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Bacillus subtilis is a model organism and the entire genome has been sequenced. However, there is an incomplete understanding of the metabolic pathways that are encoded within the genome. A *long-term goal* of the Reddick research group is to gain a more complete understanding of the *mmg* operon. The *overall objective* is to complete the syntheses of 2-methylisocitrate and develop a stereoselective synthesis of 2-methylcitrate. The *central hypotheses* of these projects are that MmgF cleaves one of the enantiomers of 2-methylisocitrate to produce succinate and pyruvate, and MmgD 2-methylcitrate synthase produces heterochiral ((2*S*-3*R*) or (2*R*,3*S*))-2-methylcitrate. The completed syntheses will facilitate new investigations furthering the understanding of the mother cell metabolic (*mmg*) operon. The synthesis of (2*RS*, 3*RS*)-2-methylisocitrate has been completed with modified literature methods, and the synthesis of (2*RS*, 3*SR*)-2-methylisocitrate has required multiple modifications in order to make the synthesis reproducible. Previous graduate students either were unable to finish the synthesis or experienced reproducibility problems with this synthesis. The stereoselective synthesis of 2-methylcitrate has been designed and the initial steps in the synthesis have begun. The progress made in these two projects will allow the Reddick research group to reach its long-term goal of obtaining more information and a better understanding of *Bacillus subtilis* and the *mmg* operon.

TOWARDS THE STEREOSELECTIVE SYNTHESES OF 2-METHYLCITRATE AND 2-METHYLISOCITRATE AND THEIR USE IN STUDYING THE METHYLCITRIC ACID CYCLE IN *BACILLUS SUBTILIS*

by

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CHAPTER I

INTRODUCTION

1.1 Review of Literature

1.1.1 Overview and Applications of *Bacillus subtilis*

Bacillus subtilis is an aerobic, rod shaped, Gram positive bacterium[1] that is industrially relevant, and used as a model organism. This can be attributed to the entire genome being sequenced[1] and its ability to undergo genetic modifications.[2] *B. subtilis* is also used to study the bacterial sporulation process.[3] *B. subtilis* inhabits different environmental conditions such as soil, within the gastrointestinal tract of specific animals,[4] and among decomposing organic matter.[4] *B. subtilis* is considered to be an aerobic bacterium, but it has been shown to function as a facultative anaerobic in the presence of nitrates.[5]

The genome of *B. subtilis* consist of over 4 million base pairs which encode for 4,100 proteins.[1] When used in industry the genetic sequence can be modified for the production of polyhydroxyalkanoates,[6] biosurfactants,[7] hyaluronic acid,[8] and bacitracin.[9] *B. subtilis* is also used in agriculture to inoculate soil.[10] The industrial efficacy of *B. subtilis* could be increased by obtaining a better understanding of the sporulation process.

1.1.2 Summary of Sporulation

B. subtilis and other rod-shaped bacteria undergo sporulation when environmental conditions are less than favorable for survival.[3,6,11] Some of these conditions include, but

are not limited to, a lack of oxygen, glucose, nitrogen, and phosphorus. The sporulation process is initiated by the *spo* genes and is regulated by transcription factors located with the developing spore and the mother cell.[3,11]

During periods of nutritional or environmental stress, *B. subtilis* will undergo an asymmetric division.[3,11] The bacterium will undergo several distinct morphological and physiological changes. During stage I, the bacterium experiences environmental stress and a cleavage furrow forms at one end of the cell. Cytokinesis occurs during stage II which creates the septum and forms the pre-spore and the mother cell. Stages III, IV, and V, involve the mother cell engulfing the spore, formation of the cell membrane, wall, and cortex, and final the spore coat. Upon maturation of the endospore cell lysis will occur and the free spore will be released. When the environmental conditions improve the spore can undergo germination to reenter the vegetative state.[3]

Before cytokinesis occurs in the early stages, the DNA of the bacterium is duplicated and both the pre-spore and mother cell receive a complete copy. Differentiation of the cells is controlled by the expression and activation of different genes. RNA polymerases contain variable sigma (σ) factors which act as transcription factors and control the transcription of genes.[3,11] The σ factors direct the RNA polymerases to the promoter region of specific genes.[3,11] This ensures that specific genes are expressed and others are not.

During the vegetative stage σ^A is the major transcription factor and works simultaneously with σ^H, the minor transcription factor. The formation of the mother cell and pre-spore is directed by the activity of σ^E and σ^F, respectively. The maturation and

formation of the spore's cell membrane, wall, and cortex, is regulated by σ^G. During late stage sporulation, genetic transcription in the mother cell is directed by σ^H. The activity of the transcription factors produces different proteins in the two chambers. Through the production of different proteins and the activation of different genes the spore and mother cell take on their own specialized functions and became differentiated from each other.

There are several other gene clusters and RNA transcription factors involved in sporulation. As a result of the discussed sigma factors along with the activation of other genes a spore will be formed and the mother cell will undergo lysis to release the spore. The spore created in the process has the ability to survive harsh environmental conditions for thousands of years. Once the environmental conditions improve the spore will undergo germination and enter the vegetative stage continuing the normal life cycle of growth and replication.

1.1.3 Genetic Regulation of the Mother Metabolic Gene Operon

During the early stage of sporulation, σ^E is active in the mother cell and is the required σ factor for the mother metabolic gene (*mmg*) operon. This operon has been shown by the Reddick Lab to encode for β-oxidation of fatty acids and the metabolism of propionyl CoA via the methylcitric acid cycle. The *mmg* operon consists of a sequence of six genes, *mmgABCDE* and *yqiQ* (now named *mmgF*). The first three genes in the sequence (*mmgABC* with additional activity from *yhaR*) encode for the metabolism of short chain fatty acids by the β-oxidation pathway. The rest of the genes in the operon, with assistance from CitB (a known aconitase enzyme in *B. subtilis*), encodes for the methylcitric acid cycle.[12]

1.1.4 Activity of the *mmg* Operon and β-oxidation

During β-oxidation, fatty acyl CoAs are shortened by a two carbon unit through a series of four reactions. First the acyl CoA **1** is oxidized to enoyl CoA **2** (an acyl-CoA dehydrogenase, MmgC), then hydrated to form β-hydroxyacyl CoA **3** (an enoyl-CoA hydratase, YhaR). A second oxidation produces β-ketoacyl CoA **4** (β-ketoacyl CoA dehydrogenase, MmgB), which is then cleaved to produce acetyl CoA **5** and an n-2 acyl CoA (thiolase, MmgA). Once the fatty acid has been completely metabolized the final cleavage will produce two molecules of acetyl CoA.[7] This β-oxidative pathway is depicted in Figure 1.

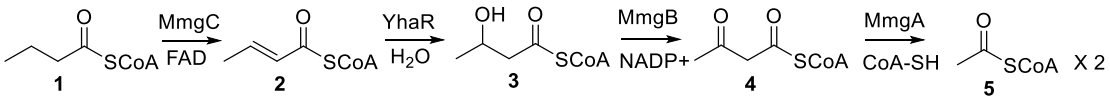


Figure 1. **β-Oxidation of Fatty Acid Acyl-CoA's**. β-oxidation of fatty acyl-CoA's occurs in four basic reactions that are repeated until the final step produces two acetyl-CoA molecules. In *Bacillus subtilis* the *mmg* operon encodes for fatty acid degradation. The genes *mmgC*, *mmgB*, and *mmgA*, with assistance from *yhaR*, are responsible for this process.

