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Enzymatic and chemical modification of fatty acid methyl esters: enzymatic catalysis of methyl linoleate using soybean lipoxygenase and chemical catalysis of methyl oleate Using Hypobromination

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ENZYMATIC AND CHEMICAL MODIFICATION OF FATTY ACID METHYL ESTERS: ENZYMATIC CATALYSIS OF METHYL LINOLEATE USING SOYBEAN LIPOXYGENASE AND CHEMICAL CATALYSIS OF METHYL OLEATE USING HYPOBROMINATION

by

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CERTIFICATE OF APPROVAL

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MASTER'S THESIS

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CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 Enzymatic and Chemical Modification of Fatty Acid Methyl Esters

Vegetable oils are a cheap and abundant chemical feedstock which can be readily broken down into fatty acid methyl esters (FAMEs) by transesterification using methanol and a base catalyst. These FAMEs contain reactive unsaturated double bonds which can be targeted for modification. In this study, enzymatic and chemical modifications of the unsaturated double bonds of FAMEs are explored with the goal of producing higher value products.

1.2 Use of Lipoxygenases in the Production of Monools

1.2.1 Introduction

It has been well documented on the lab scale that the fatty acids linoleic acid and arachidonic acid can be enzymatically modified to contain a monoperoxide by soybean lipoxygenase 1 (SLOX-1) in a buffered aqueous reaction medium.[1-3] The resulting reaction produces a high yield and specificity towards the monoperoxide, which can then be readily reduced, via a reducing agent such as tripheyl phosphine, to produce a fatty acid containing a monool.[4] However, this process is unfeasible on an industrial scale because linoleic acid is sparingly soluble in an aqueous environment, and conversion to the monoperoxide is limited by low solubility of molecular oxygen in solution. This current investigation focuses on extending the use of SLOX-1 to modify methyl linoleate, which can be obtained as derivatives from vegetable oils such as soybean oil. A proposed reaction scheme is outlined in Figure 1.1.

1.2.2 Soybean Oil and Methyl Linoleate

Soybean oil is the vegetable oil feedstock most often used to produce higher value products due to its low cost and high abundance. When breaking down soybean oil you

obtain three fatty acids and one glycerol molecule per molecule of soybean oil. Soybean oil contains the following composition of fatty acids: 51% linoleic acid, 23% oleic acid, 10% palmitic acid, 7% linolenic acid, and 4% steric acid. Methyl linoleate is the FAME of linoleic acid which can be produced by transesterification, using methanol and a base catalyst.

1.2.3 Soybean Lipoxygenase-1

SLOX-1 is a non heme iron dioxygenase. Its natural environment is in plants and mammals in which it initiates lipoperoxidation of membranes.[5] SLOX-1 is a 94 kDa enzyme, its pH optimum is 9, and its activity is independent of enzyme concentration.

Boyington et al. investigated the three dimensional structure of SLOX-1 and determined its active site as shown in Figure 1.2.[5] Three histidines and the carboxyl end of isoleucine coordinate to the iron center. The iron core interacts with the double bond of the substrate as well as catalytic oxygen to produce the resulting monool.

SLOX-1 contains two cavities of interest. Cavity one is 18 Å in length with a diameter of 8 Å. At the surface the diameter is 11 Å, reducing to 2 Å at the center. This is the channel through which molecular oxygen enters to interact with the enzyme's active site. Cavity 2 is 40 Å in length, it is a very narrow channel that has a diameter less than 3.5 Å at its narrowest point, and it contains two bends of greater than 90 degrees. This is the channel through which the substrate linoleic acid or arachidonic acid enters. The channel is very hydrophobic and contains three positively charged residues, lysine483, histidine494, and argenine707. The lysine483 is near the surface, while histdine494 and argenine707 are located in the middle of the cavity.

There is debate in literature concerning the SLOX-1 reaction mechanism.[6-7] Rickert et al. proposed a simplistic mechanism which is displayed in Figure 1.3.[6] They proposed the Iron (III)-OH first extracts a hydrogen from linoleic acid or arachidonic acid to form Iron (II)-H_2O as well as a free radical on the fatty acid molecule. Molecular oxygen then reacts with the free radical formed on the fatty acid molecule. A hydrogen

atom is then extracted from Iron (II)-H_2O to form the monoperoxide product, returning the catalytic iron complex to its original state. Schilestra et al. observed that the dioxygenation rate of lipoxygenase catalysis is determined by the amount of Iron (III) lipoxygenase in solution.[7] In their studies Iron (III) was proposed to be the catalytically active species and the initial dioxygenation rate at the beginning is linearly related to the Iron (III) to Iron (II) ratio. Changes in the distribution of these two forms have an effect on the dioxygenation rate. In addition, the substrate and the product both compete for the same active site and the reaction will not proceed in the absence of product. These findings suggest that there is a quick initial burst of enzymatic activity followed by competitive inhibition between the substrate and product.

Binding of the substrate in SLOX-1 was investigated by Ruddat et al.[8] Figure 1.4 displays the proposed binding mechanism. The following mutants were produced, tryptophan500leucine, Argenine707leucine, and lysine260leucine. It was found that lysine260leucine had no effect on activity. In the case of the mutant tryptophan500leucine, a phenyl group was replaced with a non aromatic residue. A three-fold increase in the dissociation constant (K_d) in comparison to the wild type was observed. This led to the conclusion that π-π interactions play a role in the binding of the substrate to the active site. In the case of argenine707leucine a seven-fold increase in the K_d in comparison to the wild type was observed. This led to the conclusion that the positive charge of argentine707 is necessary to bind the negatively charged carboxyl end of the substrate into the active site. One potential issue with the carboxyl acid end binding first conclusion is that the crystal structure determined by Boyington et al. found that argenine707 was located in the middle of the cavity.[5] In addition, changing an interior amino acid from a positive charge to neutral charge will likely have an effect on SLOX-1's activity regardless of whether it is directly interacting with the substrate or not.

Began et al. also performed a SLOX-1 binding study which brings Ruddat et al.'s conclusion of carboxyl end binding into question.[9] In this study, linoleic acid and

arachidonic acid were inserted into phosphatidylcholine deoxycholate mixed micelles (PDM-micelles) with their hydrophobic tail groups buried inside and carboxylic acid head groups exposed to the outside environment. A change in the positional specificity of SLOX-1 was found when inserted into the micelles. Figure 1.5 displays the structure of linoleic acid and arachidonic acid with the pertinent carbons labeled for reference. When linoleic acid was modified in a typically aqueous solution containing SLOX-1 and Tween 20, a surfactant used to solubilize linoleic acid, a high specificity was found towards a monoperoxide forming at the 13 position as expected. When linoleic acid was modified in the PDM-micelle environment, again using Tween 20 and SLOX-1, high specificity was observed towards the 9 position. This observed change in specificity leads to the possibility that in the typical aqueous environment, the hydrophobic tail end of linoleic acid enters the active site of SLOX-1 first instead of the carboxylic acid end. A similar result was observed using arachidonic acid as a substrate, while in this case the specificity changed from 15 position (aqueous environment) to the 5 position (mixed micelle environment). These results lead to the possibility of methyl linoleate being a viable substrate for SLOX-1.

1.2.4 The use of Enzymes in Organic Media and Lyoprotection

The use of organic solvents was necessary when attempting to modify methyl linoleate which is insoluble in an aqueous environment. Isooctane was chosen as the solvent because it provides a high degree of solubility for both methyl linoleate and molecular oxygen.

The use of enzymes in organic media has been studied extensively by Klibanov et al.[10-12] In their studies they have shown a variety of enzymes can be catalytically active in organic solvents when using either immobilization or lyprotection techniques. In this study, lyoprotection of SLOX-1 was used. To achieve lyprotection, the enzyme is dissolved in buffered media, at the pH optimum of the enzyme, along with a high percentage (98% w/w) of an inorganic salt such as potassium chloride. The solution is

then lyophilized using a freeze dryer to recover the resulting enzyme/inorganic salt complex. Lyoprotection allows for enzymes to be catalytically active in an unfavorable environment such as organic media because an aqueous buffered microenvironment at the enzyme's optimum pH is created. The substrate, dissolved in organic media, interacts with the insoluble catalytically active enzyme when they interact upon mixing.

1.2.5 Applications

The products from this investigation could be applicable to areas including specialty chemicals and lubricants. Production of specialty chemicals requires a high degree of specificity which enzymatic catalysis can provide. In the case of lubricants, stacking of the molecules is undesirable, and a chemical structure containing double bonds and monools is desired. An example of this is castor oil, which is a triglyceride, whose composition when broken down into fatty acids consists of 87% ricinoleic acid, which is displayed in Figure 1.6. Ricinoleic acid has a similar structure to the proposed product (Figure 1.1) that is intended to be made in this investigation. The primary difference is that the proposed product would contain two conjugated double bonds, while ricinoleic acid only contains one double bond. Castor oil is used in a variety of applications including but not limited to food additives, adhesives, lubricating grease, paints, and hydraulic fluids. It would be of great interest to develop a potential substitute for castor oil and its derivatives as it originates from castor beans which also contain the toxic substance ricin.

1.3 Epoxidation of Vegetable Oils and their Derivatives

1.3.1. Introduction

Vegetable oil is a cheap and abundant feedstock that has been increasingly targeted in recent years through innovative design to substitute for petroleum-based polymers due to its inherent low toxicity and biodegradability. The vegetable oil most often used for these applications is soybean oil due to its low price and ample supply.

There is a rapidly growing market for epoxidized soybean oil (ESBO) which is produced by converting the double bonds of soybean oil into epoxides. The major application for ESBO is as a plasticizer for polyvinylchloride (PVC). PVC resins tend to become brittle when exposed to light due to loss of hydrogen chloride. ESBO serves two purposes; it acts as both a scavenger for the hydrochloric acid released and as a plasticizer with its high oxirane oxygen content and high molecular weight.

Currently the plasticizers that dominate the PVC market are phthalates. Phthalates are currently being phased out of many products in western economies over health concerns. There are clinical studies ongoing concerning the link between phthalate exposure and health issues such as asthma and autism.[13-14] Phthalates are not chemically bonded to plastic so they can easily leach from the plastic. Ideally ESBO could be used as a replacement for phthalates. The issues to overcome as far as ESBO making a larger dent in the phthalate market are its higher cost in comparison to phthalates, potential hazards during the production of ESBO, and solubility issues limiting the conversion to ESBO.

Other applications for ESBO and its derivatives are currently being explored.[15-17] Epoxidized substances are used in a wide variety of applications including but not limited to fuel additives, agricultural and pharmaceutical molecules, flavors, fragrances, UV cure applications, surfactants, adhesives, sealants, and coatings.

1.3.2. Epoxidation Methods

The two main methods for the production of epoxides that currently exist are peroxidation and hypochlorination. The peroxidation method has been studied extensively in literature.[18-25] Peroxidation is currently the method most often employed for commercial production of ESBO. The drawbacks to this procedure are that peroxidation involves high handling and storage costs, the reagents are potentially explosive due to the use of hydrogen peroxide, purification of the metal catalysts are necessary, and there are side reactions that produce peracids. The hypochlorination

method has also been studied to a lesser extent.[26-29] A generalized reaction mechanism for the hypochlorination method is displayed in Figure 1.7. These reactions are attractive as the necessary components needed to carry out the reactions are cheap, stable, display a low degree of flammability, and do not contain volatile organic compounds.

A similar reaction method to the hypochlorination method that has displayed higher reaction rates to the desired epoxide product is the hypobromination method.[30] The only difference between these two methods is that in the hypobromination method a catalytic amount of potassium bromide (KBr) is added to the reaction solution. Klawonn et al. has shown that the oxidation rate is much higher for the hypobromite ion (BrO^-) in comparison to the hypochlorite ion (ClO^-).[30] An aqueous buffered solution was used to maintain the pH between 6 and 11 which is a pH range in which epoxide formation is favored. Under very basic conditions (pH 11-14) the epoxide will open to form diols. A generalized proposed reaction mechanism for the hypobromination method is shown in Figure 1.8. The major obstacle in using the hypobromination method to produce something such as ESBO is that the desired substrate soybean oil is insoluble in the reaction mixture thus making conversion to the desired ESBO difficult.

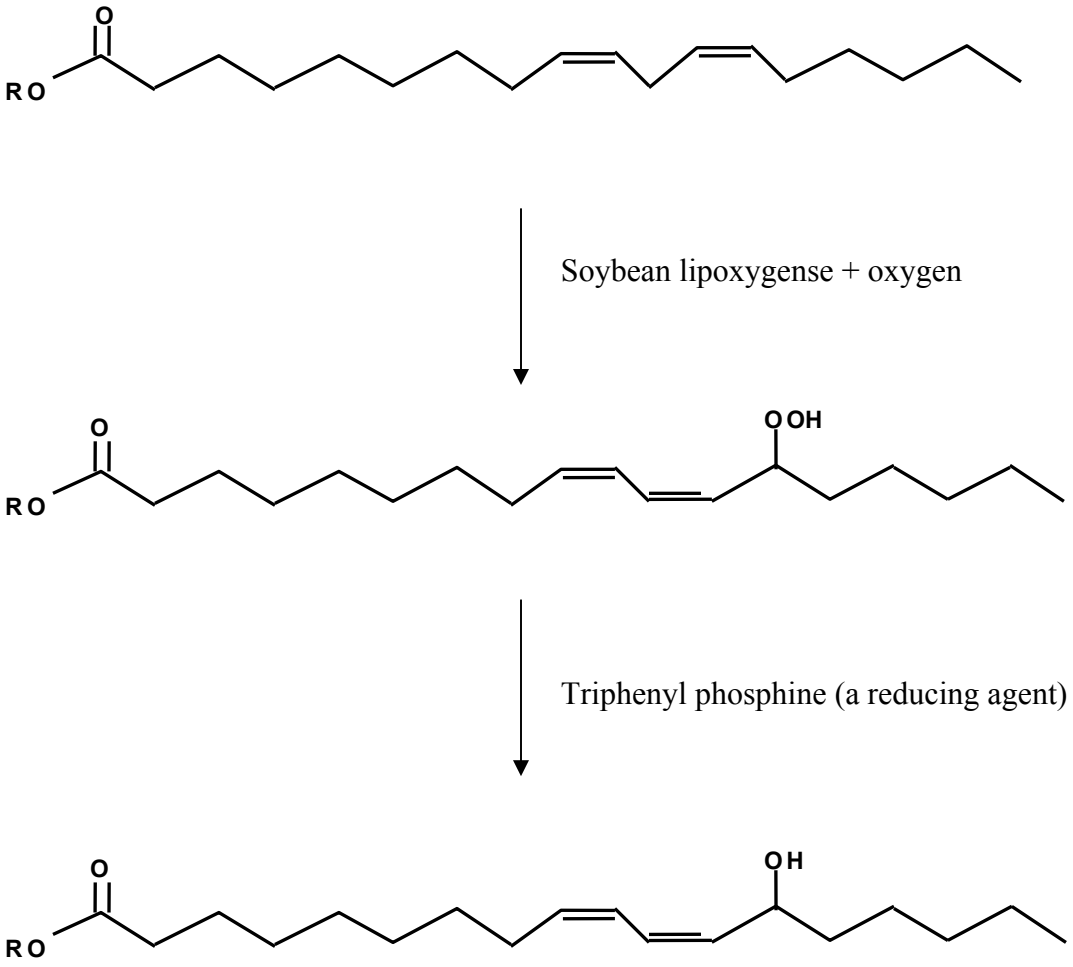
To overcome some of the solubility issues Jain performed experiments involving the use of a three phase microemulsion system (organic solvent, soybean oil substrate, and aqueous buffered hypobromination solution) along with the use of a surfactant to afford better contact between the soybean oil substrate and the oxidizing aqueous phase.[31] The system that displayed the most promise, as adapted from Klawonn et al., was as follows: tert-butanol as the organic phase; potassium bromide (KBr), sodium hypochlorite solution (11-13% available chlorine),and a buffer solution of potassium dihydrogen phosphate buffered at a pH of 8; and the soybean oil substrate as a third phase which settled between the two phases. Surfactants were also screened to optimize production to the epoxide product, it was found that the surfactant Neodol 1-5 produced the highest conversion to epoxide product (52%) as determined by ^{1}H-NMR.

