl (*R*)- and ($\pm$)-12-fluorostearates. The two enantiomers of the racemic starting material were not discriminated by the enzyme, showing almost the same reactivity. This confirms that fluorine is an unobtrusive probe as well as that the CHF is an excellent isomorph of CH₂ in mechanistic studies.
- ◆ The 16-fluorofatty acid chain can be used to directly monitor the rate of desaturation, having methyl 16-fluoropalmitate as a control fluistrates and using ¹⁹F NMR as an analytical tool.
- ◆ The % conversion of methyl ($\pm$)-10-fluoropalmitate to olefin was unexpectedly high relative to that observed for methyl 16-fluoropalmitate and also relative to the desaturation of methyl ($\pm$)-9-fluoropalmitate.
- ◆ Fluorine opens a new area for kinetic studies regarding the desaturation mechanism, since ¹⁹F NMR can be used to great advantage. Indeed, it was proven that ¹H-decoupled ¹⁹F NMR is an extremely helpful analytical tool for the elucidation of fluorocompounds.

3.2 Future work

- ◇ Chiral fluoride synthesis. It would be interesting, for example, to determine the fate of methyl (*R*)-10-fluoropalmitate after its incubation to the enzyme.
- ◇ Comparison between the 16-C and the 18-C chain. Incubation of methyl 12-fluoropalmitate becomes now necessary in order to explain the low conversion of methyl 12-fluorostearate to olefin.
- ◇ Additional model studies. Model studies for radical transfer reactions, involving fluoroalkanes as substrates will help to understand our findings.
- ◇ Applications to other systems. Fluorides can also be applied in other systems such as MMO and P₄₅₀ in order to link mechanisms.
- ◇ Use of purified enzymes. Purified enzymes are becoming available in larger quantities. Thus, the use of the purified plant Δ^9 -desaturase should be the next step for future mechanistic studies.

4.0 Experimental

4.1 General

The ^1H NMR (200MHz), ^{13}C NMR (100MHz), and ^{19}F NMR (376,5MHz, ^1H -decoupled) spectra were recorded on a Varian Gemini 200, Bruker AMX-400, and Bruker AM-400 spectrometers respectively. The following abbreviations were used: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad. J values were reported in hertz. All NMR spectra were obtained using dilute CDCl_3 solutions. All chemical shifts were quoted in δ (ppm) values and for the ^1H NMR and ^{13}C NMR spectra were reported with reference to tetramethylsilane (TMS), while for the ^{19}F NMR spectra were reported with reference to fluorotrichloromethane (CFCl_3).

The GC-MS data were collected by Dr. Clem Kazakoff of the University of Ottawa with a Hewlett Packard model HP 5980 gas chromatograph equipped with a 30m DB-5 standard capillary column. The column temperature program was initially 120°C for 2 min and then increased at 10°C per minute up to a temperature of 320°C. The mass spectrometer was a Kratos Concept model IIH equipped with a 70eV E.I ionization chamber and recorded on a SUN computer at a scan of 0.5 seconds.

The elemental analysis were obtained on a Perkin Elmer Elemental Analyzer 240C by Ann Webb at NRC. Melting point determinations were performed on a Fisher-Johns melting point apparatus. Most synthetic fatty acids and some intermediates were purified by Flash Chromatography using Merck Silica Gel 60 (230-400 mesh). Thin Layer Chromatography (TLC) was performed using Merk pre-coated Silica Gel 60 F-254 glass plates. Visualization of UV-inactive materials was accomplished by using a combination of I_2 vapor followed by a water spray.

All synthetic reagents were purchased from the Aldrich Chemical Company and used without purification, except for ricinoleic acid methyl ester and ricinelaidic acid methyl ester which were purchased from the Sigma Chemical Company. Chiral materials were used without improving their optical purity. Solvents were technical grade and distilled before use, or were 'absolute' or 'spectral grade'. All air- and moisture-sensitive reactions were performed under nitrogen. A Buchi RE 111 rotavapor was utilized for the removal of solvents *in vacuo*.

4.2 Synthesis of Substrates

4.2.1 Methyl (*S*)-12-fluorostearate

4.2.1.1 Methyl (*R*)-12-hydroxystearate from (*R*)-12-hydroxystearic acid

50mL of 10% $\text{BF}_3 \cdot \text{Et}_2\text{O}$ /methanol solution (v/v) were added to 500mg (1.67mmol) of the above hydroxyl-fatty acid and heated under reflux for 1.5h. After cooling the slightly purple mixture was transferred to a separatory funnel and was diluted with 50mL of water so as to destroy residual BF_3 . Methylene chloride (CH_2Cl_2) was used

then to extract the organic product (4×25mL). The combined organic layers were washed with saturated NaCl, dried over Na₂SO₄ and rotoevaporated under vacuum to yield 445mg (1.42mmol, 85%) of a white solid which was ~100% pure by ¹H NMR^[75]. The *R_f* factor was 0.84 on a silica gel TLC plate using 50% EtOAc/Hexanes.

m.p.: 55-56°C.

Table 14. ¹H NMR analysis of methyl (*R*)-12-hydroxystearate.