In some bacterial species, such as *B. subtilis*, the omega-3 position of the fatty acid chain has the possibility to be methylated. When this occurs the fatty acid is then referred to as an *anteiso* fatty acid. In *B. subtilis* this ω-3 methyl branch is present in greater than 80 % of the fatty acids.[13,14] In other species the methylation depends on environmental conditions and often happens 40 to 60 % of the time.[14] This affects the outcome of β-oxidation. The *anteiso* fatty acid will produce propionyl CoA **6** and acetyl

CoA **5** in the final step of the degradative pathway. This process is outlined in Figure 2 with the enzymes MmgABC and YhaR. Both acetyl-CoA **5** and propionyl-CoA **6** are condensed with oxaloacetate **7** to produce citrate **8** and 2-methylcitrate **9**, respectively. Citrate is then metabolized in the citric acid cycle, but 2-methylcitrate is utilized in the methylcitric cycle

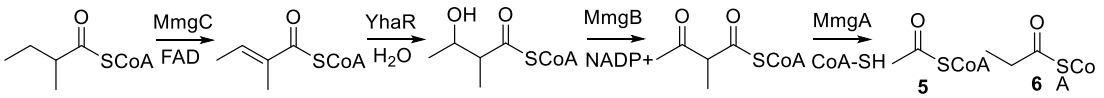


Figure 2. **β-Oxidation of ω-3 Fatty Acid Acyl-CoA's**. In *B. subtilis* approximately 85 % of fatty acids are methylated at the omega-3 position. During β-oxidation the result should be the production of acetyl-CoA and propionyl-CoA.

1.1.5 Activity of the *mmg* Operon and the Methylcitric Cycle

The methylcitric cycle is interconnected with the citric acid cycle. The methylcitric cycle is encoded in the latter half of the *mmg* operon. These genes include *mmgD*, *mmgE*, and *mmgF* with additional help from CitB, an aconitase enzyme. In our previous work, MmgD was shown to condense propionyl-CoA and oxaloacetate to form one of the "heterochiral" enantiomers (2*S*, 3*R*) or (2*R*, 3*S*)- 2-methylcitrate. MmgE then acts as a dehydratase to produce 2-methylaconitate **10**. Another enzyme, CitB then hydrates 2-methylaconitate to produce 2-methylisocitrate **11**. Once 2-methylisocitrate is formed, MmgF then acts as a methylisocitrate lyase to form pyruvate **12** and succinate **13**.[12] Pyruvate can then undergo further transformation to be converted into acetyl-CoA needed for the citric acid cycle, and succinate can then reenter the citric acid cycle as an

intermediate. The enzyme activity of MmgDEF and CitB is shown in figure 3 along with the interconnections of the citric acid cycle.

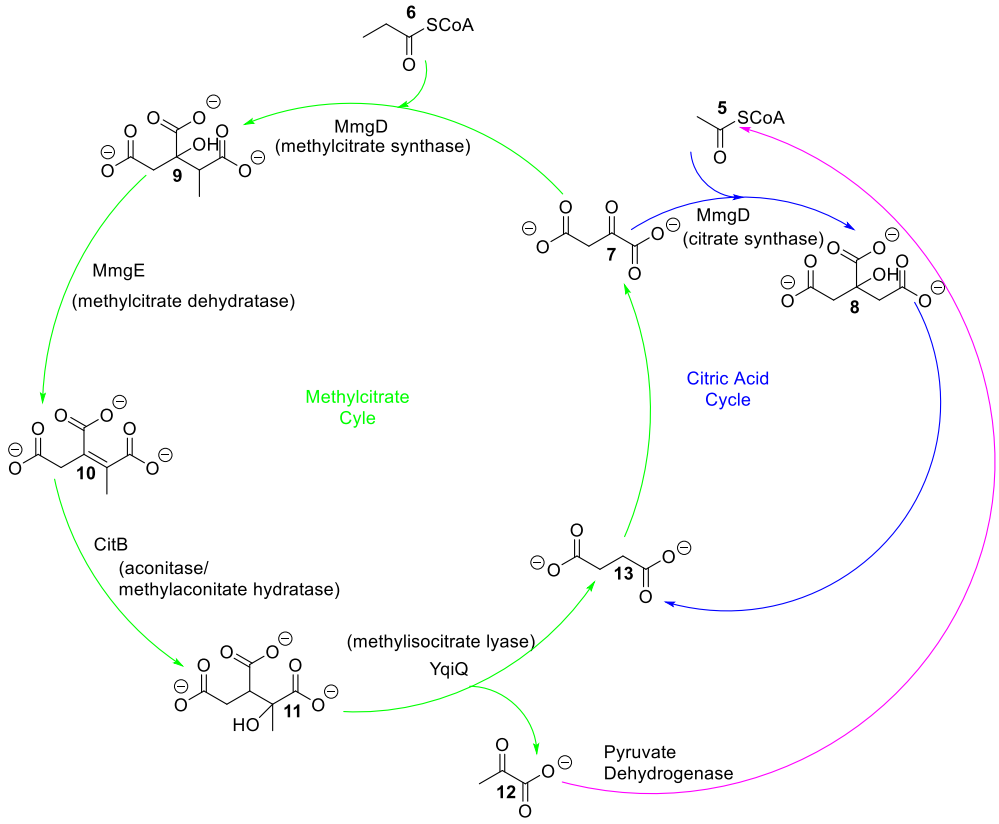


Figure 3. **The Interconnection of the Methylcitric Acid Cycle and Citric Acid Cycle Involving the Degradation of Propionyl-CoA**. The interconnection of the methylcitric cycle and the citric acid cycle is encoded in the *mmg* operon. Here the enzymes MmgDE and YqiQ (MmgF) are responsible for the observed transformations. CitB is an aconitase enzyme that hydrates the olefin **10**. The methylcitric acid cycle is activated when β-oxidation produces propionyl-CoA. At the end of the methylcitric acid cycle succinate and pyruvate are produced. Succinate will continue into the citric acid cycle and pyruvate will undergo further transformation.

1.2 Literature Review of Methylcitrate Lyase

1.2.1 Previous Methylcitric Lyase Results Obtained from *B. subtilis*

Evidence of the methylcitric acid cycle in *B. subtilis* has been previously shown by the Reddick lab. Each step was studied individually using available substrate, except MmgF. The only way to obtain 2-methylisocitrate was to reconstruct the enzymatic pathway sing MmgD, MmgE, CitB and MmgF. The activity was determined with a single enzymatic reaction by incubating MmgDEF and CitB, with propionyl CoA and oxaloacetate yielding pyruvate and succinate.[12] The methylcitric acid cycle has also been studied in other bacterial species such as *Escherichia coli* and *Salmonella enterica* through their homologous gene operons, *prp*.[15]

In the final step of the methylcitric acid cycle 2-methylisocitrate is cleaved to form pyruvate and succinate by MmgF.[12] However, it is unclear which stereoisomer is utilized by the enzyme. It has been reported that the enzyme from *Escherichia coli* PrpB uses (2*R*, 3*S*)-2-methylisocitrate.[16] PrpB and MmgF are homologous proteins with 42 % sequence identity and 61 % sequence similarity.