1.3.3 Future Studies of the Hypobromination Method

Further investigation into Jain's hypobromination of soybean oil to produce ESBO is necessary from a mechanistic, kinetic, and optimization standpoint. The two methods employed to analyze the reaction products were ^{1}H-NMR and IR spectroscopy. ^{1}H-NMR is only useful in displaying the quantitative amount of epoxide product formed as protons in alcohols are generally not visible in ^{1}H-NMR spectroscopy. IR spectroscopy is only useful in showing that alcohols exist but cannot be used for quantitative analysis. Therefore, another method of analysis is necessary to gain better understanding of this reaction system. The method most widely used for the analysis of a complex mixture of organic compounds is GC/MS. Unfortunately, GC/MS cannot be employed on a triglyceride unless you break it down into its fatty acid methyl esters (FAMEs) following epoxidation. A potential complication is that the complex product mix formed may be chemically altered prior to GC/MS analysis using the procedure to form the FAMEs. In addition, soybean oil contains a mixture of four different fatty acids each of which makes a variety of products. Due to the complex mixture of both products formed and FAMEs present accurate GC/MS analysis of something as complex as soybean oil would be extremely difficult.

An approach to the above issues is to use a simpler molecule to model the reaction. To meet this end a single FAME was chosen as a substrate. In this case methyl oleate which is 23% of soybean oil's fatty acid composition was selected as the model compound. Methyl oleate only has one double bond located between the 9 and 10 positions as shown in Figure 1.9. The presence of only one double bond reduces the number of potential products while still showing the characteristic double-bond chemistry of the more complex soybean oil system. The goal of this study was to determine the mechanism and kinetics of this reaction system in order to obtain higher conversion to the desired epoxide product. This will provide a better understanding of this reaction and

hopefully move us one step closer to being able to use the hypobromination method to commercially produce ESBO.



+ tryphenyl phosphine oxide

Figure 1.1. Proposed reaction scheme for adding a monool to vegetable oil byproducts. Methyl linoleate R = CH_3, Linoleic acid R = H.

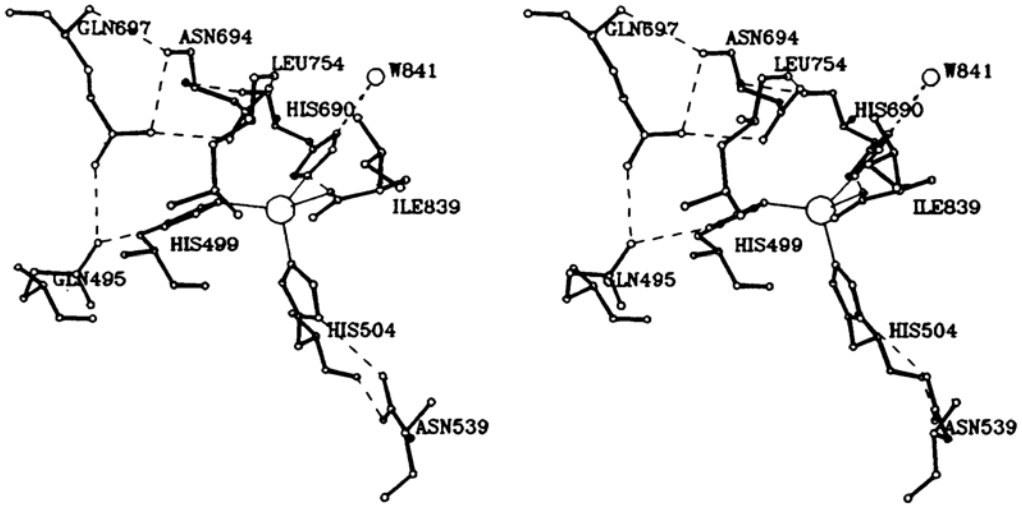


Figure 1.2. Active site of SLOX-1. HIS499, HIS690, HIS504, and ILE839 (carboxyl end) coordinated to Iron. Dotted lines are hydrogen bonds. W841 is a water molecule.

Source: Boyington J.C., Gaffney B.J., and Amzel L.M., *Science*, 260, 1482 (1993)

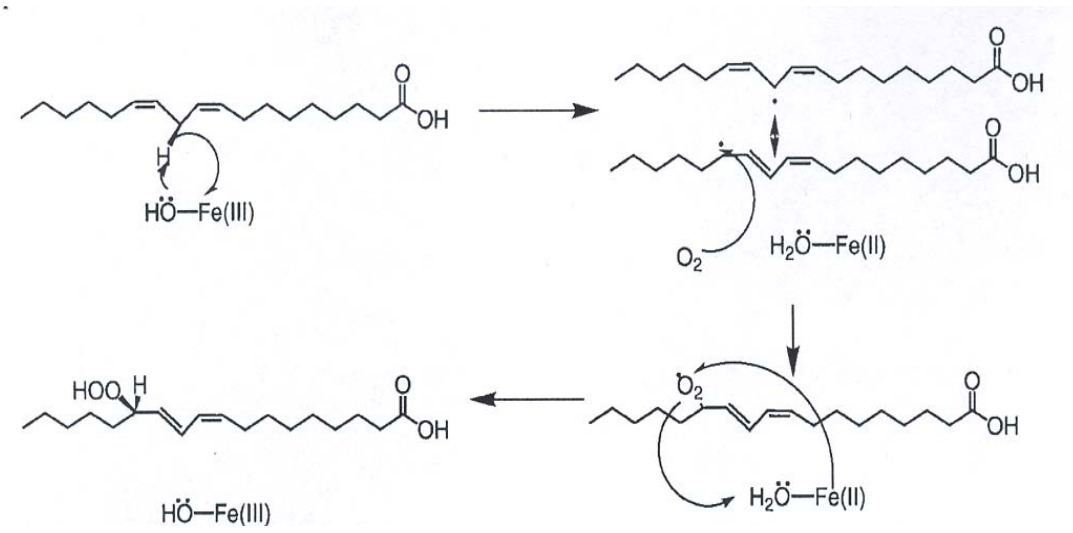


Figure 1.3. Proposed reaction mechanism for SLOX-1.

Source: Rickert K.W., and Klinman J.P., *Biochemistry*, 38, 12218 (1999)

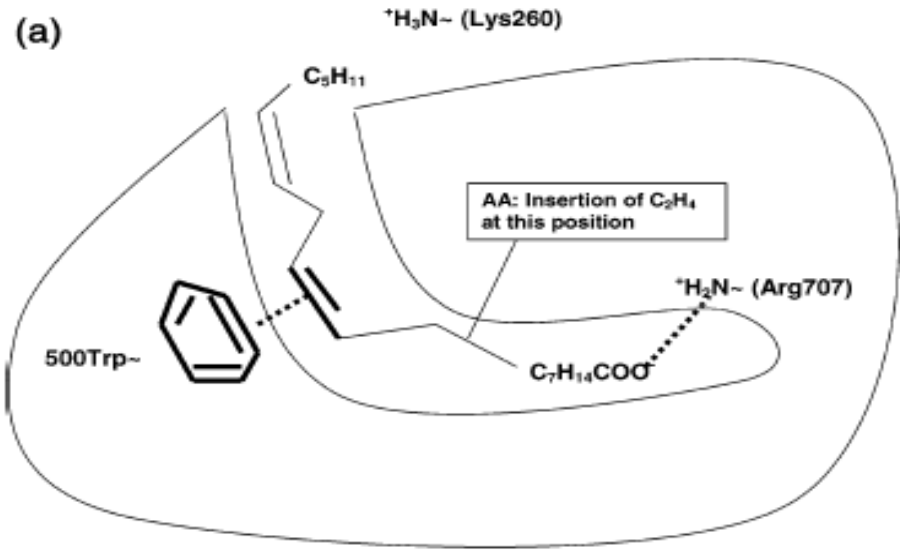


Figure 1.4. Proposed mechanism for the binding of arachidonic acid to the active site of SLOX-1.

Source: Schilstra M.J., Veldink G.A., and Vliegenthart J.F.G., *Biochemistry*, 33, 3974 (1994)

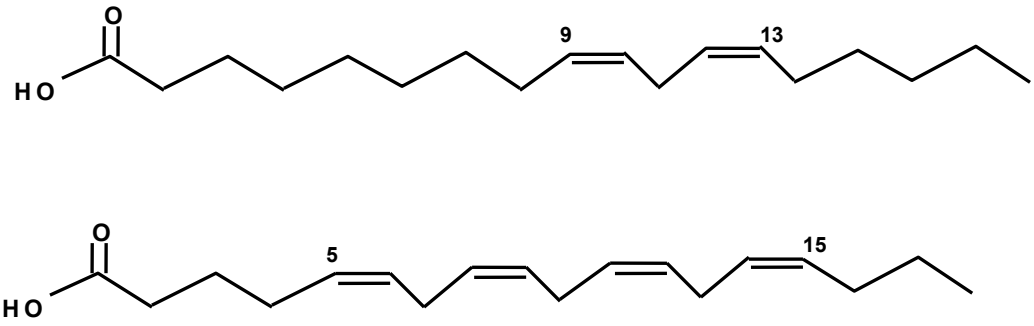


Figure 1.5. Chemical structure of linoleic acid (top) and arachidonic acid (bottom).

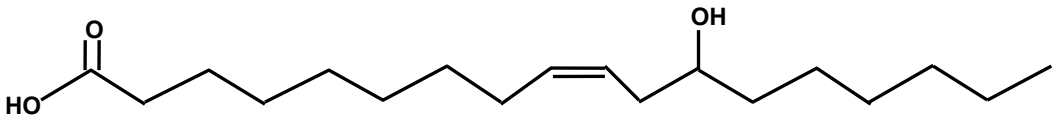


Figure 1.6. Chemical structure of ricinoleic acid.

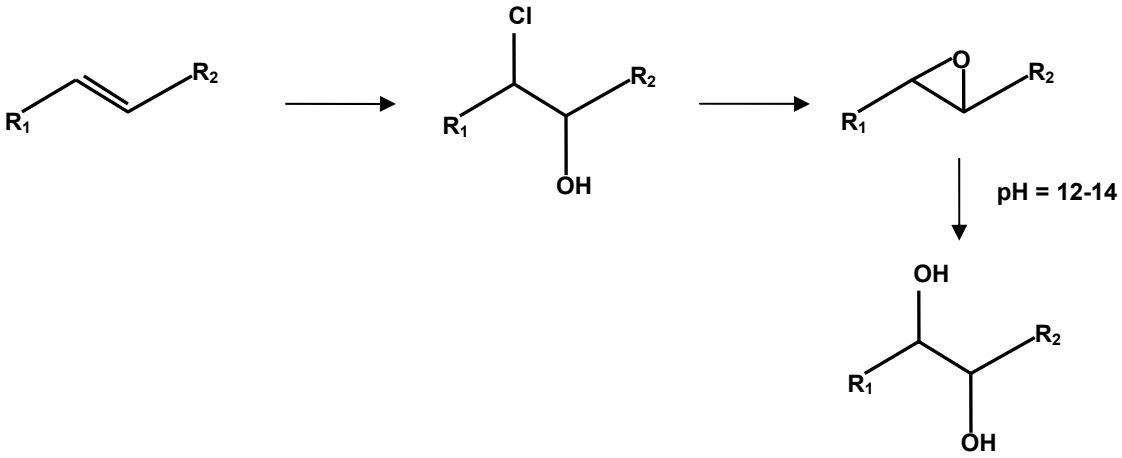


Figure 1.7. Generalized reaction mechanism for the hypochlorination method. The alkene first converts to a chlorohydrin when exposed to an oxidizing medium. These are short lived and then convert to an epoxide after giving up the chlorohydrin ion. R_1 and R_2 are alkene chains.