δ (ppm)	mult.	hydrogens (#)	hydrogens (abs.)
3.69	s	3	-COOCH ₃
3.60	m	1	-CH ₂ CHOHCH ₂ -
2.32	t	2	-CH ₂ COOCH ₃
1.64	m	4	-CH ₂ CHOHCH ₂ -
1.2-1.5	br s	24	(CH ₂) ₁₂ for C ₃ -C ₁₀ and C ₁₄ -C ₁₇
0.91	t	3	-CH ₂ CH ₃

4.2.1.2 Methyl (*S*)-12-fluorostearate from methyl (*R*)-12-hydroxystearate

To a 50mL, two-neck, round bottom flask containing 5mL methylene chloride (CH_2Cl_2) in a dry-ice/isopropyl alcohol bath at around -45°C under N_2 , diethylaminosulfurtrifluoride (DAST), (0.17mL, 1.29mmol), was added with syringe to give a clear yellow solution. Methyl (*R*)-12-hydroxystearate (400mg, 1.27mmol), dissolved in 5mL CH_2Cl_2 , was added slowly (10 min). The reaction was left stirring for 4h resulting an orange-brown liquid. This solution was quenched with cold water and then transferred to a separatory funnel along with 20mL of CH_2Cl_2 . The organic layer was washed with sat. NaHCO_3 (10mL), water (10mL), sat. NaCl (10mL), dried over Na_2SO_4 and rotoevaporated *in vacuo* to give 325mg of an orange-brown solid^[54]. The product was recrystallized three times from MeOH to yield 200mg (0.63mmol, 50% isolated yield) of a white solid pure by ^1H NMR, ^{19}F NMR and GC-MS.

m.p.: $37\text{-}39^\circ\text{C}$, lit. m.p.: 39°C ^[76],

$R_f = 0.34$ (TLC: 5.3% EtOAc/Hexanes),

R.T.: 16:09,

MS: ($\text{M}^+ - \text{HF}$) 296, [$(\text{M}^+ - \text{HF}) - 32$] 264,

^{19}F NMR: -180.43 ppm,

Anal. Calc'd for $\text{C}_{19}\text{H}_{37}\text{FO}_2$: C: 72.10, H: 11.78. Found: C: 72.09, H: 11.77.

Table 15. ^1H NMR analysis of methyl (*S*)-12-fluorostearate.

δ (ppm)	mult.	hydrogens (#)	J_{HF} value (Hz)	hydrogens(abs.)
4.45	d of m	1	48	$-\text{CH}_2\text{CHFCH}_2-$
3.67	s	3		$-\text{COOCH}_3$
2.31	t	2		$-\text{CH}_2\text{COOCH}_3$
1.63	m	4		$-\text{CH}_2\text{CHFCH}_2-$
1.2-1.5	br s	24		$(\text{CH}_2)_{12}$ for $\text{C}_3\text{-C}_{10}$ and $\text{C}_{14}\text{-C}_{17}$
0.89	t	3		$-\text{CH}_2\text{CH}_3$

4.2.2 Methyl (*S*)-12-fluorolaidate

4.2.2.1 Methyl (*S*)-12-fluorolaidate from methyl ricinelaidate

To a solution of 0.05mL (0.38mmol) of DAST in 5mL of dry hexanes, cooled to -45°C and under N_2 , 100mg (0.32mmol) of the methyl ricinelaidate, dissolved in 2mL of hexanes were added slowly. The reaction was followed by TLC (10% EtOAc/Hexanes) and it was judged to be complete after 2h. The mixture was quenched with water, diluted with hexanes (20mL), washed with sat. NaHCO_3 , water and sat. NaCl (10mL each), dried over Na_2SO_4 and rotoevaporated *in vacuo* to give 80mg of crude product. Most of the impurities were removed by flash chromatography (2.5% EtOAc/Hexanes) to yield 45mg of product (0.14mmol, 45% isolated yield) as a colorless oil.

$R_f = 0.41$ (TLC: 5.3% EtOAc/Hexanes),

R.T.: 15:50,

^{19}F NMR: -179.86 ppm,

MS: $(\text{M}^+ - \text{HF})$ 294, $[(\text{M}^+ - \text{HF}) - 31]$ 263.

Table 16. ^1H NMR analysis of methyl (*S*)-12-fluoroelaidate

δ (ppm)	mult.	hydrogens (#)	J_{HF} value (Hz)	hydrogens(abs.)
5.48	m	2	49.4	-CH=CH-
4.46	d of m	1		-CH ₂ CHFCH ₂ -
3.68	s	3		-COOCH ₃
2.30	t	2		-CH ₂ COOCH ₃
2.03	m	4		-CH ₂ CH=CHCH ₂ -
1.61	m	2		-CH ₂ CHFCH ₂ -
1.2-1.5	br s	18		(CH ₂) ₅ for C ₃ -C ₇ and C ₁₄ -C ₁₇
0.90	t	3		-CH ₂ CH ₃

Table 17. ^{13}C NMR analysis of methyl (*S*)-12-fluoroelaidate

Carbons	δ (ppm)	J_{CF} value (Hz)
C ₁	174.3	
C ₂	34.1	
C ₃	24.9	
C ₄	28.9	
C ₅ -C ₇	29.2	
C ₈	32.6	
C ₉	133.8	
C ₁₀	124.6	6.4
C ₁₁	38.4	21.4
C ₁₂	94.0	170.0
C ₁₃	34.6	21.0
C ₁₄ -C ₁₅	29.4	
C ₁₆	31.9	
C ₁₇	22.7	
C ₁₈	14.1	
-OCH ₃	51.4	

4.2.3 Methyl (*S*)-12-fluorooleate

4.2.3.1 Methyl (*S*)-12-fluorooleate from methyl ricinoleate

The reaction was performed as before (section 4.2.2.1) and the quantities of the reagents were also the same. Finally, rotoevaporation of solvent gave 90mg of crude product as a colorless liquid. Most of the impurities (cyclopropane intermediates, elimination byproduct) were removed by flash chromatography (2.5% EtOAc/Hexanes) to yield 50mg (0.16mmol, 50% isolated yield) of a colorless oil.

R_f = 0.40 (TLC: 5.3% EtOAc/Hexanes),

R.T.: 15:45,

^{19}F NMR: -179.57 ppm,

MS: ($\text{M}^+ - \text{HF}$) 294, [$(\text{M}^+ - \text{HF}) - 31$] 263.