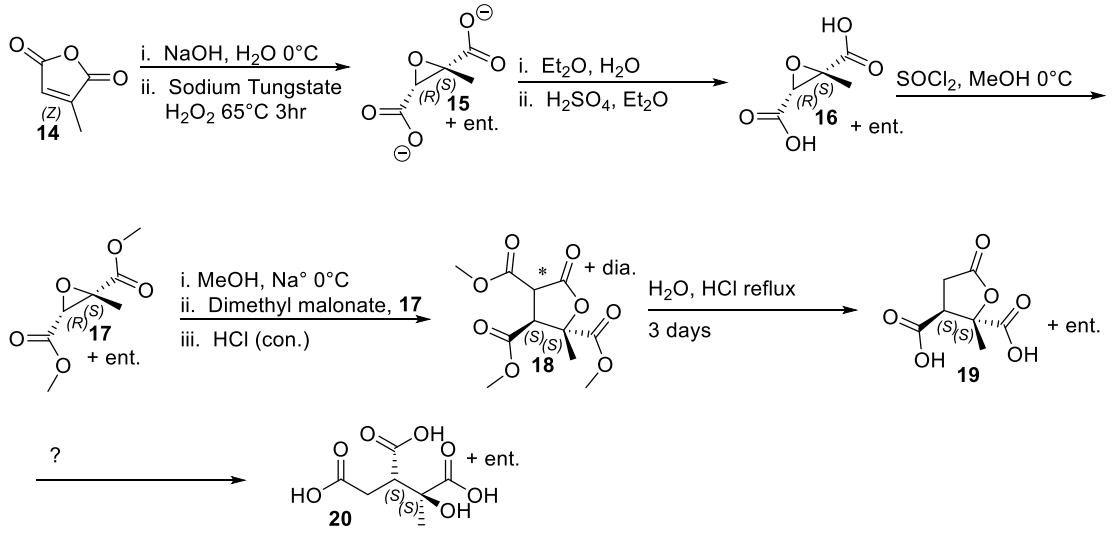
1.2.2 Previous Published Syntheses of 2-Methylisocitrate

In an effort to determine the preferred stereoisomer of the MmgF substrate in *B. subtilis*, 2-methylisocitrate must be synthesized because it is not commercially available. There are published syntheses of 2-methylisocitrate,[16, 17] but they have been problematic to reproduce in our lab (William Booth MS thesis and Nicolas Coffey MS thesis). The goal of this Master's project is to finish the synthesis of 2-methylisocitrate by modifying

the published syntheses. Upon completion of the syntheses, the desired products will be used to determine the activity of MmgF.

In the first published synthesis by Brock et al.,[16] the description of the synthesis led to a lack of reproducibility in our lab. Important details from the syntheses were lacking such as freshly distilled chemicals were required for dry conditions. Some of the terminology used in the step were incorrect or crude products were carried forward in the synthesis. The combined effects led to reproducibility problems in our lab. The published reaction sequence from Brock et al. is presented Scheme 1.

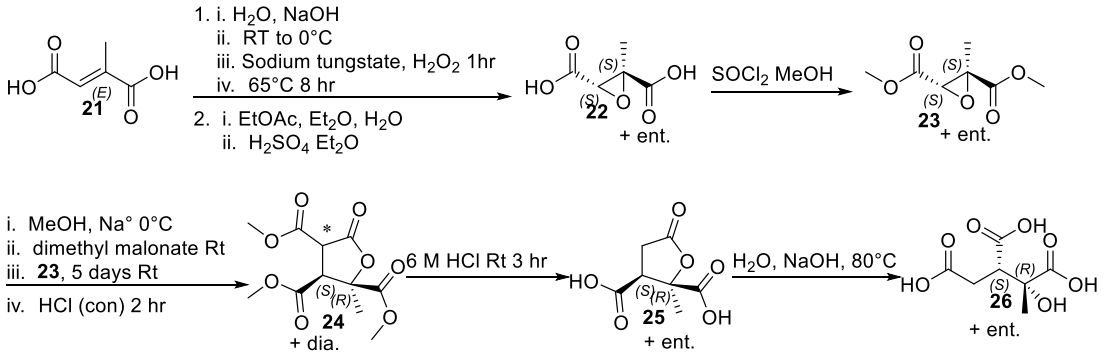
Scheme 1. Original Reaction Sequence Starting with Citraconic Anhydride[16]



The first step of the synthesis utilized a sodium tungstate dihydrate catalyst to produce an epoxide disodium salt, was then acidified. The work-up for the acidification step stated that an extraction of the residue was performed, but this terminology was incorrect. The concentrated residue was actually triturated to isolate the desired product.

The third step in the synthesis utilized thionyl chloride and anhydrous methanol to obtain the dimethyl ester. This step presented problems because both of the required reagents needed to be completely anhydrous and freshly distilled. The literature never stated that anhydrous conditions and freshly distilled reagents were needed for this step. The next reaction with sodium methoxide, dimethyl malonate, and the dimethyl ester, produced a tetrahydrofuran ring which was then treated with 6 M HCl to deprotect the methyl esters and reveal the free acids. After deprotection, the methyl ester that is closest to the carbonyl group on the tetrahydrofuran ring also underwent decarboxylation under the same reaction conditions.[16] In this published synthesis the procedure for the final step (the hydrolysis of the lactone ring) to produce the 2-methylisocitric acid was not reported.

Scheme 2. Reaction Sequence for the Mesaconic Pathway from Literature[17]



The second procedure that was followed was published by Munoz-Elias et al.[17] and is depicted in Scheme 2. Although the paper claimed to improve upon the chemistry described by Brock et al.,[16] this paper still presented us with issues of reproducibility. The sequence of reactions is shown in scheme 2. The synthesis was identical to the

above procedure, but the first step with the sodium tungstate dihydrate catalyst was problematic for us to repeat. Thionyl chloride was also used in the revised published synthesis for the esterification, followed by the deprotection and decarboxylation of the methyl ester at room temperature for 3 hours. The final step in the synthesis involved a ring opening to produce the desired 2-methylisocitrate. Our improvements and clarifications to this synthesis will be indicated in the preliminary work section.

Both Coffey and Booth were only able to get to **17** with success, but were unable attempt the completion of the pathway.