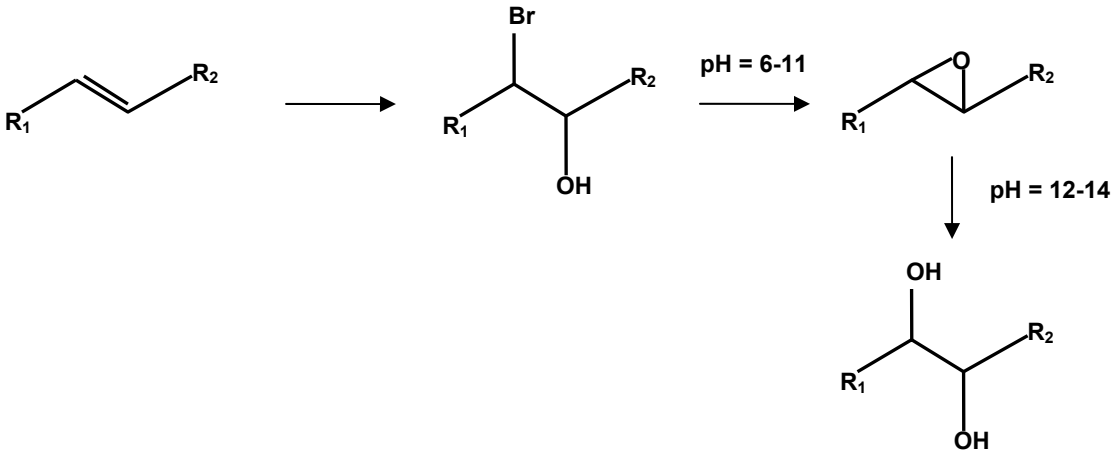


Figure 1.8. Generalized reaction mechanism for the hypobromination method. The alkene first converts to a bromohydrin when exposed to an oxidizing medium. These are short lived and then convert to an epoxide after giving up the bromohydrin ion. Under highly basic conditions, the epoxide ring opens to produce diols. R_1 and R_2 are alkene chains.

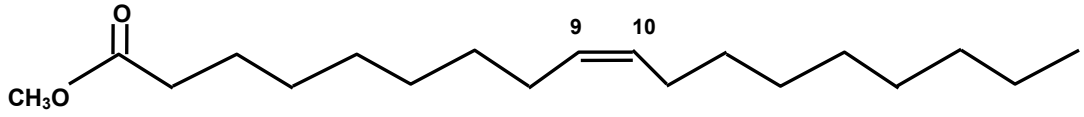


Figure 1.9. Chemical structure of methyl oleate.

CHAPTER 2

USE OF LIPOXYGENASES IN THE PRODUCTION OF MONOOLS

2.1 Introduction

As discussed in Chapter 1, the potential applications for monools produced from FAMEs are exhaustive. It has been shown that linoleic acid can be easily modified to contain a monoperoxide with a high degree of conversion and specificity to the 13 position using SLOX-1 in a buffered aqueous environment.[1-3] The resulting hydroperoxide product can then be easily reduced to a monool using a reducing agent such as triphenyl phosphine.[4]

Unfortunately, there are many limitations from the standpoint of commercial application. Linoleic acid is sparingly soluble in an aqueous environment, which also does not contain a high amount of molecular oxygen which is necessary for the reaction to be successful. Other drawbacks are a strong acid, in this case, hydrochloric acid is used to aid in recovery of the product, and an extracting solvent is necessary to recover the product.

In this study the potential for enzymatic modification of methyl linoleate in organic media was investigated. Literature results indicate, as discussed in Chapter 1, that methyl linoleate can possibly be modified to contain a monoperoxide using SLOX-1.[9] This procedure would have the advantages that methyl linoleate is highly soluble in an organic solvent such as isooctane, which also affords high molecular oxygen solubility. The organic solvent can also be recycled when removed under reduced pressure.

2.2 Materials

Diethyl ether, deuterated chloroform, chloroform, isooctane, ethyl acetate, and acetonitrile were obtained from Sigma-Aldrich. Flourinert FC-77 was obtained from 3M.

Sodium borate, potassium chloride (KCl), hydrochloric acid, and anhydrous magnesium sulfate were obtained from Fischer Scientific. The substrates linoleic acid and methyl linoleate were received from Nu-Chek Prep, Inc. All materials were used without further purification. Distilled water was used in all experiments involving aqueous conditions. Two different sources of SLOX-1 were used. SLOX-1SA was purchased from Sigma-Aldrich (131,100 units/mg), while SLOX-1BR was kindly provided by BioResearch products (18.44 IU/mg). 1 IU=100,000 sigma units

2.3 Modification Procedure

2.3.1 Synthesis of Linoleic Acid Hydroperoxide using SLOX-1

The synthesis of linoleic acid hydroperoxide in an aqueous buffered solution is a well documented procedure.[1-3] Linoleic acid hydroperoxide was produced as a reference prior to attempting to modify methyl linoleate. Linoleic acid (100 mg) and borate buffer solution at pH 9.0 (50 ml, 0.2 M, prepared using boric acid and sodium hydroxide), were placed in a 125 ml flask. The reaction was performed at room temperature (open to the air) and the sample was continuously agitated using a magnetic stirrer. Four aliquots of 50 µl each of SLOX-1 solution (Sigma, 1.5 mg/ml aqueous) were added every 30 min. After 2 h had elapsed, 1M hydrochloric acid was added to the flask until the pH of the solution decreased to 3. The pH was monitored using a Corning 220 pH meter. The addition of hydrochloric acid aids in product recovery, as it shifts the linoleic acid and products to its non-dissociated form of the product to precipitate out of the aqueous solution. The product was then extracted using 3 washes of 40 ml diethyl ether. The organic phase was collected and then dried using anhydrous magnesium sulfate. The

solution was filtered and the remaining organic phase was removed using a rotary evaporator. The recovered product was labeled and stored in a freezer until future analysis. The resulting product was characterized using ^{1}H-NMR spectroscopy using deuterated chloroform as a solvent.

2.3.2 Synthesis of Methyl Linoleate Hydroperoxide using SLOX-1

Methyl linoleate (100 mg), isooctane (30 or 50 ml), and SLOX-1 (5 mg raw enzyme or 200 mg of lyoprotected enzyme complex) were added to either a 40 ml vial (30 ml isooctane) or a 125 ml flask (50 ml of isooctane). The reactions were performed at room temperature and the sample was continuously agitated using either an incubator shaker (40 ml vial) or magnetic stirrer (125 ml flask). After the desired time period had elapsed anhydrous magnesium sulfate was added to remove any water present in the solution. The solution was filtered and the remaining organic solvent was removed using a rotary evaporator. The recovered product was labeled and stored in a freezer until future analysis. The resulting product was characterized using ^{1}H-NMR spectroscopy using deuterated chloroform as a solvent.

As a reference case, the same procedure as above was performed but in this case no SLOX-1 was added to the solution. This was done to directly compare results between when there is no SLOX-1 present to that of when SLOX-1 is present.

In the experiments which used lyoprotected SLOX-1/KCl complex, the following procedure was carried out prior to adding SLOX-1/KCl complex to the reaction media. SLOX-1 (40 mg), borate buffer solution at pH 9.0 (1 ml, 0.2 M, prepared using boric acid and sodium hydroxide), KCl (2 g, to make 98% w/w versus SLOX-1), and 19 ml of distilled water were added to a 125 ml flask. The solution was then lyophilized for 48 h

using a freeze dryer and the recovered SLOX-1/KCl complex was stored in a freezer until future use.

2.3.3. Stability Studies of SLOX-1

Methyl linoleate (100 mg), isooctance (30 ml), and lyoprotected SLOX-1/KCl complex (200 mg) were added to a 125 ml flask. The reaction was performed at room temperature and agitated using a magnetic stirrer. After 1 h the insoluble lyoprotected SLOX-1/KCl complex was recovered via filtration and placed in a freezer until future use. The typical experiment recovered roughly 150 mg of lyoprotected SLOX-1/KCl complex.

The recovered lyoprotected SLOX-1/KCl complex was then used to modify linoleic acid in an aqueous environment as follows. Linoleic acid (100 mg) and borate buffer solution at pH of 9.0 were placed in a 125 ml flask. The reaction was performed at room temperature and the sample was continuously agitated using a magnetic stirrer. 160 mg of recovered lyoprotected SLOX-1/KCl complex was added in 4 increments over 2 h. 1M hydrochloric acid was then added to the flask to decrease the pH of the solution to 3. The pH was monitored using a pH meter. The addition of hydrochloric acid aids in product recovery, as it forces the product out of solution. The product was than extracted using three washes of 40 ml diethyl ether. The organic phase was collected and dried using anhydrous magnesium sulfate. The solution was filtered and the remaining organic solvent was removed using a rotary evaporator. The recovered product was labeled and placed in a freezer until future analysis. The resulting product was characterized using ^{1}H-NMR spectroscopy using deuterated chloroform was used as a solvent.

2.3.4 Modified Linoleic Acid and Methyl Linoleate Characterization

^{1}H-NMR spectra were recorded on a Bruker Avance DRX-300 (manufactured by Bruker Analytik GnbH, Rheinstetten, Germany) operating at a ^{1}H frequency of 300 MHz. The room temperature ^{1}H-NMR spectra of linoleic acid and linoleic acid hydroperoxide are shown in Figure 2.1. The peak assignments are as follows: ^{1}H-NMR (300 MHz, CDCl3, δ=7.3 ppm)- linoleic acid: 0.9 (-$CH_2C\mathbf{H_3}$, t), 1.2-1.7 (aliphatic C$\mathbf{H_2}$, m), 2.0 (C**H2**CH=CH, q), 2.3 (C$\mathbf{H_2}$COOH, t), 2.8 (=CHC**H2**CH=, t), 5.3-5.4 (C**H**=C**H**, m), linoleic acid hydroperoxide- 0.9 ($CH_2C\mathbf{H_3}$, t), 1.2-1.7 (aliphatic C$\mathbf{H_2}$, m), 2.0 (C**H2**CH=CH, q), 2.3 (C**H**COOH, t), .4.3 (C**H**-OOH, q), 5.3-6.6 (C**H**=C**H**C**H**=C**H,** m).

The room temperature ^{1}H-NMR spectra of methyl linoleate in isooctane both with and without SLOX-1 present are shown in Figure 2.2. The peak assignments are as follows: ^{1}H NMR (300 MHz, CDCl3, δ=7.3 ppm), methyl linoleate- 0.9 (-$CH_2C\mathbf{H_3}$, t), 1.2-1.7 (aliphatic C$\mathbf{H_2}$, m), 2.0 (C**H2**CH=CH, q), 2.3 (-C$\mathbf{H_2}$$COOCH_3$, t), 2.8 (=CHC**H2**CH=, t), 3.6 (-$CH_2COOC\mathbf{H_3}$, t), 5.3-5.4 (C**H**=C**H**, m).

2.4 Results and Discussion

2.4.1. Synthesis of Linoleic Acid Hydroperoxide using SLOX-1

Linoleic acid was successfully converted to linoleic acid hydroperoxide using SLOX-1. The conversion was greater than 90% consistent with conversions reported in the literature.[1-3] Figure 2.1 shows the ^{1}H-NMR spectrum for linoleic acid hydroperoxide. These experiments were performed simply to give a reference ^{1}H-NMR spectrum prior to attempting to modify methyl linoleate. Methyl linoleate hydroperoxide should have a

similar spectrum with the only primary differences being a few minor peaks associated with the methyl ester group as described in detail in section 2.3.4.

2.4.2. Synthesis of Methyl Linoleate Hydroperoxide using SLOX-1

A variety of reactions were performed in an attempt to produce methyl linoleate hydroperoxide in isooctane using SLOX-1. Two different sources of SLOX-1 were used: SLOX-1SA and SLOX-1BR. For each source of SLOX-1, reactions were performed using both the raw SLOX-1 as received as well as the lyophilized SLOX-1/KCl complex form. Various reaction times were analyzed from as short as a few minutes to as long as 24 h, using two different mixing techniques (stir bar and incubator shaker). For all of the reactions little difference was seen when comparing the ^{1}H-NMR spectrum of the base case (no SLOX-1 present) and that when SLOX-1 was present in the reaction mixture. This result is displayed in Figure 2.2. The small amount of methyl linoleate hydroperoxide observed, as shown by the small peaks in the 5.5-6.5 ppm range, is therefore, due to autooxidation of methyl linoleate and not enzymatic catalysis.

2.4.3. Kinetic Studies

Kinetic studies were also performed as shown in Figure 2.3. The results show little change over the course of time. Again the small amount of methyl linoleate hydroperoxide formed, as shown by the small peaks in the 5.5-6.5 range, is due to autooxidation.

2.4.4. Solvent Systems besides Isooctane

Experiments were carried out utilizing different solvents besides isooctane. The solvents used included perfluorinated solvent FC-77, ethyl acetate, acetonitrile, and chloroform. FC-77 affords a very high molecular oxygen solubility which is a necessary

component of this reaction. A two-phase solvent system involving a biphasic system of distilled water and ethyl acetate was also attempted. In this particular system SLOX-1 was dissolved in the aqueous water phase, while the methyl linoleate was dissolved in the organic ethyl acetate phase with the hopes that the formation of methyl linoleate hydroperoxide occurs as these two phases interact upon mixing. In all cases no enzymatic catalysis to methyl linoleate hydroperoxide was observed.

2.4.5. Stability Studies of SLOX-1

Since no enzymatic catalysis of the methyl linoleate to the desired methyl linoleate hydroperoxide was observed, experiments were performed to ensure that SLOX-1 was not being completely deactivated due to exposure to organic media. As described in section 2.3.3., lyophilized SLOX-1/KCl complex was recovered via filtration after being placed in isooctane and was then used to enzymatically modify linoleic acid to linoleic acid hydroperoxide. The results observed displayed minimal if any enzymatic activity lost due to be exposed to organic media, the typical reaction displayed 90% conversion to linoleic acid hydroperoxide. It may be a bit surprising to observe little to no decrease in the amount of activity of SLOX-1 after lyophilization and exposure to organic media, but as described in Section 1.1.3. SLOX-1 activity is independent of enzyme concentration. As long as enough of the enzyme remains in its active form, the percent conversion should not change. This experiment ruled out that conversion of methyl linoleate to methyl linoleate hydroperoxide was not observed due to SLOX-1 being completely deactivated.

2.5 Conclusions

Unfortunately our original hypothesis that methyl linoleate could be modified to contain a monool using the reaction scheme displayed in Figure 1.1 to produce a higher value product proved to be unsuccessful. It was expected that methyl linoleate, which has a high solubility in organic media, could be converted in relatively high quantities to methyl linoleate hydroperoxide using SLOX-1.