Table 18. ^1H NMR analysis of methyl (*S*)-12-fluorooleate

δ (ppm)	mult.	hydrogens (#)	J_{HF} value (Hz)	hydrogens(abs.)
5.42	m	2	49.7	-CH=CH-
4.47	d of m	1		-CH ₂ CHFCH ₂ -
3.67	s	3		-COOCH ₃
2.30	t	2		-CH ₂ COOCH ₃
2.03	m	4		-CH ₂ CH=CHCH ₂ -
1.62	m	2		-CH ₂ CHFCH ₂ -
1.2-1.5	br s	18		(CH ₂) ₅ for C ₃ -C ₇ and C ₁₄ -C ₁₇
0.89	t	3		-CH ₂ CH ₃

Table 19. ^{13}C NMR analysis of methyl (*S*)-12-fluorooleate

Carbons	δ (ppm)	J_{CF} value (Hz)
C ₁	174.3	
C ₂	34.1	
C ₃	24.9	
C ₄	28.9	
C ₅ -C ₇	29.2	
C ₈	27.4	
C ₉	132.7	
C ₁₀	123.8	6.5
C ₁₁	33.1	22.0
C ₁₂	94.0	170.0
C ₁₃	34.7	21.0
C ₁₄ -C ₁₅	29.4	
C ₁₆	31.9	
C ₁₇	22.7	
C ₁₈	14.1	
-OCH ₃	51.4	

4.2.4 Methyl ($\pm$)-12-fluorostearate

4.2.4.1 Methyl (*R*)-12-hydroxystearate from (*R*)-12-hydroxystearic acid

The reaction is the same to this at section 4.2.1.1, so following the same procedure (*R*)-12-hydroxystearic acid (60mg, 2.0mmol) gave 535mg (1.7mmol, 85%) of methyl (*R*)-12-hydroxystearate as a white solid pure by ^1H NMR.

4.2.4.2 Methyl 12-ketostearate from methyl (*R*)-12-hydroxystearate

Acetone (35mL) and 530mg (1.69mmol) of methyl (*R*)-12-hydroxystearate were placed in a 100mL three-necked r.b. flask fitted with a magnetic stirrer, a condenser and an addition funnel. Chromic acid solution⁶¹ (2mL) was added to the stirred solution over 10 min, maintaining the temperature at 25-30°C. The color of the mixture changed to deep green and after 1h stirring at room temperature the mixture was quenched with isopropyl alcohol, diluted with 25mL of water and extracted with (3×20mL) of ether. The combined ethereal layers were washed with sat. NaHCO₃ (30mL) and water, dried over Na₂SO₄ and rotoevaporated under vacuum to yield 460mg (1.47mmol, 87%) of white solid product pure by ¹H NMR^[61].

m.p.: 42-44°C,

R_f = 0.64 (TLC: 20% EtOAc/Hexanes).

Table 20. ¹H NMR analysis of methyl 12-ketostearate.

δ (ppm)	mult.	hydrogens (#)	hydrogens (abs.)
3.67	s	3	-COOCH ₃
2.38	t	4	-CH ₂ COCH ₂ -
2.30	t	2	-CH ₂ COOCH ₃
1.60	m	4	-CH ₂ CH ₂ COCH ₂ CH ₂ -
1.2-1.5	br s	20	(CH ₂) ₁₀ for C ₃ -C ₁₀ and C ₁₄ -C ₁₇
0.89	t	3	-CH ₂ CH ₃

⁶¹The chromic acid solution was prepared by dissolving 100g (0.33mol) of sodium dichromate dehydrate (Na₂Cr₂O₇) in 300mL of water. Sulfuric acid (96%), 136g (1.34mmol), was then added. The solution was then diluted to 500mL total volume. This solution was prepared to oxidized 1.00mol of secondary alcohol.

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4.2.4.3 Methyl (±)-12-hydroxystearate from methyl 12-ketostearate

To a 100mL r.b. flask, equipped with a stirring bar, NaBH_4 (222mg, 5.8mmol) was added and it dissolved in abs. MeOH. To this solution, methyl 12-ketostearate (455mg, 1.46mmol) dissolved in 22mL of abs. MeOH, were added immediately. The mixture was stirred for 1h under N_2 . Drops of dilute HCl were added to this solution to consume any excess of NaBH_4 . The mixture then was diluted with 25mL of water and extracted with (3×20mL) of CH_2Cl_2 . The combined organic layers were washed with water, dried over Na_2SO_4 and rotoevaporated under vacuum to yield 375mg (1.2mmol, 82%) white solid product pure by ^1H NMR^[77].

m.p.: 52-54°C,
 R_f = 0.46 (TLC: 20% EtOAc/Hexanes).

Table 21. ^1H NMR analysis of methyl (±)-12-hydroxystearate.

δ (ppm)	mult.	hydrogens (#)	hydrogens (abs.)
3.67	s	3	-COOCH ₃
3.58	m	1	-CH ₂ CHOHCH ₂ -
2.30	t	2	-CH ₂ COOCH ₃
1.62	m	4	-CH ₂ CHOHCH ₂ -
1.2-1.5	br s	24	$\text{-(CH}_2\text{)}_{12}$ for C ₃ -C ₁₀ and C ₁₄ -C ₁₇
0.89	t	3	-CH ₂ CH ₃

4.2.4.4 Methyl (±)-12-fluorostearate from methyl (±)-12-hydroxystearate

A solution of 370mg (1.18mmol) of methyl (±)-12-hydroxystearate, in 5mL of CH_2Cl_2 was slowly added to a solution of 0.16mL (1.21mmol) of DAST, in 5mL of CH_2Cl_2 , cooled at -45°C . The reaction mixture was warmed to room temperature and mixed with cold water. The lower layer was separated, diluted with 20mL CH_2Cl_2 , washed with sat. NaHCO_3 (10mL), water (10mL) and sat. NaCl (10mL), dried over Na_2SO_4 and rotoevaporated under vacuum to give 275mg of crude product as an orange-brown solid. The product was recrystallized three times from MeOH to yield 150mg (0.47mmol, 40% isolated yield) of a white solid pure by ^1H NMR, ^{19}F NMR and GC-MS.

m.p.: $37-38^\circ\text{C}$, lit. m.p.: 39°C ^[76],

$R_f = 0.61$ (TLC: 10% EtOAc/Hexanes),

R.T.: 16:10,

MS: ($\text{M}^+ - \text{HF}$) 296, [$(\text{M}^+ - \text{HF}) - 32$] 264,

^{19}F NMR: -180.43 ppm,

Anal. Calc'd for $\text{C}_{19}\text{H}_{37}\text{FO}_2$: C: 72.10, H: 11.78. Found: C: 72.38, H: 11.94.