1.3 Literature Review of Methylcitrate Synthase

1.3.1 Previous 2-Methylcitrate Synthase Work

In previous studies by Brock et al.,[15] the stereochemistry of the intermediates of the methylcitric cycle from *E. coli* were investigated. They reported that the "homochiral" (2*S*, 3*S*)-2-methylcitrate was produced by PrpC and this product was isomerized to (2*R*, 3*S*)-2-methylisocitrate by the enzymes methylcitrate dehydratase (PrpD) and aconitase (AcnB), respectively.[15] This isomerization would require a *syn* elimination followed by an *anti* addition of water across the double bond. Our laboratory has observed results that conflict with these literature reports. Our lab has shown that MmgD from *B. subtilis* and PrpC from *E. coli* produce one of the heterochiral enantiomers ((2*S*, 3*R*) or (2*R*, 3*S*)) of 2-methylcitrate and has also shown evidence that MmgE and PrpD use only the *anti* elimination mechanism to produce *(Z)*-2-methylaconitate **27** from either of the heterochiral 2-methylcitrate enantiomers, or (*E*)-2-

methylaconitate **28** from either of the homochiral 2-methylcitrate enantiomers. The dehydration and addition of water across the double bond can be seen in Figure 4.

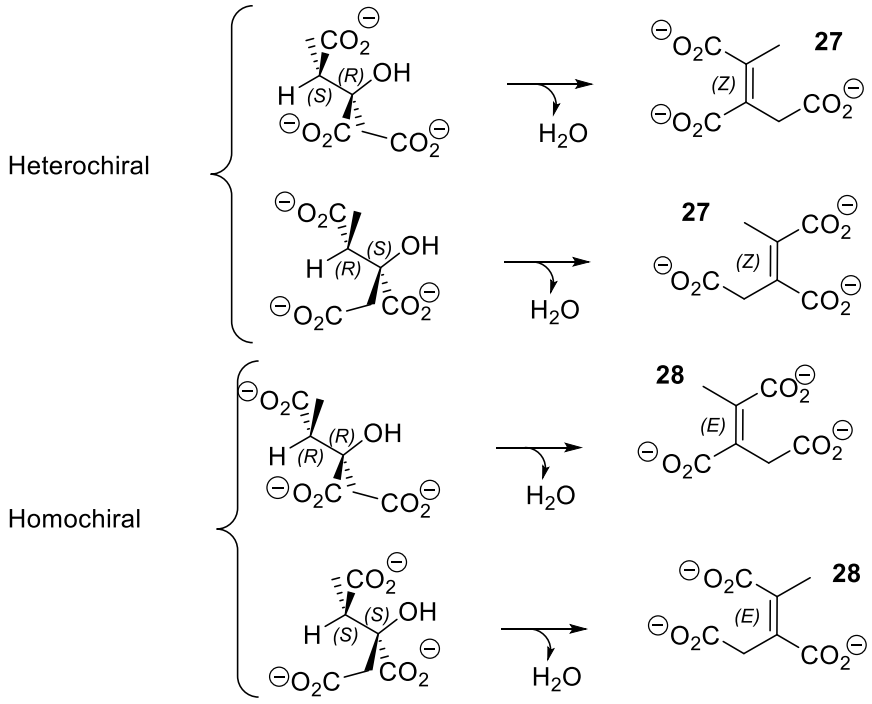


Figure 4. Dehydration of Heterochiral and Homochiral 2-Methylcitrate. Possible dehydration results of the homochiral and heterochiral 2-methylcitrate via the *trans* elimination of water has been observed in previous studies. Here both the heterochiral and homochiral diastereomers are represented with their appropriate eliminations. With the *trans* elimination of the heterochiral enantiomers the *Z*-alkene will be produced and the homochiral enantiomers will afford the *E*-enantiomers.

The assignments of the stereochemistry by Brock et. al.[15] were based on comparisons that were reported in previous journal articles. This might be faulty when trying to determine the absolute stereochemistry of the product, because no structural elucidation was performed in any of these studies.

In a more recent paper by Krawczyk and Martyniuk,[18] the NMR data of the homochiral and the heterochiral 2-methylcitrate were reported. In order to make these assignments they used two synthetic organic methods to produce different diastereomers. In their reported synthesis they took advantage of enolate chemistry and the selectivity associated with the different types of reagents. The first method utilized zinc as a chelating metal to obtain a racemic mixture of the homochiral enantiomers. The second method produced the heterochiral enantiomers with lithium diisopropylamide.

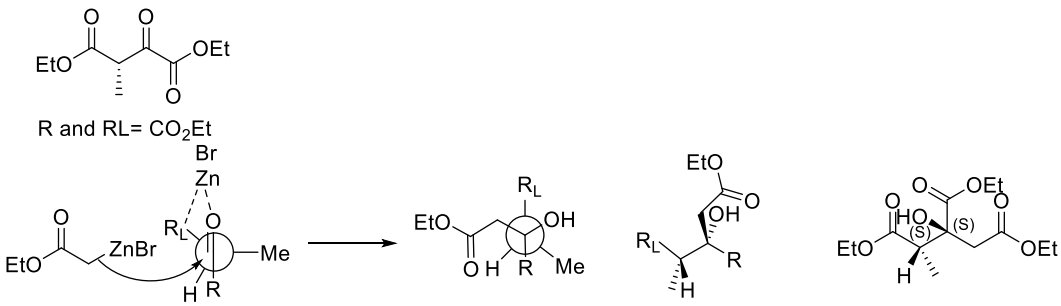


Figure 5. Cram Chelating Metal Transition State Model. Cram chelating metel transition state model is used to control the stereochemistry with the Reformatsky reaction. Since the nucleophile attacks from the least sterically hindered side of the electrophile this leads to the homochiral set of enantiomers

The different stereoisomers can be determined by considering the transition states in the mechanism and the different types of control. Since zinc is a chelating metal the Reetz-Cram chelating metal model[19] should be used, and depicted in Figure 5. With this model, 1,3-asymmetric induction occurs by minimizing the steric hindrance between the 1,3-carbonyl-containing compound and the organozinc reagent leading to a Reformatsky reaction and ultimately the homochiral enantiomers. The lithium diisopropylamide

reaction is stereochemically controlled with the Zimmerman-Traxler model[20] as seen in Figure 6. The transition state minimizes 1,3-diaxial clashing in the chair conformation, which eventually leads to the heterochiral enantiomers.

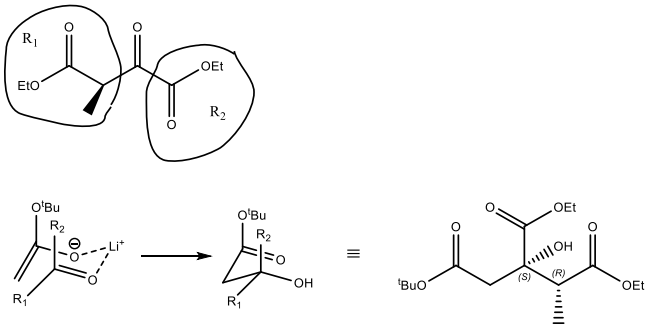


Figure 6. Zimmerman-Traxler Transition State Model. Zimmerman-Traxler transition state with the use of Lithium diisopropylamide leads to the heterochiral set of enantiomers by reducing the steric hindrance of the 1,3-diaxial interactions.