The results however can lead to some conclusions regarding the binding mechanism of linoleic acid in SLOX-1. As discussed in Chapter 1, there are conflicting reports in literature regarding the binding of linoleic acid to SLOX-1.[8-9] The mutant experiments carried out by Ruddat et al. concluded that the linoleic acid binds carboxylic acid end first and is a necessary component to the binding mechanism for linoleic acid to SLOX-1.[8] However, the PDM-micelle experiments performed by Began et al. displayed a difference in positional specificity of monoperoxide formation when comparing reactions run in an aqueous environment versus the mixed micelle environment.[9] This suggested that in an aqueous environment linoleic acid potentially binds to SLOX-1 hydrophobic tail first.

After further analysis, the results of our studies are consistent with the scientific findings found by Began et al. In their study they reported that in the PDM-micelle environment linoleic acid was modified in the 9 position while in the typical aqueous environment it was modified in the 13 position. Based on the results found in this study that no enzymatic catalysis of methyl linoleate was observed, this suggest that in both cases linoleic acid attaches to the SLOX-1 active site carboxyl end first. In the case of the PDM-micelle environment the hydrophobic tail of linoleic acid are partially

entrapped by the PDM-micelles. This creates a situation in which the carboxylic end of linoleic acid is partially obstructed from fully entering the active site of SLOX-1. Therefore, the site of enzymatic catalysis shifts to the 9 position which is closer to the carboxylic end of linoleic acid.

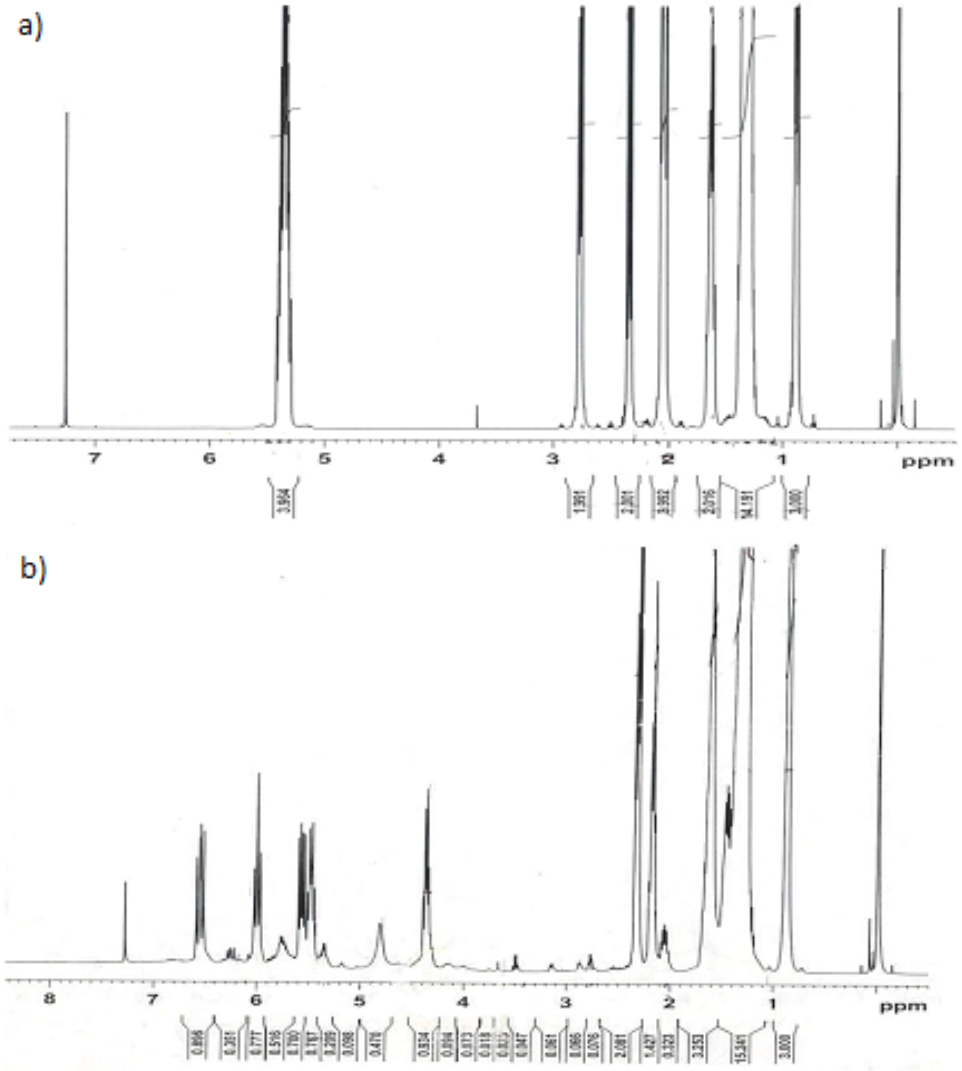


Figure 2.1. Room temperature ^{1}H-NMR spectra of a) linoleic acid and b) linoleic acid hydroperoxide. The peak assignments are as follows: ^{1}H-NMR (300 MHz, CDCl3, δ=7.3 ppm), linoleic acid- 0.9 (-$CH_2C\mathbf{H_3}$, t), 1.2-1.7 (aliphatic C$\mathbf{H_2}$, m), 2.0 (C**H2**CH=CH, q), 2.3 (C$\mathbf{H_2}$COOH, t), 2.8 (=CHC**H2**CH=, t), 5.3-5.4 (C**H**=C**H**, m), linoleic acid hydroperoxide- 0.9 ($CH_2C\mathbf{H_3}$, t), 1.2-1.7 (aliphatic C$\mathbf{H_2}$, m), 2.0 (C**H2**CH=CH, q), 2.3 (C**H**COOH, t), .4.3 (C**H**-OOH, q), 5.3-6.6 (C**H**=C**H**C**H**=C**H,** m). The peak at 2.8 ppm represents in a) the interior CH_2 group between the non-conjugated double bonds of linoleic acid in b) the double bonds shift and become conjugated due to hydroperoxidation and therefore the peak at 2.8 ppm associated with the interior CH_2 group between the double bonds of linoleic acid shrinks to almost zero when converted to linoleic acid hydroperoxide.

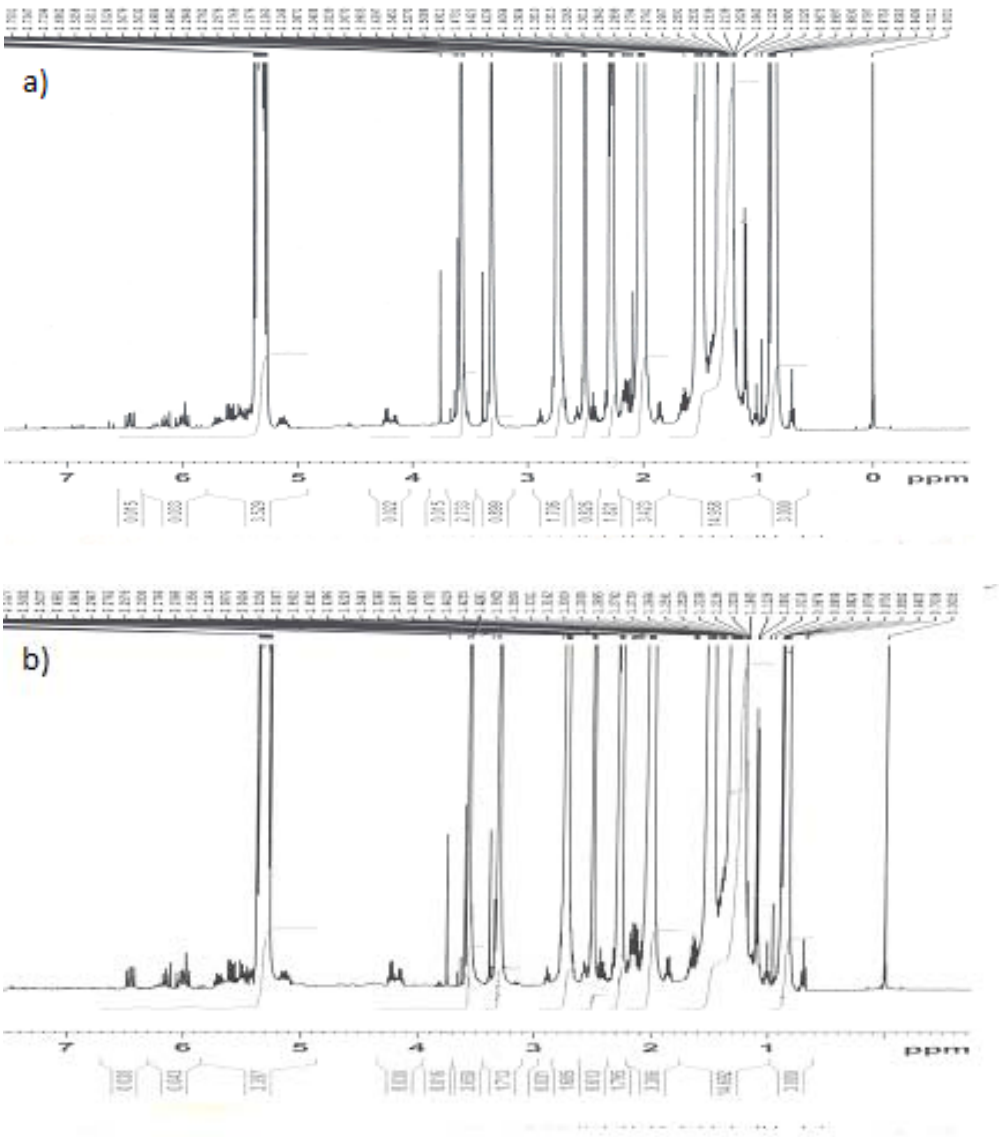


Figure 2.2. Room temperature ^{1}H-NMR spectra of a) methyl linoleate in isooctane no SLOX-1 present and b) methyl linoleate reacted with SLOX-1 in isooctane. The peak assignments are as follows: ^{1}H NMR (300 MHz, CDCl3, δ=7.3 ppm), methyl linoleate- 0.9 (-$CH_2C\mathbf{H_3}$, t), 1.2-1.7 (aliphatic C$\mathbf{H_2}$, m), 2.0 (C**H2**CH=CH, q), 2.3 (-C$\mathbf{H_2}COOCH_3$, t), 2.8 (=CHC**H2**CH=, t), 3.6 (-$CH_2COOC\mathbf{H_3}$, t), 5.3-5.4 (C**H**=C**H**, m). The peak at 2.8 ppm represents in the interior CH_2 group between the non-conjugated double bonds of methyl linoleate. One would observe a significant decrease in this peak from a) to b) if methyl linoleate was being enzymatically converted methyl linoleate hydroperoxide. The spectrum between the base case and that containing SLOX-1 are almost identical, a small amount of autooxidation is taking place observed by the small peaks in the 5.3-6.6 ppm range.

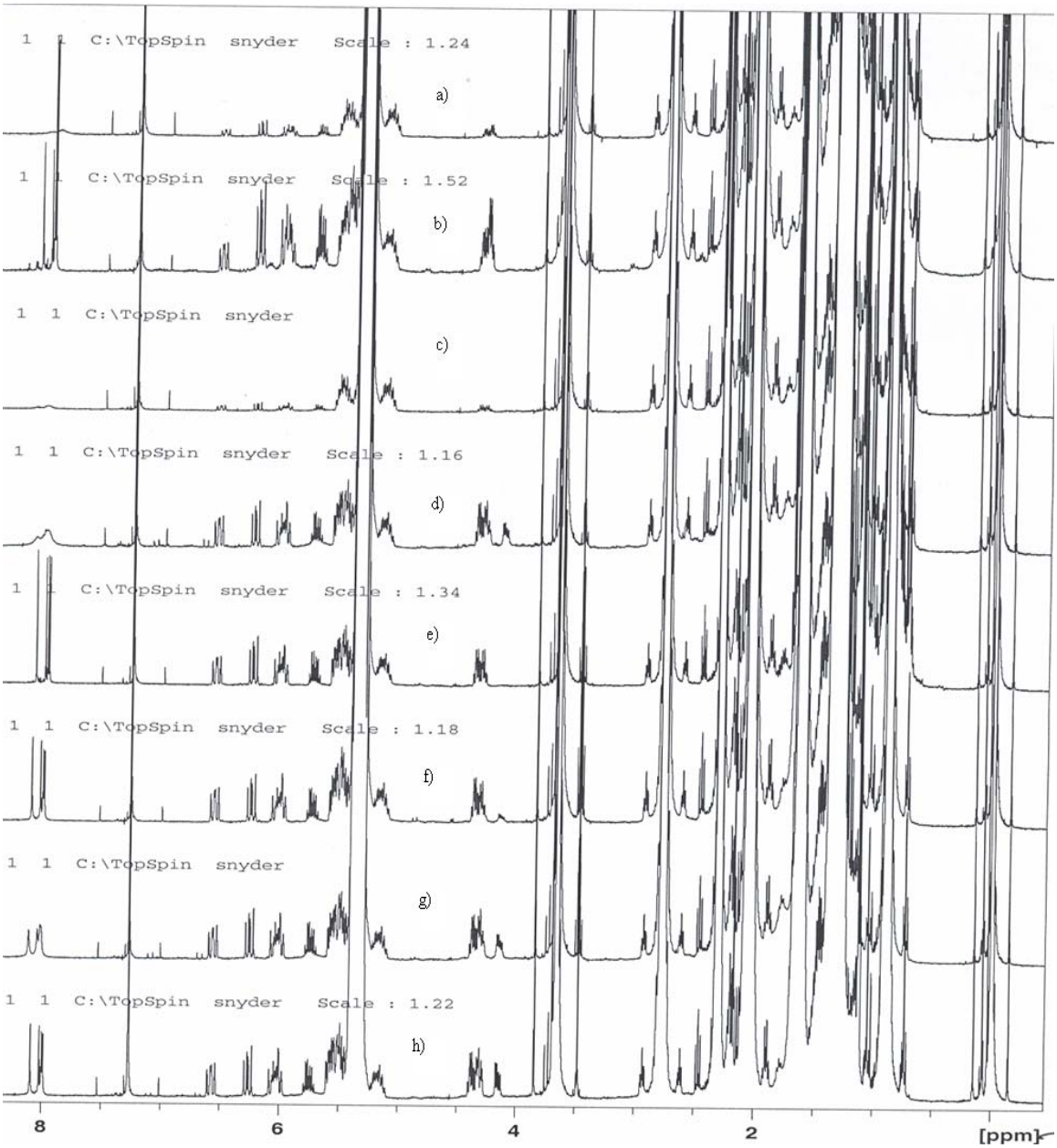


Figure 2.3. Kinetic studies. Room temperature ^{1}H-NMR spectra of methyl linoleate reacted with SLOX-1 in isooctane. a) 10 min reaction time, 10 mg of SLOX-1, b) 30 min reaction time, 5 mg of SLOX-1, c) 10 min reaction time, 5 mg of SLOX-1, d) 2 min reaction time, 5 mg of SLOX-1, e) 18 h reaction time, 200 mg lyoprotected SLOX-1/KCl complex, f) 7 h reaction time, 200 mg SLOX-1/KCl complex, g) 3 h reaction time, 200 mg SLOX-1/KCl complex, h) 1 h reaction time, 200 mg SLOX-1/KCl complex.