Table 22. ^1H NMR analysis of methyl ($\pm$)-12-fluorostearate.

δ (ppm)	mult.	hydrogens (#)	J_{HF} value (Hz)	hydrogens(abs.)
4.45	d of m	1	48	$-\text{CH}_2\text{CHFCH}_2-$
3.67	s	3		$-\text{COOCH}_3$
2.30	t	2		$-\text{CH}_2\text{COOCH}_3$
1.62	m	4		$-\text{CH}_2\text{CHFCH}_2-$
1.2-1.5	br s	24		$(\text{CH}_2)_{12}$ for C_3-C_{10} and $\text{C}_{14}-\text{C}_{17}$
0.89	t	3		$-\text{CH}_2\text{CH}_3$

4.2.5 Methyl 16-fluoropalmitate

4.2.5.1 Methyl 16-hydroxypalmitate from 16-hydroxypalmitic acid (juniperic acid)

Juniperic acid (915mg, 3.36mmol) was treated with a $\text{BF}_3 \cdot \text{Et}_2\text{O}$ in methanol (10% v/v, 55mL) under reflux for 1.5h. Excess of BF_3 was destroyed by the addition of water (40mL) and the organic layer was extracted with CH_2Cl_2 (5×20mL). The combined organic layers were washed with sat. NaCl solution (30mL) and then dried over Na_2SO_4 . The solvent was removed under reduced pressure, affording the methyl 16-hydroxypalmitate (880mg, 3.08mmol, 92%) which was pure by ^1H NMR. The R_f factor was 0.26 on a silica gel TLC plate using 20% EtOAc/Hexanes.

Table 23. ^1H NMR analysis of methyl 16-hydroxypalmitate.

δ (ppm)	mult.	hydrogens (#)	hydrogens (abs.)
3.65	s	3	$-\text{COOCH}_3$
3.63	t	2	$-\text{CH}_2\text{CH}_2\text{OH}$
2.29	t	2	$-\text{CH}_2\text{COOCH}_3$
1.57	m	2	$-\text{CH}_2\text{CH}_2\text{OH}$
1.2-1.4	br s	24	$(\text{CH}_2)_{12}$ for $\text{C}_3\text{-C}_{14}$

4.2.5.2 Methyl 16-fluoropalmitate from methyl 16-hydroxypalmitate

To a solution of 0.45mL (3.41mmol) of DAST, in 5mL of CH_2Cl_2 , cooled at -45°C and under N_2 , 870mg (3.04mmol) of the hydroxyl-fatty acid, dissolved in 5mL of CH_2Cl_2 were added slowly. After 3h the mixture was warmed to room temperature, quenched with water, diluted with 20mL of CH_2Cl_2 , transferred to a separatory funnel, washed with sat. NaHCO_3 ($2 \times 10\text{mL}$), water (10mL) and sat. NaCl (10mL), dried over Na_2SO_4 and rotoevaporated under vacuum to give 800mg of crude product as a yellowish solid. Flash chromatography was performed to purify the product (2% EtOAc/Hexanes) and gave 450mg (1.56mmol, 51% isolated yield) of a white solid pure by ^1H NMR, ^{19}F NMR and GC-MS.

m.p.: 32-34°C,

$R_f = 0.36$ (TLC: 5.3% EtOAc/Hexanes),

R.T.: 14:21,

MS: M^+ 288, ($M^+ - 31$) 264,

^{19}F NMR: -218.47 ppm,

Anal. Calc'd for $\text{C}_{17}\text{H}_{33}\text{FO}_2$: C: 70.79, H: 11.53. Found: C: 70.42, H: 11.63.

Table 24. ^1H NMR analysis of methyl 16-fluoropalmitate.

δ (ppm)	mult.	hydrogens (#)	J_{HF} value (Hz)	hydrogens(abs.)
4.44	d of t	2	47.36	$-\text{CH}_2\text{CH}_2\text{F}$
3.67	s	3		$-\text{COOCH}_3$
2.30	t	2		$-\text{CH}_2\text{COOCH}_3$
1.62	m	2	-	$-\text{CH}_2\text{CH}_2\text{F}$
1.2-1.4	br s	24		$(\text{CH}_2)_{12}$ for $\text{C}_3\text{-C}_{14}$

4.2.6 Methyl ($\pm$)-10-fluoropalmitate

4.2.6.1 ($\pm$)-11-heptadecen-1-ol from undecylenic aldehyde and hexylmagnesium bromide

In a 250mL, two-necked, thoroughly dry, r.b. flask, equipped with a 50mL dropping funnel (with calcium chloride guard-tube on the top) and nitrogen gas, 15mL of Grignard reagent (hexylmagnesium bromide, 2M solution in ether, 30mmol) were placed and the temperature was brought to ca. -8°C in a freezing mixture of crushed ice and anhydrous calcium chloride 4mL (29.7mmol) of the aldehyde along with 15mL of ether (freshly distilled over LiAlH₄) were placed into the separatory funnel, and they were added slowly over a period of 30 min. When all the undecylenic aldehyde had been added the reaction product was poured on 25g of crushed ice and then 50mL 3M H₂SO₄ was added to dissolve the basic magnesium bromide. The mixture was transferred into a separatory funnel and the two layers were separated. The aqueous layer was extracted with three 20mL portions of ether. The combined ethereal layers were washed with 3M H₂SO₄ (40mL), sat. NaCl, dried over Na₂SO₄ and rotoevaporated under vacuum to give 4.20g of crude product as a yellowish solid^[78]. Flash chromatography was performed to purify the product (5% EtOAc/Hexanes) and gave 3.15g (12.4mmol, 42% isolated yield) of a white solid, pure by ¹H NMR.

m.p.: 35-37°C,

R_f = 0.61 (TLC: 20% EtOAc/Hexanes),

R.T.: 13:00,

MS: (M⁺-H₂O) 236.