The information presented by Krawcyzk et al.[18] and the analysis of the transition state models led our lab to conduct further analysis of the enzymatic products from MmgD and PrpC by HPLC and compare the enzymatic results to NMR experiments of the commercial standard. When MmgD and PrpC were separately incubated with propionyl-CoA and oxaloatate it was observed that they produced the same substance which co-eluted with the minor product from the commercially available standard as seen in Figure 7.

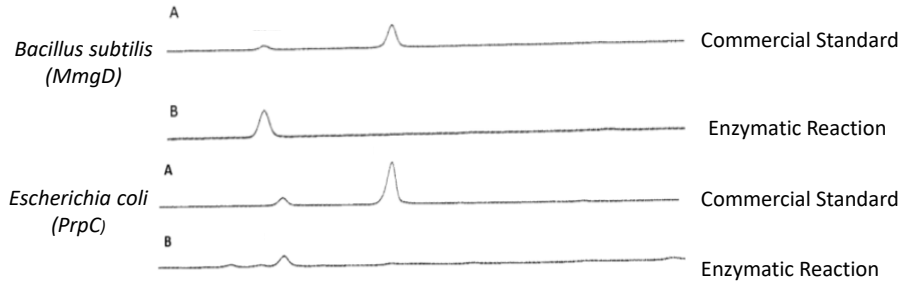


Figure 7. Comparison of Enzymatic Products from MmgD and PrpC with the Commercial Standard. When comparing the enzymatic products from MmgD and PrpC from *B. subtilis* and *E. coli*, respectively, they line up with the minor product from the commercially available standard from Sigma-Aldrich. The enzymatic reactions are shown in line A and the commercial standards are in line B of both chromatograms obtained from HPLC analysis.

Through personal communications with Sigma-Aldrich it was disclosed that the standard was synthesized through the Reformatsky reaction. Considering the transition state models the major component of the reaction would have the stereochemistry of (2*RS*, 3*RS*)-2-methylcitrate and the minor product would take on the (2*RS*, 3*SR*)-2-methylcitrate stereochemistry.

The commercial standard was further analyzed by NMR correlared with the results obtained by Krawczyk et al.[18] According to their NMR results the C2-methyl peak from the homochiral set of enantiomers would be further downfield from the heterochiral enantiomers.[18] Our NMR analysis of the commercial standard showed the major product peaks shifted further down field seen in Figure 8. Utilizing the disclosed reaction conditions from Sigma-Aldrich, it was determined that the major component of the standard was the (2*RS*, 3*RS*) enantiomers. These results were consistent with Krawcyzk's data. The ratio of the major to minor product is 9 to 1. According to the

Krawczyk et al.[18] the products from the Reformatsky reaction would have a methyl group located on carbon-2 with a multiplicity of a double at 1.07 ppm and the other` stereochemical outcome from this reaction would be (2*RS*, 3*RS*)-2-methylcitrate. The doublet at 0.97 ppm belongs to the heterochiral enantiomers (2*RS*, 3*SR*)-2-methylcirtate.

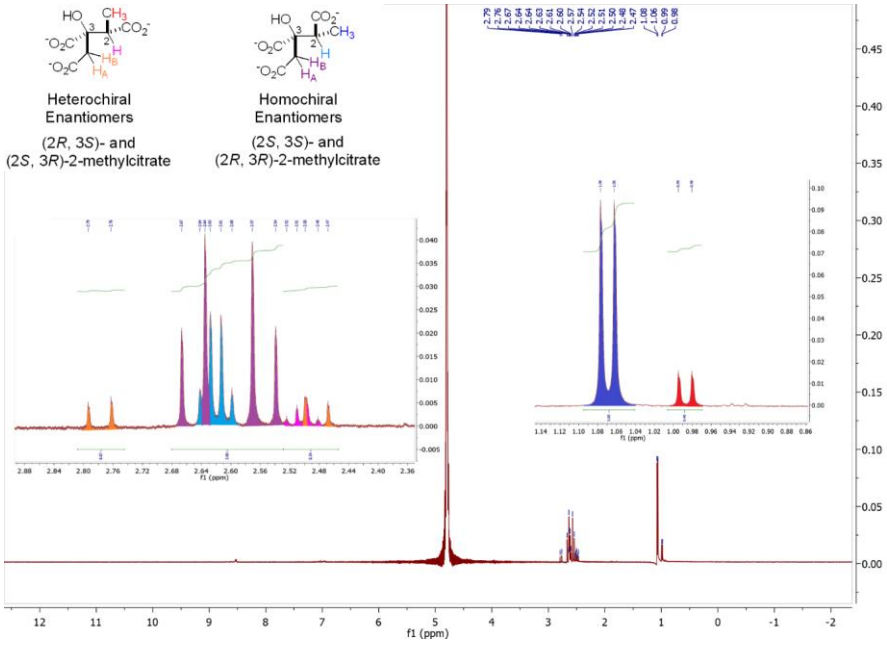


Figure 8. NMR Analysis of the Commercially Available 2-Methylcitrate. The NMR analysis of the commercially available standard from Sigma-Aldrich synthesized through the Reformatsky reaction shown two different sets enantiomers were produced. The major component at 1.07 ppm is the homochiral enantiomers and the minor product would be the heterochiral enantiomers. The ratio of major to minor products is 9 to 1.

Based on this NMR data and our HPLC analysis with the enzymatic reactions compared to the commercial standard, we have concluded that the product produced by MmgD and PrpC is one of the heterochiral enantiomers and not (2*S*, 3*S*)-2-methylcitrate as stated by Brock et al.[17] The HPLC analysis with stereochemical analysis is shown in

Figure 9. The integrated ratios of the major or minor products is 9 to 1 like the NMR analysis.

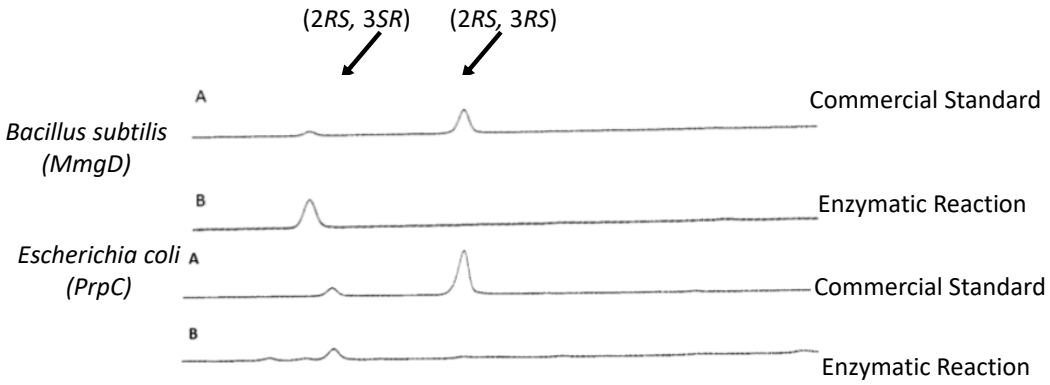


Figure 9. HPLC Stereochemical Comparison of the Enzymatic Reaction with the Commercial Standard of 2-Methylcitrate. With the results from the NMR analysis the product peaks from the commercial standard were assigned. The minor commercial product which lines up with the results from the enzymatic reactions can be assigned as the heterochiral ((2*RS*, 3*SR*)-2-methylcitrate) enantiomers. The integrated values of the peaks are 1 to 9, minor to major.