CHAPTER 3

EPOXIDATION OF METHYL OLEATE USING HYPOBROMINATION

3.1 Introduction

The goal of this study was to gain a better understanding of the products produced, mechanism, kinetics, and means to optimize production of epoxide as it applies to the hypobromination of soybean oil method previously studied by Jain.[31] The hypobromination method is very similar to the hypochlorination method. The only difference is that a catalytic amount of KBr is added to the reaction mixture. This is done as the hypobromite ion (BrO^-) has been shown to provide higher reaction rates than the hypochlorite ion (ClO^-).

Soybean oil and the many products produced would be far too complex to analyze using GC/MS and would first have to be broken down into FAMEs using transesterification. In addition, the transesterification process could prove to be ineffective as the product compounds produced could be modified by the transesterification process. Therefore, a simpler molecule, methyl oleate, was investigated. This was done as methyl oleate (23% of soybean oil's fatty acid composition) and the complex mixture of products formed during this reaction can be characterized using GC/MS.

3.2 Materials

Methanol, chloroform, carbon tetrachloride, ethyl acetate, dimethylformamide (DMF), tetrahydrofuran (THF), sodium hypochlorite (11-13% available chlorine), and

methyl oleate were all obtained from Sigma-Aldrich and used without further purification. Potassium bromide (KBr), tert-butanol, and potassium dihydrogen phosphate, were obtained from Fischer Scientific. Distilled water was used in all formulations.

3.3 Modification procedure

3.3.1 Synthesis of Epoxidized Methyl Oleate using Hypobromination

KBr (21 mg, 0.176 mmol), aqueous buffer solution at pH 8.0 (0.5 ml, prepared by adjusting a 0.5 M solution of potassium dihydrogen phosphate with 2.0 M sodium hydroxide), sodium hypochlorite at room temperature (0.282 ml, 0.357 g), and tert-butanol at 35°C (0.5 mL) were placed in 1.5 ml vials in that order. The samples were than incubated in a New Brunswick incubator shaker at 35°C for 10 min with a shaking speed of 250 rpm. After 10 min the shaker was switched off with the temperature control still set at 35°C with the vials remaining in the incubator for an additional 2 min to allow time for the phases to separate.

The vials were then immediately removed and methyl oleate at 35°C (35.7 mg, 0.12 mmol) was added to the reaction mixture. The vials were then immediately placed back in the incubator shaker set at 35°C at 250 rpm mixing speed and allowed to react for the allotted reaction time ranging from as short as 1 min to as long as 48 h. Following the desired reaction time, the shaker was switched off with the temperature control still set at 35°C, allowing the vials to remain in the incubator for an additional 2 min to allow time for the phases to separate. The vials were then immediately removed and the top tert-butanol phase containing the products was extracted from the reaction mixture using a

Pipetman and placed in a new 2 ml vial. 1.2 ml of DMF at 35°C was then immediately added to the recovered tert-butanol phase in 0.2 ml increments. 0.6 ml of the newly created tert-butanol/DMF solution was then placed in a GC/MS compliant vial, to which an additional 0.6 ml of DMF at 35°C was slowly added to the mixture in 0.2 ml increments. This was done as no precipitation of products was observed. When diluting the sample further with DMF, adding DMF rapidly, or adding DMF at room temperature, precipitation of products was observed at times. The samples were then analyzed using GC/MS.

In addition, a sample using the hypochlorination method was prepared as a comparison reference in which the above procedure was followed exactly the same way with the only difference being that no KBr was added to the reaction solution. This sample was run for 1 h.

3.3.2 Epoxidized Methyl Oleate characterization

GC/MS spectra were recorded on a Shimadzu GC/MS-QP 5000 (manufactured by Shimadzu Corporation, Kyoto, Japan) operating using a Rtx XTI-5 column (5% diphenyl, 95% dimethyl polysiloxane, manufactured by Restek Corporation, Bellefonte, PA). The injector and interface temperatures were set at 300°C. The column temperature program consisted of the following: 85°C held for 5 min, increase of 10°C/min to 200°C, increase of 2°C/min to 255°C, and finally an increase of 10°C/min to 290°C. The GC/MS full chromatogram for modified methyl oleate using the hypobromination method, 2 min reaction time, is displayed in Figured 3.1. The GC/MS full chromatogram for the modified methyl oleate using the hypochlorination method, 1 h reaction time, is shown in Figure 3.2. The mass spectra for the five major peaks produced from the modified

methyl oleate using the hypobromination method, as well as reference spectra, if available, are displayed in Figures 3.3-3.10.

3.4 Results and Discussion

3.4.1. Epoxidation of Methyl Oleate using Hypobromination

3.4.1.1. Investigation of Reaction Procedure

` The reaction system was devised to maintain the same molar ratios of the various components, as used in the soybean oil modification procedure devised by Jain[31], but the individual reactions were scaled down (500 mg of soybean oil versus 32.7 mg methyl oleate) to minimize the amount of substrate (methyl oleate) used as a large number of reactions needed to be prepared and only small amounts are needed for GC/MS analysis.

There were a few issues that had to be overcome during the course of investigating this reaction. This reaction is sensitive to reaction conditions due to the rapid and complex nature of the reaction. Almost all of the methyl oleate double bonds are consumed and converted to a variety of products within the first few minutes. To overcome this issue a carefully calculated procedure was devised to maintain constant conditions as described in Section 3.3. The second problem was finding a solvent which didn't precipitate any of the products produced. After the reaction was allowed to react for the desired time period, the tert-butanol phase containing the modified methyl oleate products had to be diluted with a solvent in order to reduce the product concentrations to a level that did not produce saturated GC/MS signal intensities. Many solvents were investigated including methanol, chloroform, carbon tetrachloride, ethyl acetate, DMF, THF, and tert-butanol. It was found that all of the solvents except DMF (discovered both

visually and via GC/MS analysis) rapidly precipitated some of the modified methyl oleate products. The best solvent was found to be DMF. Even with DMF it was found that if more than 2 ml of DMF is added, if it was not at 35°C, or if DMF was added rapidly to the recovered tert-butanol phase, products precipitate out of solution.

3.4.1.2 Characterization of the Reaction Products

As seen in Figure 3.1 five major product peaks are observed along with several minor product peaks. The methyl oleate (retention time of 23.4 min) was almost completely consumed after 2 min reaction time.

The product with a retention time of 28.3 min is consistent with the desired methyl-9,10-epoxyoctadecanote (epoxide product). Figures 3.3 and 3.4 display the observed mass spectrum of the proposed product and that of the reference spectra matches closely. The characteristic fragmentation ions for the epoxide product with m/z = 155 and 199 are observed in Figures 3.3 and 3.4. This is the major product being produced in this reaction, and it was found to increase as time increases as expected.

The product with a retention time of 28.6 min is proposed to be methyl-9- and/or 10-oxo-octodecanote (ketone product). There were no reference spectra available that matched the proposed product, but similar structures were found in the NIST62 library and literature. The NIST62 library is software that is provided with the GC/MS-QP 5000 system. It provides the closest matches to the collected spectrum on a percent similarity basis. Figures 3.5 and 3.6 show that the mass spectra observed and that of the reference spectra match quite closely leading to the conclusion that this particular product peak is a ketone product. The collected data does not allow for determination of whether the ketone is being formed in the 9 and/or 10 position.

The product with a retention time of 36.4 min is proposed to be methyl 9,10-dihydroxyoctodecanote (diol product). Figures 3.7 and 3.8 show that a diol product gives a unique mass spectral signature displaying a strong abundance percentage at fragmentation ions m/z = 155 and 187. The fragmentation ion at m/z = 155 results from the splitting of the C-C bond between the 8 and 9 positions, while the fragmentation ion at m/z = 187 is due to splitting of the C-C bond between the two alcohol groups in the 9 and 10 positions.

The product with a retention time of 37.1 min is proposed to be methyl 9-bromo, 10-hydroxyoctodecanoate and/or methyl 9-hydroxy, 10-bromo-octodecanote (bromohydrin product). A reference spectrum for a bromohydrin with a similar structure was not available. Figure 3.9 indicates that the molecular weight of the molecule has to be at least 343 g/mol. This product being a ketone, epoxide, or diol is ruled out because the fragmentation ions observed are larger than the molecular weight of those three compounds. This leads to the conclusion that this product must contain either Cl or Br. Br should give a very characteristic signature as the precise isotropic composition for Br is ^{79}Br 50.5% and ^{81}Br 49.5%. Unfortunately this could not be readily observed in the collected spectra. This is likely due to other fragmentation ions with the same m/z overlapping the fragmentation ions associated with ^{79}Br and ^{81}Br. In the other products mass spectra (Figures 3.3, 3.5, and 3.7) fragmentation ions at 79 and 81, are also found so any fragmentation ions associated with ^{79}Br and ^{81}Br will be masked by these other fragmentation ions. The overall observed abundances in Figure 3.9 are slightly higher at 79 and 81 than in the spectra collected for the other products (Figures 3.3, 3.5, and 3.7), allowing one to propose that the increase in the percent abundance is observed due to the

additional ^{79}Br and ^{81}Br fragmentation ions. Figure 3.9 also displays fragmentation ions at m/z = 93 and 95 which could coincide with the fragmentation ions of $+CH_2$-^{79}Br and $+CH_2$-^{81}Br. The higher relative abundance percentages at m/z = 93 and 95 in Figure 3.9 as compared to the other products spectra (Figures 3.3, 3.5, and 3.7) are observed, allowing again for the proposal to be made that an increase in the percent abundance is observed due to the $+CH_2$-^{79}Br and $+CH_2$-^{81}Br fragmentation ions . Fragmentation of the bromohydrin product removing both the hydroxyl group associated bromohydrin and the $–OCH_3$ group associated with the methyl ester would produce fragmentation ion of m/z of 343, which is the highest fragmentation ion shown in the spectrum in Figure 3.9. Further evidence of Br being present can be found by examining Figure 3.2 which is the GC/MS full chromatogram for the reaction run for 1 h using the hypochlorination method (no KBr present). There is no product peak in close vicinity of 37.08 min leading to the hypothesis that this molecule contains Br.

This narrows the possibilities for the product peak at 37.1 min to either a dibromo or a bromohydrin of methyl oleate. The bromohydrin product would have a molecular weight of 393 g/mol. While a dibromo product would have a molecular weight of 456 g/mol. It would be expected that m/z signals higher than 343 would be observed if a dibromo product were present. Also previous experiments carried out by Klawonn et al. concluded that the bromohydrin species is present in large quantities early in the reaction.[30] All of the above are consistent with a hypothesis that this particular product is the bromohydrin product. The specificity (Br in the 9 or 10 position, and hydroxyl in the 9 or 10 position) cannot be determined from the collected data.

The product at retention time of 37.7 min is unknown. None of the NIST62 library hits gave close matches to what would be an expected potential product for this reaction and no close match in other literature sources were found. Figure 3.10 shows high fragmentation ion abundances at m/z = 155 and 187 which is characteristic of a diol product. It also displays high fragmentation ions abundances at m/z = 171 and 199 which is characteristic of an epoxide product. One possible conclusion is that an epoxide between the 9 and 10 positions and a hydroxyl at the 9 or 10 position is being formed. The problem with this conclusion is this molecule is extremely unlikely to be stable. The fragmentation ions observed for this product display similarity to that of the other products produced, but due to the large differences in relative abundances of each fragmentation ion it is difficult to come to any conclusion as to the exact structure of this molecule.

3.4.1.3. Kinetic Studies and Reaction Mechanism

Figures 3.1 and 3.2 show the impact that the addition of KBr (hypobromination versus hypochlorination) has in increasing the kinetic rate of this reaction. Figure 3.2 shows that a significant amount of methyl oleate (retention time of 23.438 min) is still present at 1h reaction time when using the hypochlorination method, while Figure 3.1 shows that the double bonds of the methyl oleate (retention time of 23.367 min) have almost completely been consumed and converted into a different product within 2 min of reaction time when using the hypobromination method. These results show that the hypobromite ion (BrO^-) plays a critical role in increasing the reaction rate. An increase in the reaction rate of converting the double bond in methyl oleate to a complex mixture of

products is observed when comparing with the results found when only the hypochlorite ion (ClO^-) is present.

The reaction kinetics for the epoxidation of methyl oleate using the hypobromination method were followed for the time period of 1 min to 24 h. The results are displayed in Figures 3.11-3.13. The percent composition of the products was determined by integrating the areas of the products, and dividing by the total integrated area. The percent compositions calculated are based on percent signal not molar percentages and based only on relative changes in integrated signal intensities. As a result they provide a semi-quantitative understanding of the overall kinetics of this reaction. As discussed previously, there were five major products produced, four of which have been determined with one being an unknown. Minor products produced were summed together as a group, since they were insignificant on an individual level as far as the overall kinetics of reaction are concerned.

The double bonds of the methyl oleate are completely consumed and converted to a diol, epoxide, bromohydrin, ketone, unknown, or minor products within the first few min of reaction. As time increases the ketone and diol products initially produced are almost completely consumed (0-2% percent composition at 24 h), while a significant increase in epoxide product formation are observed from time = 2 min to time = 24 h as shown in Figures 3.11-3.13. After 19 h reaction time the yield of epoxide product was 69.4%, while for 24 h it was 70%. After that time no changes in the percent compositions of the products are observed. Samples reacted for 48 h displayed 70% conversion but were not included in the kinetic figures. It is unlikely that the ketone product is being directly converted to the epoxide product, and is proposed to be in

equilibrium with the diol product which is then converted to the epoxide product after a H_2O molecule has been removed.