Table 25. ¹H NMR analysis of (±)-11-heptadecen-1-ol.

δ (ppm)	mult.	hydrogens (#)	hydrogens (abs.)
5.82	m	1	-CH=CH ₂
4.96	m	2	-CH=CH ₂
3.59	br s	1	-CH ₂ CHOHCH ₂ -
2.07	m	2	-CH ₂ CH=CH ₂
1.61	m	4	-CH ₂ CHOHCH ₂ -
1.2-1.5	br s	20	(CH ₂) ₁₀ for C ₄ -C ₉ and C ₁₃ -C ₁₆
0.89	t	3	-CH ₂ CH ₃

4.2.6.2 ($\pm$)-11-heptadecen-1-yl fluoride from ($\pm$)-11-heptadecen-1-ol

1.5g of 11-heptadecen-1-ol (5.9mmol) were dissolved in 5mL of CH_2Cl_2 , and they were added to a DAST solution (0.80mL, 6.10mmol, in 5mL of CH_2Cl_2) which was previously cooled in a dry ice-isopropyl alcohol bath ($T \sim -45^\circ\text{C}$) under N_2 . The mixture was stirred in low temperature for 3h and after it warmed to room temperature. Excess of DAST was quenched by the addition of cold water and then the mixture was diluted with 20mL of CH_2Cl_2 , washed with sat. NaHCO_3 (10mL), water (10mL) and sat. NaCl (10mL), dried over Na_2SO_4 and rotoevaporated under vacuum to give 1.36g of crude product as a yellowish solid. ^1H NMR showed that the product was about 60% pure and the major impurity was the elimination byproduct. The two components could not be separated efficiently by flash chromatography (the R_f factor of the desirable component was 0.56, using pentane as a solvent) and gave 1.18g (4.61mmol, 78%) of a colorless liquid.

Table 26. ^1H NMR analysis of ($\pm$)-11-heptadecen-1-yl fluoride

δ (ppm)	mult.	hydrogens (#)	J_{HF} value (Hz)	hydrogens(abs.)
5.82	m	1	48	$-\text{CH}=\text{CH}_2$
5.39	m	2		$-\text{CH}=\text{CH}-$, elim. byprod.
4.95	m	2		$-\text{CH}=\text{CH}_2$
4.46	d of m	1		$-\text{CH}_2\text{CHFCH}_2-$
2.03	m	2		$-\text{CH}_2\text{CH}=\text{CH}_2$
1.61	m	4		$-\text{CH}_2\text{CHFCH}_2-$
1.2-1.5	br s	20		$(\text{CH}_2)_{10}$ for $\text{C}_4\text{-C}_9$ and $\text{C}_{13}\text{-C}_{16}$
0.90	t	3		$-\text{CH}_2\text{CH}_3$

4.2.6.3 ($\pm$)-10-fluoropalmitic acid from ($\pm$)-11-heptadecen-1-yl fluoride

A 10mL r.b. flask was charged with KMnO_4 (3.5g, 21mmol) and 33mL of water and then it was immersed in an ice-water bath and stirred vigorously via a magnetic stirrer. A solution of 11-heptadecen-1-yl fluoride (815mg, 3.18mmol), 5.2mL glacial acetic acid and 37mg (0.07mmol) of the phase-transfer agent hexadecyltributylphosphonium bromide were added in one portion. Stirring was continued without any further addition of ice for overnight. The mixture was recooled and 30mL of hexanes

were added. Sodium sulfite (Na_2SO_3) was added to destroy the MnO_2 until the color changed from purple to colorless and then a solution of 5mL of concentrated HCl in 5mL of water was added slowly. Two clear layers resulted. The layers were separated and the organic layer was washed with 20mL of water. The aqueous layer was washed with hexanes (3×20mL). Finally the combined organic layers were dried over Na_2SO_4 and the solvent was removed by rotoevaporation *in vacuo* to give 480mg (1.75mmol, 55%) of white crystals as a product^[63].

Table 27. ^1H NMR analysis of ($\pm$)-10-fluoropalmitic acid.

δ (ppm)	mult.	hydrogens (#)	J_{HF} value (Hz)	hydrogens(abs.)
4.45	d of m	1	48	$-\text{CH}_2\text{CHFCH}_2-$
2.35	t	2		$-\text{CH}_2\text{COOH}$
1.62	m	4		$-\text{CH}_2\text{CHFCH}_2-$
1.2-1.5	br s	20		$(\text{CH}_2)_{10}$ for $\text{C}_3\text{-C}_{10}$ and $\text{C}_{14}\text{-C}_{17}$
0.89	t	3		$-\text{CH}_2\text{CH}_3$

4.2.6.4 Methyl ($\pm$)-10-fluoropalmitate from ($\pm$)-10-fluoropalmitic acid

470mg (1.70mmol) of the above acid was dissolved in 45mL of a 10% (v/v) solution of $\text{BF}_3 \cdot \text{Et}_2\text{O}$ in methanol and refluxed for 1.5h. Excess of BF_3 was destroyed by the addition of 40mL of water. This mixture was extracted with CH_2Cl_2 (5×20mL). These extracts were combined, washed with sat. NaCl solution (30mL), dried over Na_2SO_4 and the solvent was rotoevaporated under vacuum to give 380mg of crude product. The product then was purified by flash chromatography (2% EtOAc/Hexanes) to give ~93% pure methyl ($\pm$)-10-fluoropalmitate (major impurity was the overoxidation by-product) as a low melting point white solid.

m.p.: 24-25°C,

R_f = 0.52 (TLC: 10% EtOAc/Hexanes),

R.T.: 14:06,

MS: ($\text{M}^+ - \text{HF}$) 268, [$(\text{M}^+ - \text{HF}) - 32$] 236,

^{19}F NMR: -180.49 ppm,

Anal. Calc'd for $\text{C}_{17}\text{H}_{33}\text{FO}_2$: C: 70.78, H: 11.53. Found: C: 70.71, H: 11.53.