1.3.2 Plan to Synthesize Enantiopure 2-Methylcitrate

In order to determine the absolute stereochemistry of 2-methylcitrate formed by MmgD (and PrpC from *E. coli*), the four diastereomers will be synthesized in an enantioselective manner. Many reactions in organic synthesis produce a racemic mixture of desired products. This arises from the reagents and the starting material reacting in a non-selective manner. Many molecules can interact from two sides. This occurs most often when the reaction happens at a planar functionality such as an alkene. Depending on the type of reaction a *trans* or *syn*-addition will be observed. Reactions such as halogenation or any reaction that involves the formation of a bromonium ion, add across

the double bond in an *anti*-manner. The initial step in the formation of the bromonium ion is *syn* addition, but the following step is anti-addition. Epoxidations, hydroboration oxidation, and hydrogenations via Pd/C and the Lindlar catalyst, interact with the planar functionality in a *syn*-fashion. This allows for the stereochemistry of the product to be predicted but not controlled, thus producing two enantiomers as a racemic mixture. If a carbocation is formed during the reaction the stereochemical outcome will be a mixture of diastereomers.

The desire to control the stereochemical outcome of a reaction was driven by the pharmaceutical industry. One of the best known examples for the need of enantiopure medications is Thalidomide. In the mid 1950's racemic Thalidomide was prescribed to pregnant women to help control morning sickness. The women that took Thalidomide began to give birth to babies with birth defects. It was later determined that only one of the enantiomers of Thalidomide showed teratogenic effects.[21]

Enantioselective synthesis was led by three chemists, William Knowles, K. Barry Sharpless, and Ryoji Noyori. Their research revolved around alkenes and adding substituents to a desired face of the functional group. In 2001 these three chemists were awarded a Nobel prize for the use of metal catalysts in asymmetric synthesis. Sharpless developed catalysts for asymmetric epoxidations,[22] dihydroxylations,[23] and oxyaminations.[24] Noyori and Knowles worked on enantioselective cyclopropanations[25] and asymmetric hydrogenation,[26] respectively.

The stereochemical outcome of a reaction can be controlled by many different methods. Some methods include, but are not limited to, chiral catalysts, steric hindrance,

the formation of solvent cage effects, and addition of chiral axillary groups, biocatalysts, chiral resolution, enantioselective organometallic catalysts, and functional groups that act as coordinating groups.

Epoxides are useful organic synthesis tools when an alcohol group must be added to a double bond along with another functional group. This is due to the control that can be obtained when the epoxide is opened under acidic or basic conditions. When the epoxide is open under acidic conditions the most sterically hindered side will be attacked because of the formation of a carbocation at that position. Under alkaline conditions the epoxide will be attacked on the less sterically hindered side. The traditional methods of forming epoxides are not enantioselective and will lead to a racemic mixture of products being obtained. If an enantiopure alcohol is needed these methods are not acceptable and the need for asymmetric epoxidations arises.

In order to determine the absolute stereochemistry of 2-methylcitrate synthase, an asymmetric epoxidation will be utilized to set the two stereocenters. There are several modern organic chemistry methods that have the ability to form asymmetric epoxides, and they all work best with different types of alkenes. The Sharpless asymmetric epoxidation requires an allylic alcohol to be present in the substrate.[27] The Jacobsen-Katsuki asymmetric epoxidation works best with *cis*-olefins, but is a poor candidate for epoxidation to *trans*-olefins and tri-substituted olefins due to the steric hindrance of the salen catalyst.[28] More recently, R. Curci and co-workers reported the ability of dioxiranes to produce epoxides on a variety of substrates.[29] Dioxirane can be produced *in situ* from a ketone and an oxidant. Wanting to harness the ability of dioxirane, Y. Shi and

co-workers developed a highly selective method of epoxidation utilizing fructose derivatives as chiral catalysts.[30-33] The Shi epoxidation method is able to form enantioenriched products on a wide variety of olefins.

When the chiral ketone catalyst was being developed, it was determined that the best candidate would contain chiral centers in close proximity to the reactive ketone, have a rigid, fused ring structure, and possess a bulky structural component that would only allow access to the dioxirane from one direction. With these elements in mind carbohydrates were the chosen starting material for the catalyst design. Ultimately, D-fructose was chosen as the building block for the catalyst, because it is readily available, inexpensive, and possesses several chiral centers with known configurations. It was also important that the catalyst contain multiple oxygen atoms to increase the reactivity of the ketone through induction. In 1996, Y. Shi and co-workers developed a fructose derived ketone catalyst that successfully formed epoxides on *trans* and tri-substituted olefins.[30]

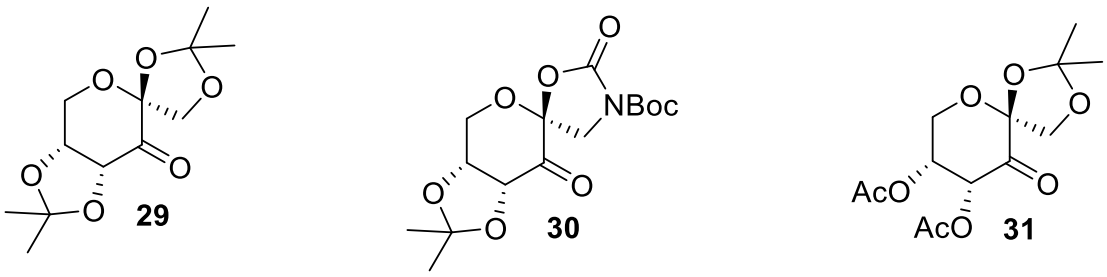


Figure 10. The Original Shi Ligand and Its Derivatives. The original Shi ligand ligand **29** was developed from D-fructose due to the chiral centers already present in the molecule, but it was not effective at epoxidizing *cis* or α,β-unsaturated olefins. Derivative **30** is effective epoxidizing *cis* olefins and **31** harnesses the ability to form an epoxide on α,β-unsaturated esters.

The original Shi ligand **29** derived from D-fructose only possessed the ability to form an epoxide on *trans* or tri-substituted olefins.[30-32] Other types of olefins were determined to be poor substrates for the original ligand.[30] In an effort to resolve this issue, two similar chiral ketone ligands were developed. These derivatives are shown in Figure 10.