The bromohydrin and unknown products remained essentially constant throughout the course of this reaction with minimal variation from 2 min to 24 h as shown in Figure 3.11-3.13. Based on these findings the bromohydrin and unknown product were proposed to have negligible impact on the formation of epoxide.

The minor products displayed a slight decrease over the time course of this reaction. For the minor products: at time = 2 min percent composition is 9%; at time = 24 h percent composition is 4.7%. It was also observed that there was minor amount of cleavage of methyl oleate between the 9 and 10 positions of methyl oleate forming two smaller sized alkenes at longer time periods which is also included in the minor products but this only makes up about 1-2% of the overall composition. Conversion of the minor products as a group to the epoxide product over time is minimal and was treated as negligible.

The above observations are in contrast to that reported by Klawonn et al. and Jain as in their reactions they proposed that a large amount of bromohydrin was initially being formed and was then converted into epoxide over the course of time.[30-31] Klawonn et al. were investigating shorter chain substrates and this could be a cause for the disagreement. It was found using GC analysis that for the substrate α-methylstyrene a greater than 95% conversion to 2-phenyl-2-hydroxy-1-propyl bromide (bromohydrin) is observed within the first minute. This bromohydrin product is then converted to the desired epoxide product with a maximum yield of 83% found between 30 and 60 min. In α-methylstyrene the double bond is located in an exterior position. In methyl oleate the

double bond is embedded within two long alkane chains. Klawonn et al. studied other substrates including 5-decene, the most similar substrate structurally to methyl oleate. In that reaction they reported that the reaction rate to epoxide product decreased (in relation to α-methylstyrene) and a maximum yield of 75% to the desired epoxide product was observed at 24 h reaction, on par with the results observed using methyl oleate as a substrate. However, the reaction mechanism for 5-decene was not studied and their mechanisms were assumed to be similar to that observed for α-methylstyrene.

When using the substrate methyl oleate, it was found that the bromohydrin product remained at a constant level throughout the time course of the reaction. After the initial rapid consumption of the methyl oleate double bond producing a complex mixture of products, it is the diol and ketone products formed which are then converted to desired epoxide product to obtain a 70% yield of desired methyl 9,10 epoxyoctadecanote at 24 hours reaction time. The ketone is proposed to be in equilibrium with the diol product, and the diol product is converted to an epoxide after losing a H_2O molecule. A generalized schematic for the mechanism of the epoxidation of methyl oleate via hypobromination is proposed in Figure 3.14.

3.5 Conclusions

The epoxidation of methyl oleate using the hypobromination method was studied to gain a better understanding of the products formed, mechanism, kinetics, and means to increase yield of epoxide as it applies to soybean oil modification to produce ESBO. It was observed that the hypobromite ion (BrO^-) plays a significant role in increasing the rate of conversion of the methyl oleate double bonds in comparison to when only the hypochlorite ion (ClO^-) is present. Literature results suggest that a large amount of

bromohydrin product is initially produced in the reaction which was then converted to the desired epoxide product over the course of time. In the case of methyl oleate, using GC/MS analysis, it was observed that the bromohydrin product remains unchanged throughout the time course of reaction, and it is the diol and ketone products formed which are converted to the desired epoxide product over the course of 24 h to yield 70% epoxide product. It is proposed that the ketone product does not directly convert into epoxide product, but rather it is in equilibrium with the ketone product which is then converted to the desired epoxide product after losing a H_2O molecule. These findings give a better understanding of the kinetics and mechanism involved in the hypobromination of long chain alkenes such as soybean oil. It is expected these results will aid in the development of processes to increase epoxide yields using the hypobromination method in long chain alkenes such as soybean oil.

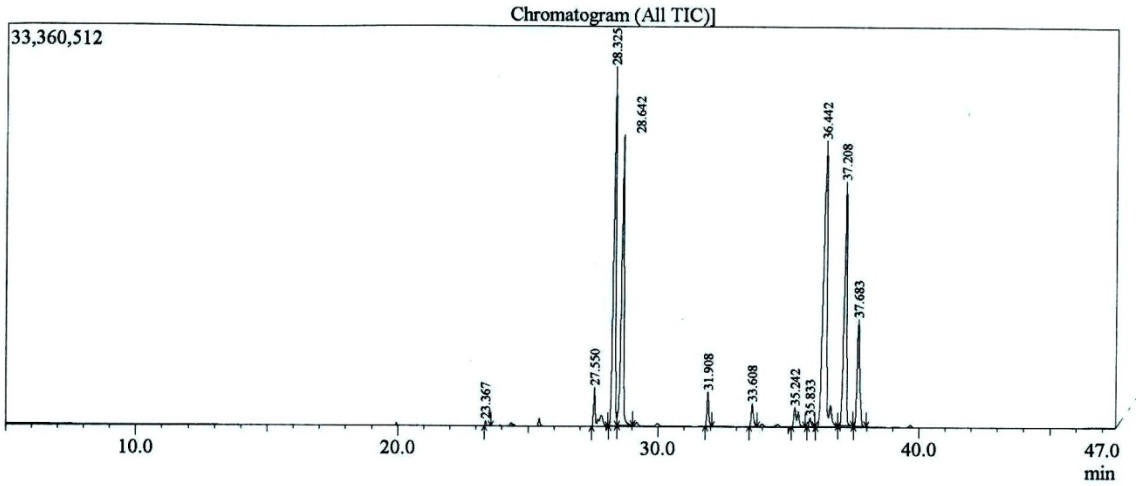


Figure 3.1. GC/MS full chromatogram of modified methyl oleate using the hypobromination method after 2 min reaction time at 35°C. The reaction was performed by agitating a mixture containing 21 mg KBr, 0.5 M potassium phosphate with 1M NaOH added till a pH of 8 was reached, 0.5 ml tert-butanol, 0.284 ml sodium hypochlorite (11-13% available chlorine), and 35.7 mg methyl oleate. Proposed identities of the major product peaks observed are as follows: 28.3 min = epoxide product, 28.6 min = ketone product, 36.4 min = diol product, 37.2 min = bromohydrin product.

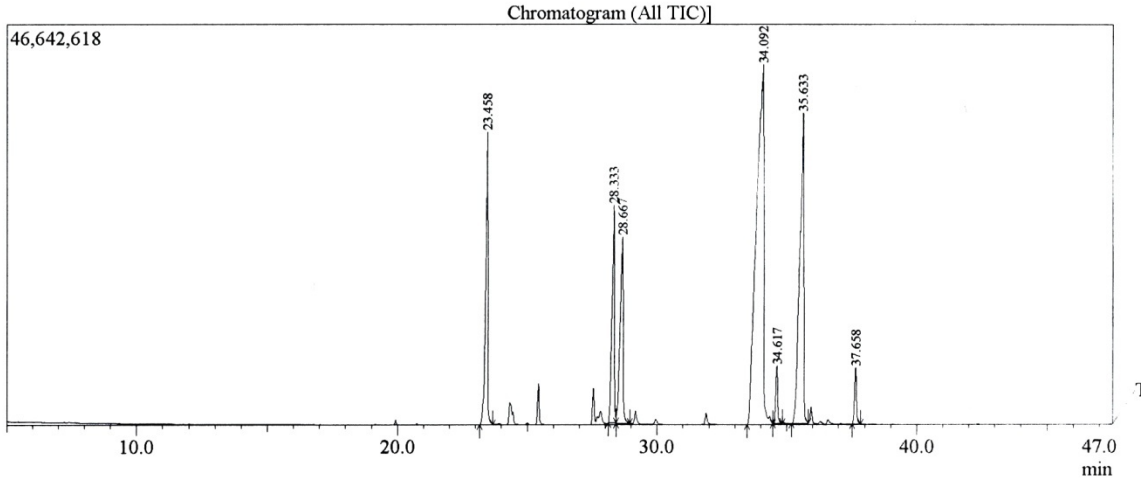


Figure 3.2. GC/MS full chromatogram of modified methyl oleate using the hypochlorination method after 1 h reaction time at 35°C. The reaction was performed by agitating a mixture containing 0.5 M potassium phosphate with 1M NaOH added till a pH of 8 was reached, 0.5 ml tert-butanol, 0.284 ml sodium hypochlorite (11-13% available chlorine), and 35.7 mg methyl oleate.

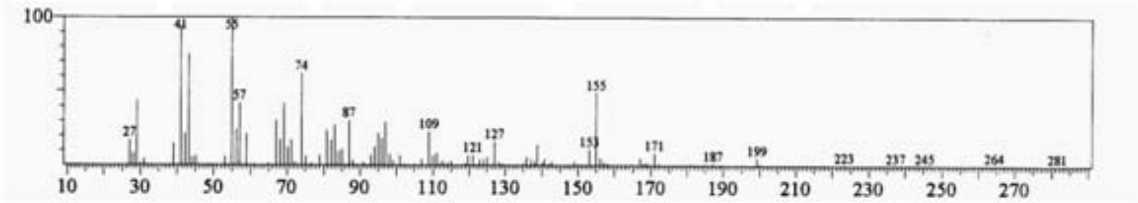


Figure 3.3. Mass spectrum of product with retention time of 28.3 min.

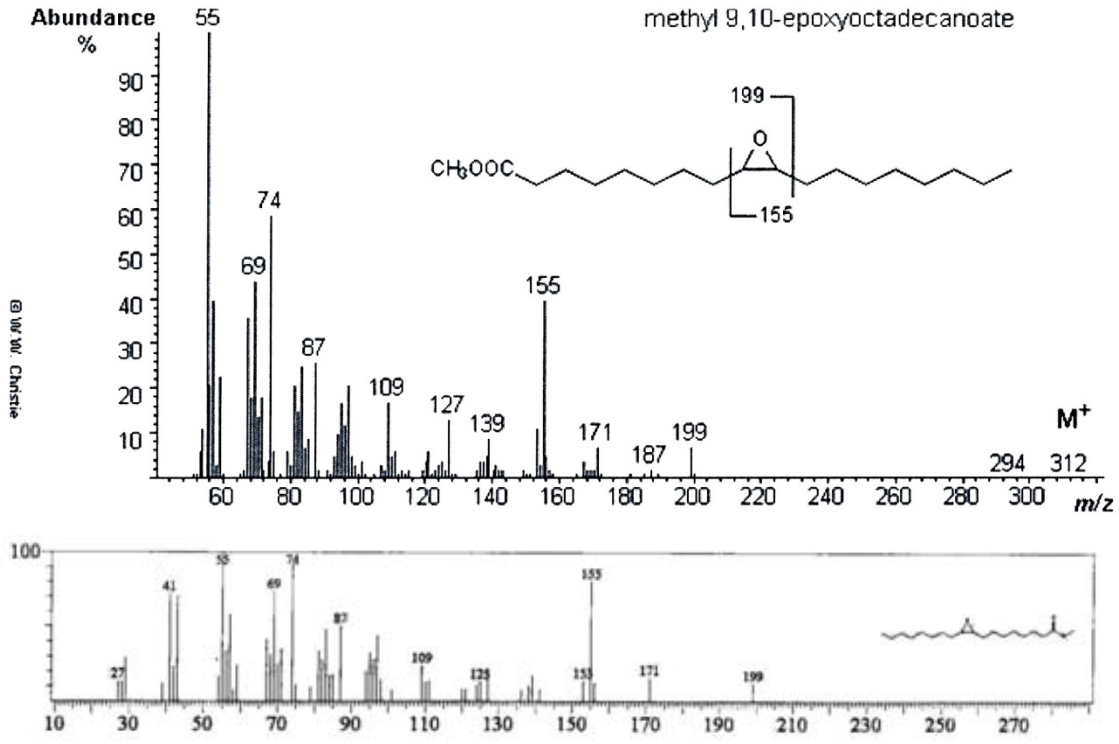


Figure 3.4. Reference mass spectra for the product with retention time of 28.3 min.

Source: (Top) Christie, W.W., *The Lipid Library*, <http://lipidlibrary.aocs.org>, The American Oil Chemists' Society (2010). (Bottom) NIST62 library Shimadzu Corporation.

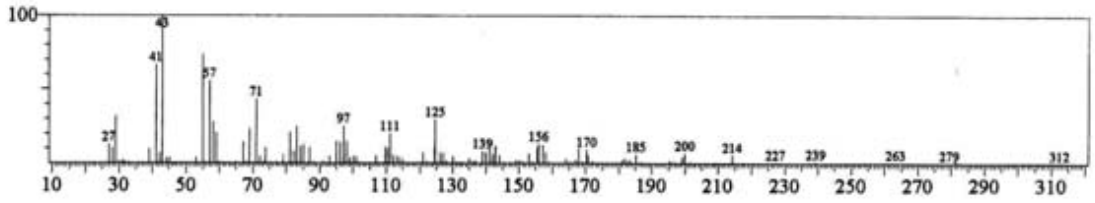


Figure 3.5. Mass spectrum for product with retention time of 28.6 min.