Table 28. ^1H NMR analysis of methyl ($\pm$)-10-fluoropalmitate.

δ (ppm)	mult.	hydrogens (#)	J_{HF} value (Hz)	hydrogens(abs.)
4.45	d of m	1	48	$-\text{CH}_2\text{CHFCH}_2-$
3.67	s	3		$-\text{COOCH}_3$
2.31	t	2		$-\text{CH}_2\text{COOCH}_3$
1.63	m	4		$-\text{CH}_2\text{CHFCH}_2-$
1.2-1.5	br s	20		$(\text{CH}_2)_{12}$ for $\text{C}_3\text{-C}_7$ and $\text{C}_{12}\text{-C}_{15}$
0.89	t	3		$-\text{CH}_2\text{CH}_3$

4.2.7 Methyl (±)-9-fuoropalmitate

4.2.7.1 9-decen-1-al from 9-decen-1-ol

A solution of CH₂Cl₂ (42mL) and oxalyl chloride (3.6mL, 42mmol) was placed in a 250mL three-necked r.b. flask equipped with a magnetic stirrer, a drying tube with molecular sieves, two dropping funnels containing DMSO (7.0mL, 90mmol) dissolved in 13mL of CH₂Cl₂ and the 9-decen-1-ol (6.5mL, 36.5mmol) in 26mL of CH₂Cl₂ respectively and under nitrogen gas. The DMSO solution was added to the stirred oxalyl chloride solution at around -45°C. The reaction mixture was stirred for 2 min and the alcohol was added within 5 min; stirring was continued for an additional 15 min. 30 mL (215mmol) of triethylamine (TEA) were added and the reaction mixture was stirred for 5 min and then allowed to warm to room temperature. Water (100mL) was then added to hydrolyze the oxalyl chloride and dissolve the excess of DMSO. The aqueous phase was reextracted with additional CH₂Cl₂ (2×50mL). The organic layers were combined, washed with 1% HCl, water, 5% NaCO₃, water, dried over Na₂SO₄ and rotoevaporated under vacuum to give a slightly colored oil (4.57g, 29.7mmol, 82%) as a product (~90% pure by ¹H NMR^[67]).

R_f = 0.35 (TLC: 5% EtOAc/Hexanes).

Table 29. ^1H NMR analysis of 9-deacon-1-al.

δ (ppm)	mult.	hydrogens (#)	hydrogens (abs.)
9.71	t	1	-CHO
5.76	m	1	-CH=CH ₂
4.90	m	2	-CH=CH ₂
2.37	t of d	2	-CH ₂ CHO
2.00	m	2	-CH ₂ CH=CH ₂
1.60	m	2	CH ₂ CH ₂ CHO
1.2-1.5	br s	8	(CH ₂) ₄ for C ₄ -C ₇

4.2.7.2 ($\pm$)-10-heptadecen-1-ol from 9-decen-1-al and heptylmagnesium bromide

A 100mL, three-neck, thoroughly dry, r.b. flask was equipped with a stirring bar, two 50mL dropping funnels (with CaCl_2 guard-tubes on the top), a condenser and nitrogen gas. Magnesium turnings (1.1g, 45mmol), previously crushed, washed with acetone, ether and dried, were placed in the flask along with 6mL of ether (anhydrous, freshly distilled over LiAlH_4) and a crystal of iodine. 6.3mL (40mmol) of heptylbromide and 10mL of ether were placed in one of the dropping funnels. 2-3mL of this solution was introduced into a flask and the mixture was heated in a water bath so as the ethereal solution was refluxing gently. When the reaction started (decolorization of I_2 , cloudiness, bubbling released from the Mg) the hot water was removed and the stirrer was set in

motion. The rest of the solution was added slowly over a period of 30 min. The water bath was placed again to help the reaction and after 30 min the reaction was judged to be complete (brown color of the solution and a few Mg turnings). The Grignard reagent was cooled around -10°C (in an ice-anhydrous CaCl_2 bath) and the 9-decen-1-al (4.57g, 29.7mmol) along with 15mL of ether were placed into the second dropping funnel. The solution of the aldehyde was added into the flask slowly over a period of 30 min giving a slightly green color. After the addition of the aldehyde the reaction product was poured upon 25g of crushed ice and then 50mL 3M H_2SO_4 were added to dissolve the basic magnesium bromide. The mixture was transferred into a separatory funnel and the two layers were separated. The aqueous layer was extracted with three 25mL portions of ether. The combined ethereal layers were washed with 3M H_2SO_4 (40mL), sat. NaCl, dried over Na_2SO_4 and rotoevaporated under vacuum to give 6.30g of crude product as a yellowish solid. Flash chromatography was performed to purify the product (5% EtOAc/Hexanes) and gave 3.95g (15.43mmol, 52% isolated yield) of a white solid pure by ^1H NMR.

m.p.: $41-43^{\circ}\text{C}$,

$R_f = 0.60$ (TLC: 20% EtOAc/Hexanes),

R.T.: 13:01,

MS: ($\text{M}^+ - \text{H}_2\text{O}$) 236.

Table 30. ^1H NMR analysis of ($\pm$)-10-heptadecen-1-ol.