The first derivative **30** that was developed possessed the ability to form an asymmetric epoxide on *cis* olefins. This catalyst was modified at the 1,3-oxirane spiro cycle moiety to include a carbamate where the nitrogen is protected by a Boc group.[33]

The epoxidation of electron poor olefins such as α,β-unsaturated esters have been troublesome for traditional methods. It is possible to use a nucleophilic epoxidizing agent such as hydrogen peroxide treated with sodium hydroxide, but this method is not stereoselective. To overcome this obstacle Shi and coworkers developed a derivative of their original catalyst by removing the ketal moiety and replacing it with two acetate groups **31**. This increased the electrophilicity of the dioxirane ring formed and enabled the epoxidation of α,β-unsaturated esters.[34]

The Shi epoxidation is not without its faults. The reaction conditions must be strictly regulated to produce the desired results. The pH of the reaction has been shown to affect the reaction profoundly.[35] The optimum pH for the Shi epoxidation is approximately 10.5. If the epoxidation is conducted at a lower pH, the Shi catalyst will undergo a Baeyer-Villiger oxidation when it interacts with Oxone.[34, 35] The Baeyer-Villiger reaction pathway will eventually lead to hydrolysis and produce acetone subsequently leading to a competing reaction. The acetone produced through hydrolysis

would interact with Oxone producing dimethyldioxirane *in situ* that would have to ability to form an epoxide with the olefin.[29] The product from this reaction would not be enantioenriched. This would cause a decreased enantiomeric excess due to the structural simplicity of dimethyldioxirane produced.[29]

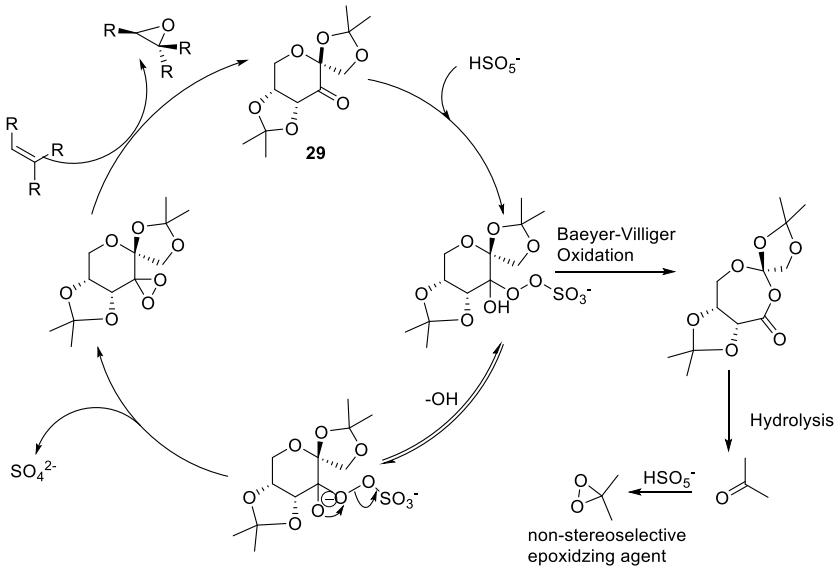


Figure 11. The Shi Catalytic Cycle. The D-fructose derived Shi ligand does not possess the ability itself to form an epoxide upon an olefin. Oxone acts as an oxidant on the ketone of D-fructose to form a dioxirane ring which is then the epoxidizing agent of the Shi ligand. If the pH is not strictly controlled a Baeyer-Villiger oxidation will occur and the ligand will be degraded to acetone. The acetone can then react with oxone and lead to a non-stereoselective epoxidation.

If the reaction is conducted at a higher pH, Oxone will undergo auto decomposition.[35] The pH of the reaction can be controlled with the addition of potassium carbonate or the reaction can be carried out in an EDTA solution buffered to the desired pH.[35] The auto decomposition of Oxone can also be reduced by decreasing the

temperature of the reaction to 0 °C. The catalytic cycle and possible side reactions are shown in Figure 11.

When the Shi ligand is used as an epoxidizing agent the stereochemistry of the products can be predicted by considering the sterics between the catalyst and the substrate. It has been shown that the Shi ligands prefer a spiro transition state compared to the planar transition state.[30] The spiro transition state is produced when the dioxirane is orthogonal to the olefin, and the planar transition states would occur when the dioxirane is parallel to the olefin. Figure 12 depicts a simplified version of possible transition states with *trans* olefins.

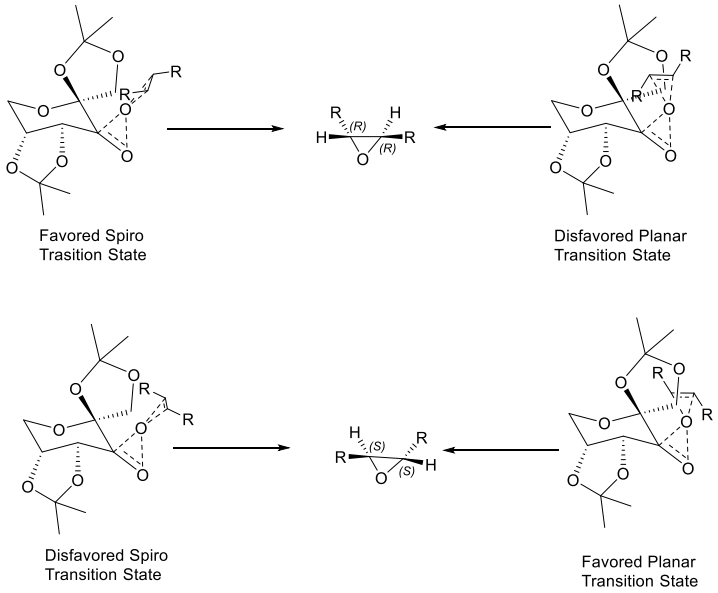
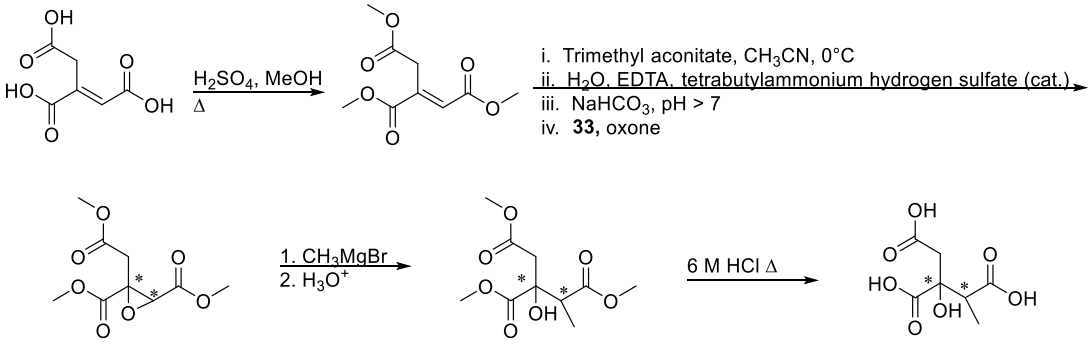


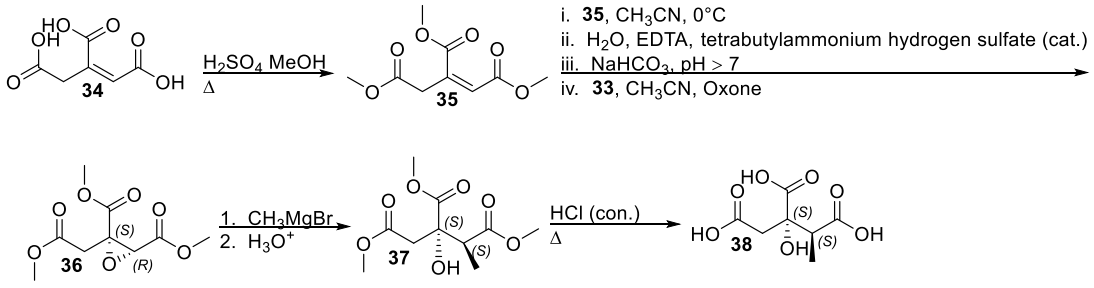
Figure 12. Possible Transition States of the Shi Ligand. After the dioxirane ring has been formed on the Shi ligand there are four possible transition states, but they can be broken down into two types, spiro and planar. The spiro transition state occurs when the olefin is orthogonal to the dioxirane ring and the planar forms when the olefin is parallel to the ring. The favored transition state happens when steric hindrance is minimalized. The favored spiro transition state produces the (*R*,*R*) configuration with a simple *trans* olefin.