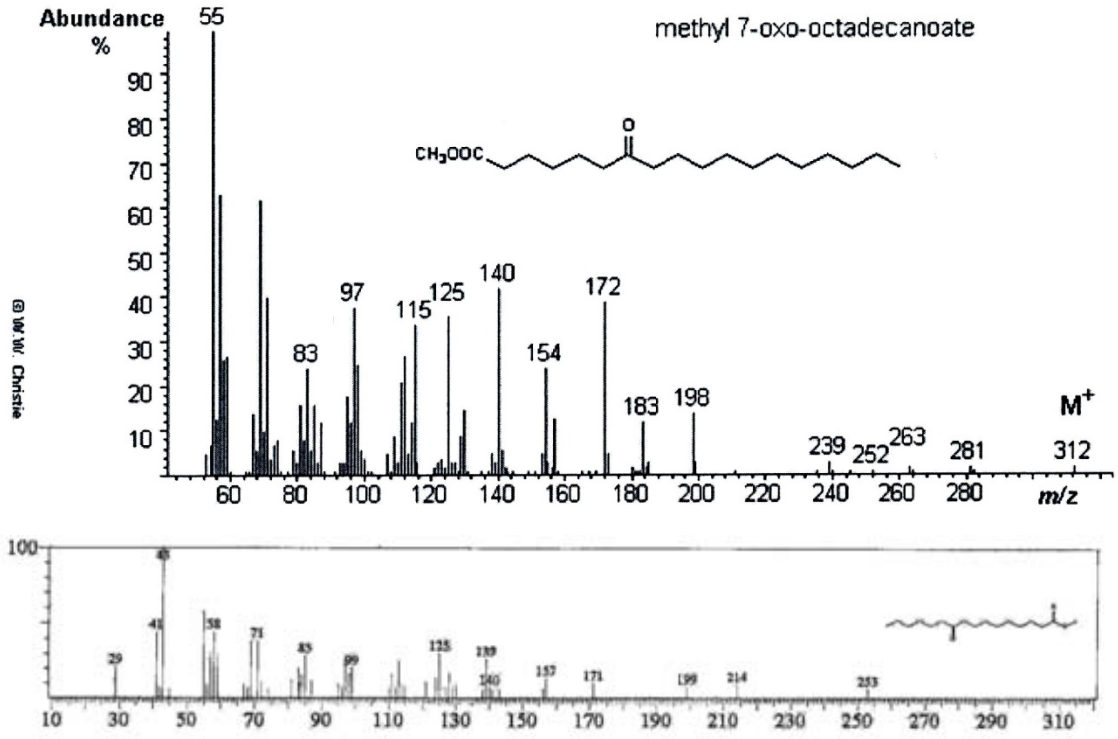


Figure 3.6. Reference mass spectra for the product with retention time of 28.6 min.

Source: (Top) Christie, W.W., *The Lipid Library*, <http://lipidlibrary.aocs.org>, The American Oil Chemists' Society (2010). (Bottom) NIST62 library Shimadzu Corporation.

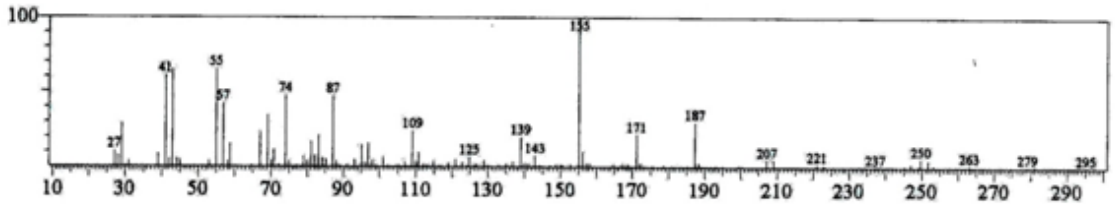
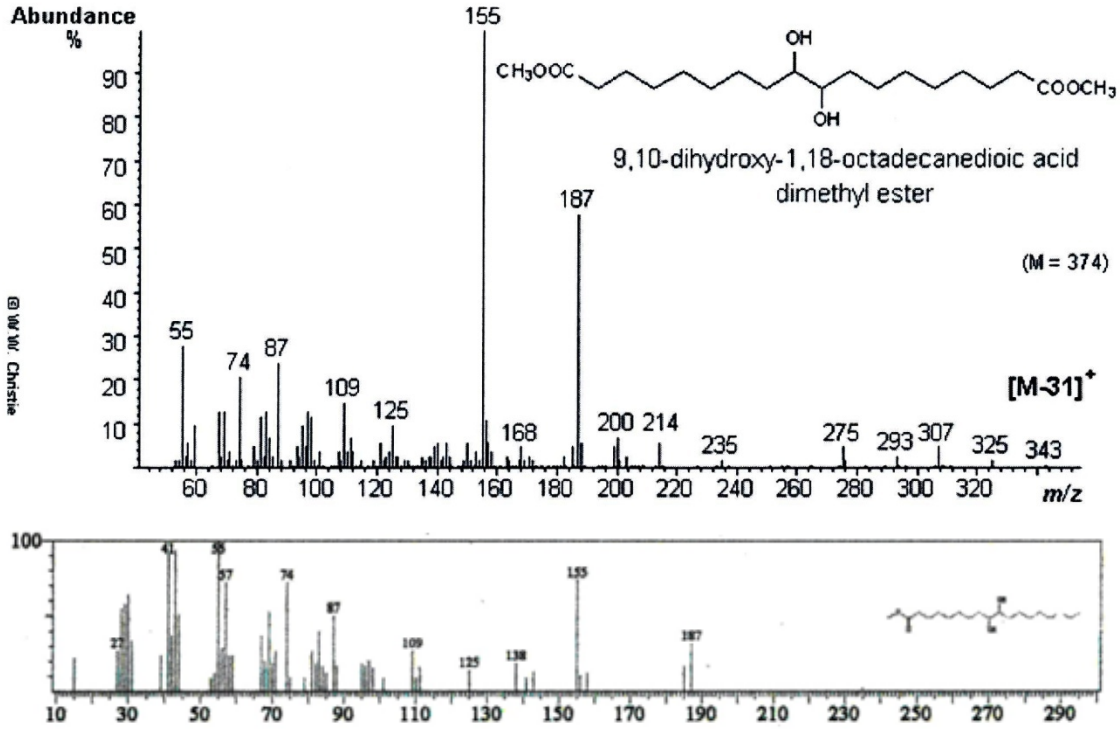


Figure 3.7. Mass spectrum for product with retention time of 36.4 min.



3.8. Reference mass spectra for the product with retention time of 36.4 min.

Source: (Top) Christie, W.W., *The Lipid Library*, <http://lipidlibrary.aocs.org>, The American Oil Chemists' Society (2010). (Bottom) NIST62 library Shimadzu Corporation.

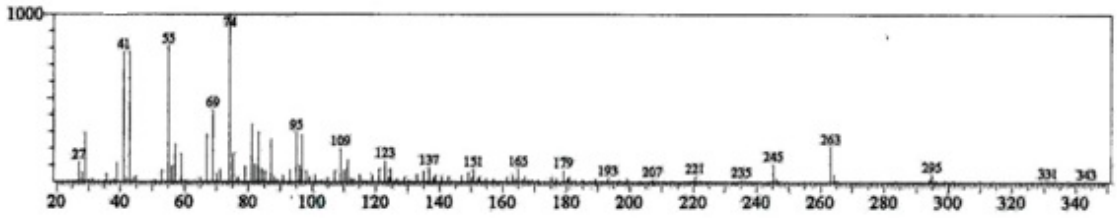


Figure 3.9. Mass spectrum of product with retention time of 37.2 min.

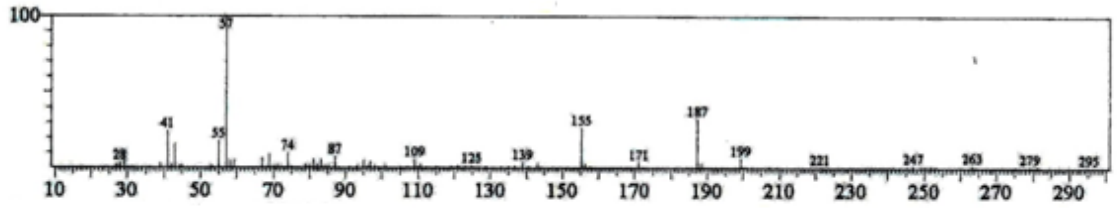


Figure 3.10. Mass spectrum of product with retention time of 37.7 min.

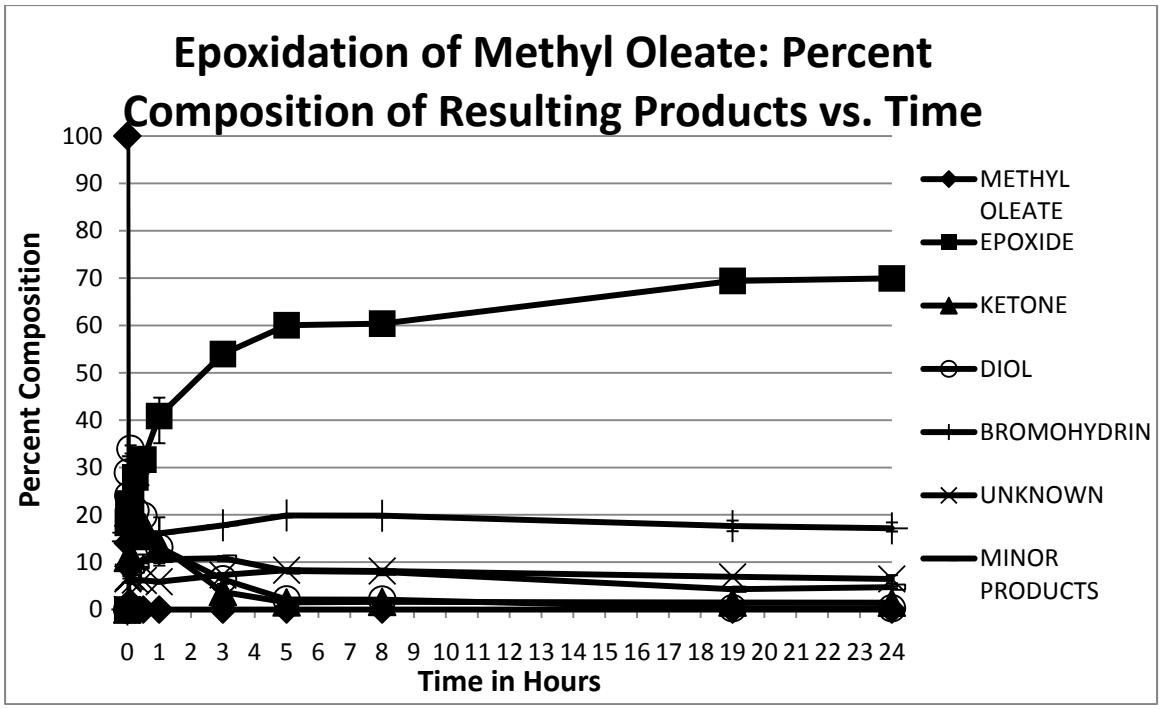


Figure 3.11. Kinetics of the epoxidation of methyl oleate using the hypobromination method: percent composition of resulting products vs. reaction time varying from 1 min to as high as 24 h at 35°C. The reaction mixture contained 21 mg KBr, 0.5 M potassium phosphate with 1M NaOH added till a pH of 8 was reached, 0.5 ml tert-butanol, 0.284 ml sodium hypochlorite (11-13% available chlorine), and 35.7 mg methyl oleate. The percent composition was determined by integrating the numerous product peaks and divided by the total area. Three samples were taken at each time point with error bars are shown. Minor products are the sum of all other minor peaks found on the GC/MS chromatogram.

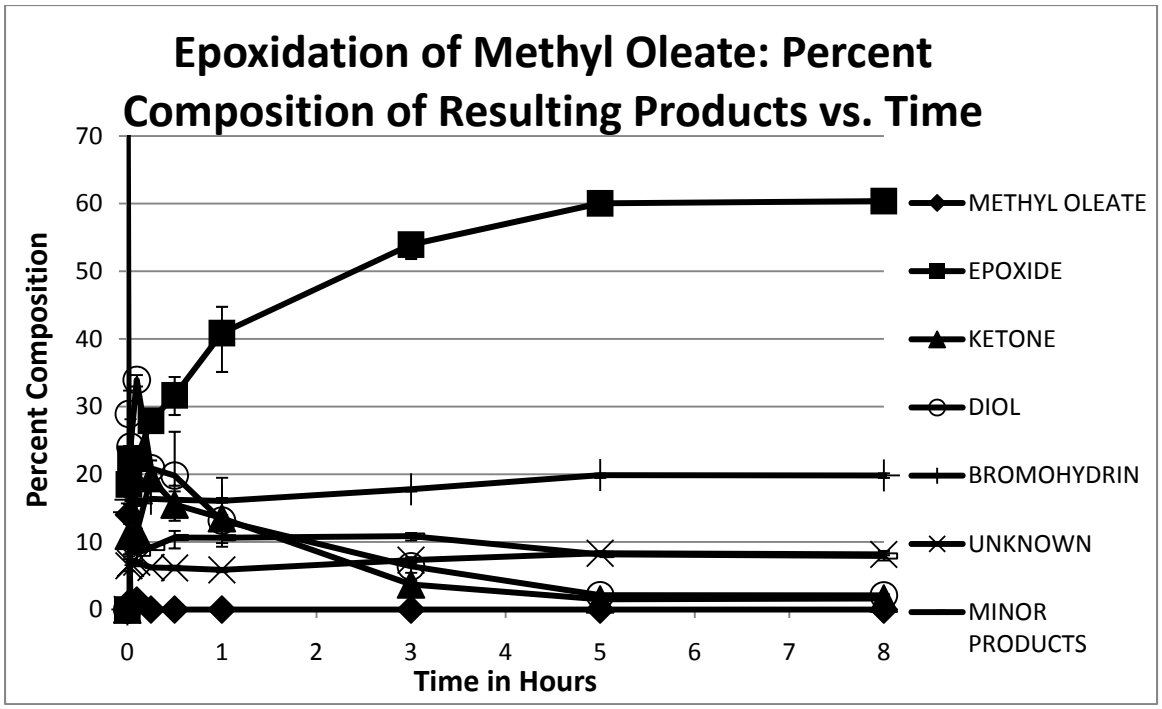


Figure 3.12. Kinetics of the epoxidation of methyl oleate using the hypobromination method: percent composition of resulting products vs. time varying from 1 min to as high as 5 h at 35°C. The reaction mixture contained 21 mg KBr, 0.5 M potassium phosphate with 1M NaOH added till a pH of 8 was reached, 0.5 ml tert-butanol, 0.284 ml sodium hypochlorite (11-13% available chlorine), and 35.7 mg methyl oleate. The percent composition was determined by integrating the numerous product peaks and divided by the total area. Three samples were taken at each time point and error bars are shown. Minor products are the sum of all other minor peaks found on the GC/MS chromatogram.