δ (ppm)	mult.	hydrogens (#)	hydrogens (abs.)
5.79	m	1	$-\text{CH}=\text{CH}_2$
4.93	m	2	$-\text{CH}=\text{CH}_2$
3.57	br s	1	$-\text{CH}_2\text{CHOHCH}_2-$
2.03	m	2	$-\text{CH}_2\text{CH}=\text{CH}_2$
1.61	m	4	$-\text{CH}_2\text{CHOHCH}_2-$
1.2-1.5	br s	20	$(\text{CH}_2)_{10}$ for C_4-C_9 and $\text{C}_{13}-\text{C}_{16}$
0.89	t	3	$-\text{CH}_2\text{CH}_3$

4.2.7.3 (±)-10-heptadecen-1-yl fluoride from (±)-10-heptadecen-1-ol

To a solution of 1.35mL (10.2mmol), in 5mL of CH_2Cl_2 , cooled to -45°C in a dry ice-isopropyl alcohol bath and under N_2 , 2.3g (9.05mmol) of the alcohol, dissolved in 5mL of CH_2Cl_2 , were added slowly. After 3h the reaction mixture was warmed to room temperature, quenched with water, diluted with 20mL of CH_2Cl_2 , transferred to a separatory funnel, washed with sat. NaHCO_3 (10mL), water (10mL) and sat. NaCl (10mL), dried over Na_2SO_4 and rotoevaporated *in vacuo* to give 1.75g (6.8mmol, 75%) of crude product as a yellowish solid. The product was about 75% pure by ^1H NMR and the major impurity was the elimination byproduct.

Table 31. ^1H NMR analysis of (±)-10-heptadecen-1-yl fluoride

δ (ppm)	mult.	hydrogens (#)	J_{HF} value (Hz)	hydrogens(abs.)
5.80	m	1	48	$-\text{CH}=\text{CH}_2$
5.37	m	2		$-\text{CH}=\text{CH}-$, elim. byprod.
4.94	m	2		$-\text{CH}=\text{CH}_2$
4.44	d of m	1		$-\text{CH}_2\text{CHFCH}_2-$
2.02	m	2		$-\text{CH}_2\text{CH}=\text{CH}_2$
1.62	m	4		$-\text{CH}_2\text{CHFCH}_2-$
1.2-1.5	br s	20		$(\text{CH}_2)_{10}$ for $\text{C}_4\text{-C}_9$ and $\text{C}_{13}\text{-C}_{16}$
0.89	t	3		$-\text{CH}_2\text{CH}_3$

4.2.7.4 (±)-9-fluoropalmitic acid from (±)-10-heptadecen-1-yl fluoride

Aqueous KMnO_4 (6.32g, 40mmol) in 60mL of water was placed in a 250mL r.b. flask and cooled with stirring in a ice bath. 10-heptadecen-1-yl fluoride (1.75g, 6.8mmol), glacial acetic acid (12mL) and phase-transfer agent (50mg) were added in one portion. The reaction was allowed to proceed overnight without any further addition of ice. The mixture was recooled and 60mL of hexanes were added. Sodium sulfite (Na_2SO_3) was added to destroy the MnO_2 and then a solution of 8mL of concentrated HCl in 8mL of water was added slowly. Two clear layers were formed; the organic layer was washed once with 50mL of water and the aqueous layer was washed twice with 40mL of hexanes. The combined organic layers were dried over Na_2SO_4 and the solvent was removed by rotoevaporation *in vacuo* to give 800mg (2.92mmol, 43%) of white crystals as a product.

Table 32. ^1H NMR analysis of (±)-9-fluoropalmitic acid.

δ (ppm)	mult.	hydrogens (#)	J_{HF} value (Hz)	hydrogens(abs.)
4.44	d of m	1	48	$-\text{CH}_2\text{CHFCH}_2-$
2.33	t	2		$-\text{CH}_2\text{COOH}$
1.62	m	4		$-\text{CH}_2\text{CHFCH}_2-$
1.2-1.5	br s	20		$(\text{CH}_2)_{10}$ for $\text{C}_3\text{-C}_{10}$ and $\text{C}_{14}\text{-C}_{17}$
0.89	t	3		$-\text{CH}_2\text{CH}_3$

4.2.7.5 Methyl (±)-9-fluoropalmitate from (±)-9-fluoropalmitic acid

To a 100mL r.b. flask, 800mg (2.9mmol) of the above fluorofatty acid were dissolved in 45mL solution of 10% (v/v) of $\text{BF}_3 \cdot \text{Et}_2\text{O}$ in methanol and refluxed for 1.5h. The reaction mixture was cooled to room temperature and then transferred to a separatory funnel. 40mL of water were added to destroy any excess of the catalyst and the solution was extracted with CH_2Cl_2 (5×20mL). The combined organic layers were washed with sat. NaCl solution (30mL), dried over Na_2SO_4 and rotoevaporated under vacuum to give 750mg of crude product. The product then was purified by flash chromatography (2% EtOAc/Hexanes) to give ~94% pure methyl (±)-9-fluoropalmitate (major impurity was the overoxidation byproduct) as a low melting point white solid. Further purification was accomplished by recrystallization (twice from MeOH).

m.p.: 26-28°C,

R_f = 0.51 (TLC: 10% EtOAc/Hexanes),

R.T.: 14:02,

MS: ($\text{M}^+ - \text{HF}$) 268, [$(\text{M}^+ - \text{HF}) - 32$] 236,

^{19}F NMR: -180.50 ppm,

Anal. Calc'd for $\text{C}_{17}\text{H}_{33}\text{FO}_2$: C: 70.78, H: 11.53. Found: C: 70.95, H: 11.46.

Table 33. ^1H NMR analysis of methyl ($\pm$)-9-fluoropalmitate.

δ (ppm)	mult.	hydrogens (#)	J_{HF} value (Hz)	hydrogens(abs.)
4.45	d of m	1	48	-CH ₂ CHFCH ₂ -
3.66	s	3		-COOCH ₃
2.30	t	2		-CH ₂ COOCH ₃
1.63	m	4		-CH ₂ CHFCH ₂ -
1.2-1.5	br s	20		(CH ₂) ₁₂ for C ₃ -C ₇ and C ₁₂ -C ₁₅
0.88	t	3		-CH ₂ CH ₃

4.3 Incubation experiments

4.3.1 General

The yeast *Saccharomyces cerevisiae* ATCC #12341 was obtained from the Yeast Genetic Stock Center at the University of California-Berkeley. All biological media were purchased from Difco with the exception of D-glucose which was obtained from BDH. Distilled deionized water (ddH₂O) was used in all media and growth preparations. All cultures were grown at ~30°C, 150rpm for 24h in a New Brunswick incubator-shaker. Centrifugation was performed in a Sorvall RC-5B refrigerated centrifuge at ~5°C, 5500rpm, for 10 min. All biological transfers were performed in a sterile environment (Laminar flow fumehood) in close proximity to a Bunsen burner flame. All media and glassware were sterilized by autoclaving in a Amso Eagle Series Gravity Autoclave (Cycle: 30 min at 120°C). The necks of all glassware were flamed before and after transfer.