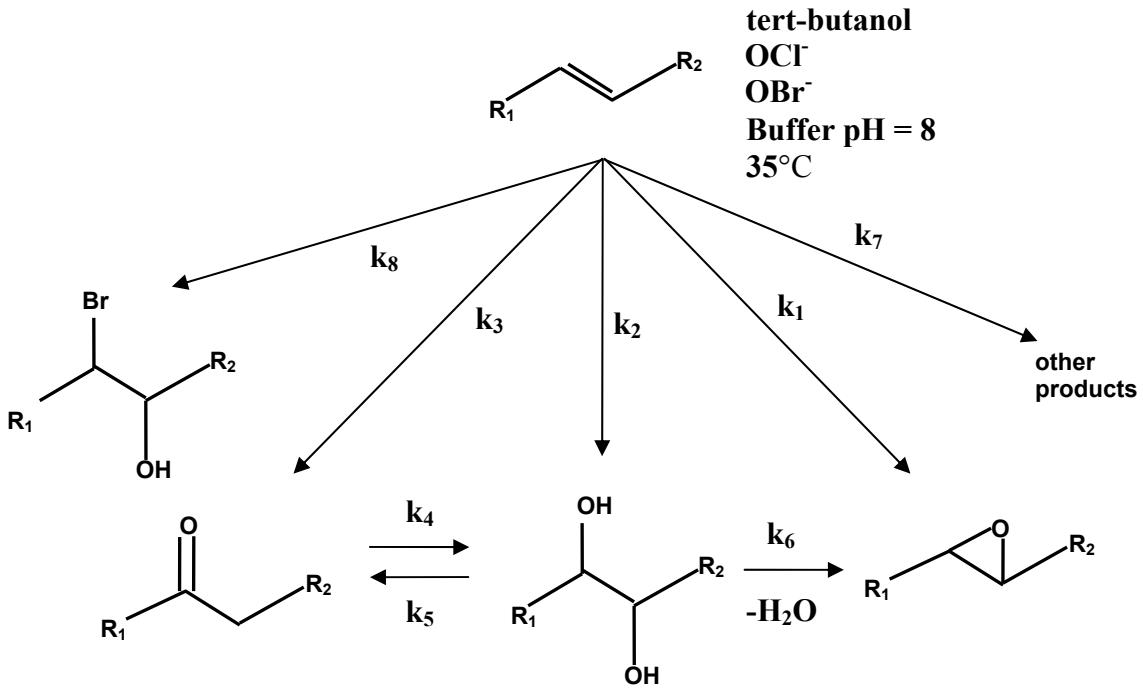


Figure 3.13. Generalized reaction mechanism for the epoxidation of methyl oleate using the hypobromination method. Very rapidly (first 2 min) the double bonds of methyl oleate are almost completely consumed and converted into either an epoxide, ketone, diol, bromohydrin, or other products. From time = 2 min to time = 24 h, 90% of the increase in epoxide product to the final yield of 70% results due to conversion of the diol and ketone products into the desired epoxidized product. It is proposed that the ketone and diol products are in equilibrium, and the ketone is not directly converted to epoxide product but rather the diol product is converted to an epoxide after losing a H_2O molecule. $R_1 = C_9H_{17}O_2$, $R_2 = C_7H_{15}$.

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

4.1 Summary of Research

Enzymatic and chemical modifications of fatty acid methyl esters were investigated. The enzyme SLOX-1, for which linoleic acid is a known substrate, was used to attempt to modify methyl linoleate to a desired hydroperoxide product in organic media. The resulting hydroperoxide product, if formed, could then be easily reduced to a monool using the reducing agent triphenyl phosphine. This product would have industrial applicability, if the devised reaction system could be used produce this product in significant quantities. The aqueous enzymatic conversion of linoleic acid to contain a monoperoxide is not a viable process on an industrial scale as linoleic acid is sparingly soluble in an aqueous environment, conversion is hindered by the amount of molecular oxygen present in solution, and both a strong acid and ether are needed to recover the resulting product. Methyl linoleate is highly soluble in organic media, and enzymes have been shown to have effective activities in organic solvent especially when they are first lyoprotected prior to exposure to organic media.[10-12] The solvent isooctance was chosen as an ideal solvent as it affords high dissolved molecular oxygen concentrations, which is necessary to make the SLOX-1 enzymatic modification procedure successful.

Unfortunately enzymatic modification of methyl linoleate to the resulting hydroperoxide product was not achieved. A wide variety of solvents (other than isooctance) and a two phase solvent system were attempted to no avail. Studies were also performed to ensure SLOX-1 was not being completely deactivated due to exposure to organic media. These results, however, provide further evidence that the carboxylic acid group of the fatty acid plays a necessary role in obtaining conversion to the desired hydroperoxide product.

Chemical means to modify methyl oleate were also investigated, more specifically the hypobromination of methyl oleate. This was done in order to gain a better understanding of the products produced, mechanism, kinetics, and ways to increase yields of the epoxide product as it applies to the hypobromination of soybean oil. Results displayed that the hypobromide ion (BrO^-) plays a crucial role in increasing the reaction rate of the conversion of the methyl oleate double bond into products when compared to that of the hypochlorination method when only the hypochloride ion (ClO^-) is present.

Five major products were produced along with several minor products when using the hypobromination method to modify the substrate methyl oleate. The products produced consisted of a mixture of diol, ketone, epoxide, bromohydrin, unknown, and minor products. After the initial rapid conversion of the methyl oleate double bond (first two minutes) to a complex mixture of products, the diol and ketone products initially produced (significant quantities formed at short time periods) are then converted to the desired epoxide product at longer reaction times. It is proposed that the ketone product does not directly convert into epoxide product, but rather it is in equilibrium with the diol product which is then converted to the desired epoxide product after losing a H_2O molecule. The bromohydrin product formed was found to stay steady throughout the time course of the reaction. A proposed mechanism for this reaction is displayed in Figure 3.14. This is in contrast with results reported by Klawonn et al. when using α-methylstyrene as a substrate.[30] For α-methylstyrene Klawonn et al. found that a bromohydrin product is formed in large quantities (95%) at short time periods (first minute) and is than converted to the desired epoxide product over the course of time (30 to 60 minutes to reach maximum yield of epoxide). Differences in the mechanism and kinetics of the reaction could be due to the difference in substrates being used. In the case of α-methylstyrene the double bond is located in an exterior position, while for methyl oleate the double bond is embedded between long alkane chains. The yield of the desired 9,10-epoxyoctadecanote product was 70% at 24 hours reaction time.

4.2 Recommendations for Future Work

Future studies involving the use of SLOX-1 to produce higher value products should focus on using linoleic acid as a substrate since methyl linoleate is not a viable substrate. As discussed previously, there are many obstacles concerning applicability to industry when using the typical aqueous environment. BioResearch Products (North Liberty, Iowa), which kindly provided SLOX-1, showed great interest in finding a use for SLOX-1 as it is cheap and readily available so there is definitely potential in this area of research.

The focus of future research in this area should investigate the use of non-aqueous media and/or a two phase (aqueous and organic) system in order to attempt to find a solvent system which affords a high solubility of linoleic acid. Common organic solvents, such as isooctane, ethyl acetate, and acetonitrile, were attempted but linoleic acid was found to be insoluble in these solvents. When choosing a solvent system the toxicity of the solvent chosen needs to be taken into consideration. Past experiments involving the use of Novoyzme 435 in pyridine to attempt to modify complex carbohydrates such as starch (which is insoluble in most organic solvents) found that Novozyme 435 readily loses its activity due to its exposure to pyridine.

Further investigation into the hypobromination of methyl oleate is needed to provide more concrete evidence of the products produced. Standards such as the diol and bromohydrin of methyl oleate should be synthesized. The diol can be made using osmium tetroxide, while the bromohydrin can be made using N-bromosuccinimide. These standards when produced can be used to give both further proof of identity of the products, as well as be used to determine more accurately the quantitative amounts of these products in solution. After collecting the necessary quantitative data, the rate constants (k_1 through k_8) for the mechanism displayed in Figure 3.14 as well as kinetic rate equations could be developed in order to quantitatively access the kinetics of this

reaction. Also it would be ideal to indentify the fifth major product produced in this reaction, which is currently unknown. ^{1}H-NMR spectroscopy should be employed to determine more accurate quantification of epoxide yield. When using GC/MS to determine yield, the assumption made is that the products produced will give similar relative integrated areas.

The ultimate goal of investigating the hypobromination of methyl oleate, beyond the kinetics and mechanism of the reaction, is to determine ways to optimize the yield of epoxide as it applies to the epoxidation of soybean oil. Varying the pH of the solution by using different buffered media is a possible way to meet this end. Klawonn et al. found that varying the pH did not affect the overall yield when using α-methylstyrene as a substrate.[30] However, since the observed reaction mechanism when using methyl oleate as a substrate appears to be different, varying the pH could have a positive effect on the yield of epoxide product. The oxirane ring closure of the diol product to form an epoxide product is pH dependent. At high pH the diol product is favored, while at a lower pH the epoxide product is favored. The optimal KBr concentration for the epoxidation of soybean oil using the hypobromination method has previously been determined by Jain so varying the KBr concentration is unlikely to have any positive influence on epoxide yield, and will not be studied.[31]

Another recommendation is to attempt to form a large amount of diol product at short time periods, which can then be converted to the desired epoxide product as the time course of the reaction progresses. It is known that diols are more stable under basic conditions and proceeds to close in an oxirane ring to form the epoxide product under more acidic conditions. Therefore, a hypobromination system which has a high pH at short time periods with pH being decreased over the course of time could possibly produce a higher yield of desired epoxide product. This could be accomplished by beginning the reaction at an elevated pH and then adding an acidic compound to the reaction solution after being allowed to react for a few minutes. There could be some

possible complications with this procedure as it applies to the substrate methyl oleate, as it is believed that the main product precipitating out of solution is the diol product. Increasing the amount of diol formed initially in the reaction could precipitate product from the tert-butanol phase. This recommendation may be more applicable to the soybean modification procedure where a three phase system is present, in which the soybean oil and the products produced are insoluble.

REFERENCES

1. Piazza G.J., Foglia T.A., and Nunez A., *Journal of the American Oil Chemists Society*, 76, 551 (1999)

2. Piazza G.J., Foglia T.A., and Nuñez A., *Journal of the American Oil Chemists Society*, 74, 1385 (1997)

3. Schilstra, M.J., Veldink, G.A., Verhagen, J., Vliegnthart J.F.G., *Biochemistry*, 31, 7692 (1992)

4. Martini, D., Buono, G., Montillet, J., et al., *Tetrahedron : Asymmetry*, 7, 1489 (1996)

5. Boyington J.C., Gaffney B.J., and Amzel L.M., *Science*, 260, 1482 (1993)

6. Rickert K.W., and Klinman J.P., *Biochemistry*, 38, 12218 (1999)

7. Schilstra M.J., Veldink G.A., and Vliegenthart J.F.G., *Biochemistry*, 33, 3974 (1994)

8. Ruddat V.C., Mogul R., Chorny I., et al., *Biochemistry*, 43, 13063 (2004)

9. Began G., Sudharshan E., and Rao A.G.A., *Biochemistry,* 38, 13920 (1999)

10. Klibanov, A.M., *Nature*, 409, 241 (2001)

11. Klibanov, A.M., *Current Opinion in Biotechnology*, 14, 427 (2003)

12. Zaks, A., Klibanov A.M., *The Journal of Biological Chemistry*, 263, 8017 (1988).

13. Jaakkola, J.J.K., and Knight, T.L., *Environmental Health Perspectives*, 116, 845 (2008).

14. Larsson, M., Weiss, B., Janson, S., Sundell, J., and Bornehag, C.G., *Neurotoxicology*, 30, 822 (2010).

15. Crivello, J.V., Narayan, R., Sternsteain, S.S., *Journal of Applied Polymer Science*, 64, 2073 (1997).

16. Adhvarya, A., Erhan, S.Z., *Industrial Crops and Products*, 15, 247 (2002)

17. Can, E., Kuseufoglu, S., Wool, R.P., *Journal of Applied Polymer Science*, 81, 69 (2001).

18. Sack, M., Wohlers, H.C., *Journal of the American Oil Chemists' Society*, 36, 623 (1959)

19. Kamata, K., Yonehara, K., Sumida, Y., Yamaguchi, K., Hikichi, S., Mizunol, N., *Science*, 300, 964 (2003)

20. Rangarajan, B., Havey, A., Grulke, E.A., Culnan, P.D., *Journal of the American Oil Chemists' Society*, 72, 1161 (1995)

21. Wiebe, M. Kuemmel, J., Pruess, J., Warnecke, H.J., *Lipid*, 100, 404 (1998)

22. Sinadinovic-Fiser, S., Jankovic, M., Petrovic, Z.S., *Journal of the American Oil Chemists' Society*, 78, 725 (2001)

23. Traylor, T.G., Tsuchiya, S., Byun, Y., Kim, C., Journal *of American Chemical Society*, 115, 2775 (1993)

24. Shu, L., Shi, Y., *Tetrahedron*, *57*, 5213 (2001)

25. Lane, B.S., Vogt, M., DeRose, V.J., Burgess, K., *Journal of American Chemical Society*, 124, 11946 (2002)

26. S. Krishnan, D. G.Kuhn, G. A. Hamilton, *Journal of American Chemical Society* 99, 8121 (1977).

27. Amatsu, H., Miyamoto, T.K., Sasaki, Y., *Bulletin of the Chemical Society of Japan*, 61, 3193 (1988)

28. Collman, J.P., Brauman, J.I., Hampton, P.D., Tanaka, H., Bohle, D.S., Hembre, R.T., *Journal of American Chemical Society*, 112, 7980 (1990)

29. Monti, D., Pastorini, A., Mancini, G., Borocci, S., Tagliatesta, P., *Journal of Molecular Catalysis A: Chemical*, 179, 125 (2002)

30. Klawonn, M., Bhor, S., Mehltretter, G., Dobler, C., Fischer, C., Beller, M., *Advanced Synthesis and Catalysis*, 345, 389 (2003)

31. Jain, K., *Applications of Microemulsions in Producing Monomers and Polymers: Epoxidation of Soybean Oil and Design of Polymers with Regulated Molecular Weight and Specified End-Groups*., Ph.D. Thesis, University of Iowa (2005)

32. Christie, W.W., *The Lipid Library*, <http://lipidlibrary.aocs.org>, The American Oil Chemists' Society (2010)