4.3.2 Preparation and incubation of yeast

The yeast, *Saccharomyces cerevisiae*, was grown on previously prepared slant cultures^[79]. Every three incubations a new slant was inoculated from a stock culture of *Saccharomyces cerevisiae*, and incubated at 30°C for 24h. The culture medium was prepared (for incubation of 50mg of substrate) as follows: D-glucose (13g) was dissolved in 250mL of ddH₂O in a 1L Erlenmeyer flask; Bacto peptone (13g), and Bacto yeast extract (6.5g) were dissolved in 400mL of ddH₂O in a 5L beaker. 19mL of the D-glucose solution and 31mL of the Bacto peptone and Bacto yeast extract solution were added to a 225mL Erlenmeyer flask and they were used as the 'starting culture'. The rest of the Bacto peptone and Bacto yeast extract solution was then shared into two 1L Erlenmeyer flasks. These four flasks were stoppered with cotton wool, capped with a double layer of tinfoil and sterilized by autoclaving along with a 10mL graduated cylinder, three cotton-plugged pipettes and a 140mL beaker. After cooling, the D-glucose solution was added to each 1L flask (equally divided) which were left to stand overnight, while the 'starting culture' solution was inoculated with the yeast and incubated at 30°C for 24h.

In a sterile environment, 8mL of the 'starting culture' solution was transferred to each of the two 1L Erlenmeyer flasks and then 50mg of the substrate, dissolved in 1mL of abs. ethanol, were added to one flask and 1mL of abs. ethanol was added to the other flask (control). These two flasks were incubated (30°C, 150rpm) for overnight. After growth all solutions appeared turbid. The yeast cultures were poured into four pre-chilled polyethylene centrifuge bottles and centrifuged at 5500rpm for 11 min at 5°C. The supernatants were discarded and to each pellet 20mL of ddH₂O were added and they were recentrifuged. The cells were then hydrolyzed, while the medium was extracted as follows.

4.3.3 Hydrolysis and extraction of fatty acids

4.3.3.1 Yeast extract

The harvested yeast cells were hydrolyzed with 50mL of a hydrolysis solution (1M KOH in 1:1 H₂O/ethanol) in a 100mL round bottom flask. This mixture was refluxed and stirred for three hours under nitrogen. After cooling, the cell debris was filtered off by vacuum and rinsed with water. This solution then was carefully rotoevaporated to remove the ethanol. The resulting brown mixture was diluted with 100mL of water, transferred to a separatory funnel, acidified to pH $\approx$ 2 with 5mL of 50% H₂SO₄ and extracted with CH₂Cl₂ (4 $\times$ 30mL). The combined organic layers were washed with water (20mL), saturated NaCl solution (20mL), dried over anhydrous Na₂SO₄ and rotoevaporated *in vacuo* to give a slightly yellow solid as a product.

4.3.3.2 Culture medium

The medium was acidified to pH $\approx$ 2 with 5mL of 50% H₂SO₄, and extracted with CH₂Cl₂ (40mL), CHCl₃ (40mL) and hexanes (40mL). The organic layers were combined, washed with saturated NaCl solution, dried over anhydrous Na₂SO₄ and the solvent was removed by rotoevaporation to yield a slightly yellow residue.

4.3.4 Derivatization of extracts with diazomethane

Diazomethane was prepared for the esterification of fatty acid extracts by the following method. A 50% aqueous solution of KOH (3mL) and 15mL of diethyl ether was placed in a 25mL test tube. Nitrosomethylurea (500mg) was added gradually, waiting until it had reacted in between additions. Diazomethane gas bubbled into the ether layer and turned it yellow in color. The aqueous layer was removed with a pipet and anhydrous Na₂SO₄ added to the tube to remove any remaining water molecules. The ethereal diazomethane solution was added to the pre-cooled (in a ice bath) fatty acid

extracts, dissolved in ether, until no bubbling (occurring from the release of N_2) was observed. The ether was then removed by rotoevaporation and the resultant product was stored into Reactivials (1mL) at $0^\circ C$. TLC analysis of the extract was carried out on silica gel plates (80% EtOAc/Hexanes).

4.3.5 Oxidative cleavage of fatty acids

A stock oxidant solution of $KMnO_4$ (0.0025M) and KIO_4 (0.01M) was prepared by dissolving crystalline $KMnO_4$ (39.5mg) and KIO_4 (230mg) in distilled water in a 100mL volumetric flask. A stock solution of Na_2CO_3 (0.002M) was prepared by dissolving 21.1mg of the solid in water in a 100mL volumetric flask.

About 20mg of the methylated fatty acids were dissolved in t-butyl alcohol (20mL), water (5mL), 0.002M Na_2CO_3 (5mL) and stock oxidant solution (10mL) in a 100mL round bottom flask. This viscous purple mixture was stirred at around $28^\circ C$ for 48h. More stock oxidant was added to this solution during the reaction time if change of the color (yellowish with a brown precipitate of MnO_2 at the bottom) was observed until the purple color persisted again. When the reaction was complete, excess of oxidant was destroyed by addition of $Na_2S_2O_5$ giving a clear, colorless solution. 1M NaOH (5mL) were added to the flask and the t-BuOH was removed carefully by rotoevaporation. The cloudy solution was then diluted with water (5mL), acidified to pH = 2 with 50% H_2SO_4 , and extracted with hexanes (4×5mL). The combined extracts were dried over Na_2SO_4 and the solvent was removed by rotoevaporation *in vacuo* to give a colorless liquid as a product.

5.0 References

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