An asymmetric epoxidation was chosen to set the stereocenters of 2-methylcitrate, because of the chiral alcohol present on carbon 3. The available starting materials for the synthesis of enantiopure 2-methylcitrate by this method are *E*-aconitic and and *Z*-aconitic acid. By undergoing a Fischer esterification trimethyl *E*-aconitate and *Z*-aconitate will be obtained. These compounds are then candidates for the Shi asymmetric epoxidation for α,β-unsaturated esters. Once the epoxide is obtained a Grignard reaction will be used to install the methyl group at carbon 2, then the free acids can be revealed by deprotection of the esters. The general linear synthesis is presented in Scheme 3.

Scheme 3. Proposed Synthesis of Enantiopure 2-Methylcitrate

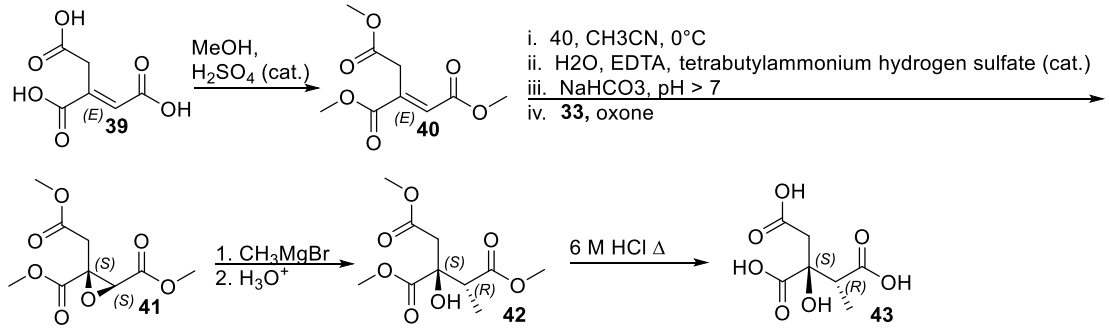


Scheme 4. Reaction Sequence to Obtain (2*S,* 3*S*)-2-Methylcitrate.



With the modified commercial Shi ligand only two of the four diastereomers of 2-methylcitrate will be obtained, (2*S*, 3*S*)-2-methylcirate and (2*R*, 3*S*)-2-methylcirate. The synthetic plan for these diastereomers is shown in Schemes 4 and 5, respectively. The predicted stereochemistry of the epoxide products **36** and **41** was obtained through transition state models where the favored spiro transition state reduced the steric hindrance of the Shi ligand and the substrate for the epoxidation. The transition state model to produce **36** is seen in Figure 13 and the transition state model for **41** is depicted in Figure 14.

Scheme 5. Reaction Sequence to Obtain (2*R*, 3*S*)-2-Methylcitrate.



In order to obtain the (2*R*, 3*R*)-2-methylcitrate and (2*S*, 3*R*)-2-methylcitrate diastereomers the enantiomer of the commercially available Shi ligand must be synthesized through total synthesis, then modified to include the acetyl groups instead of the bicyclic ketal moiety.

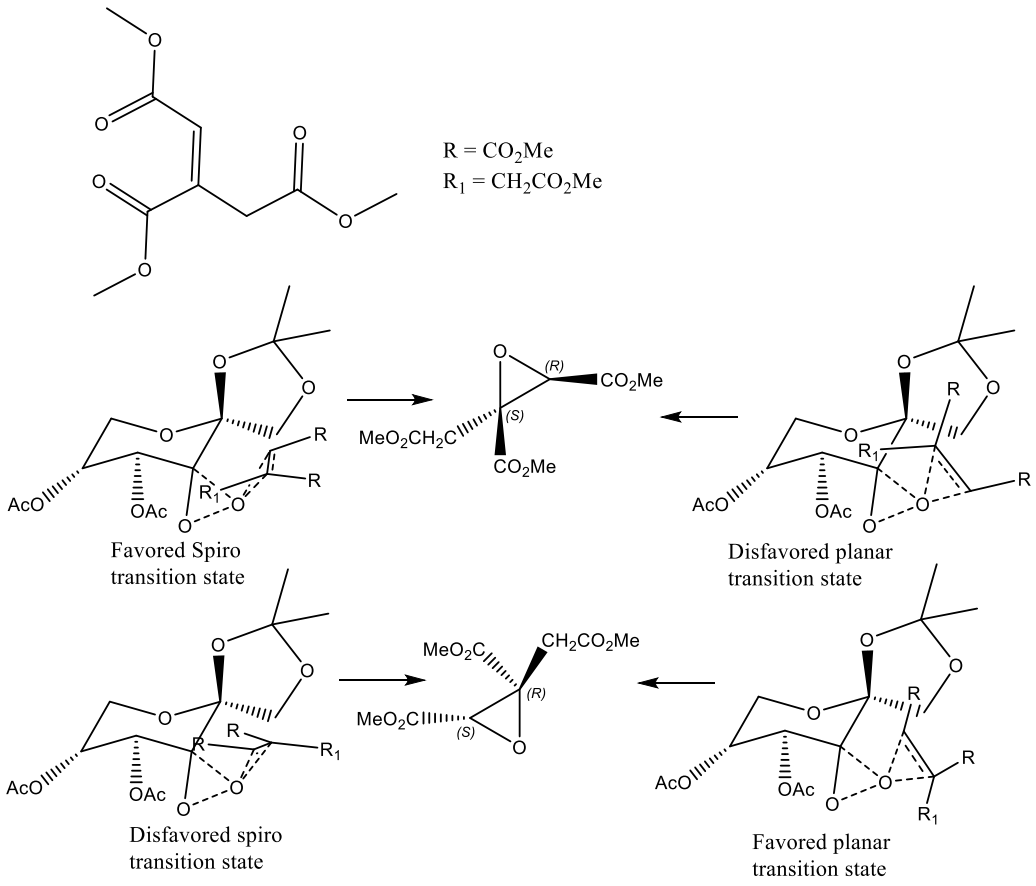


Figure 13. Transition States with Trimethyl *E*-Aconitate. The transition state models with trimethyl *E*-aconitate and the modified Shi ligand for α,β-unsaturated ester was used to determine the stereochemical out and the epoxide produced during the reaction.

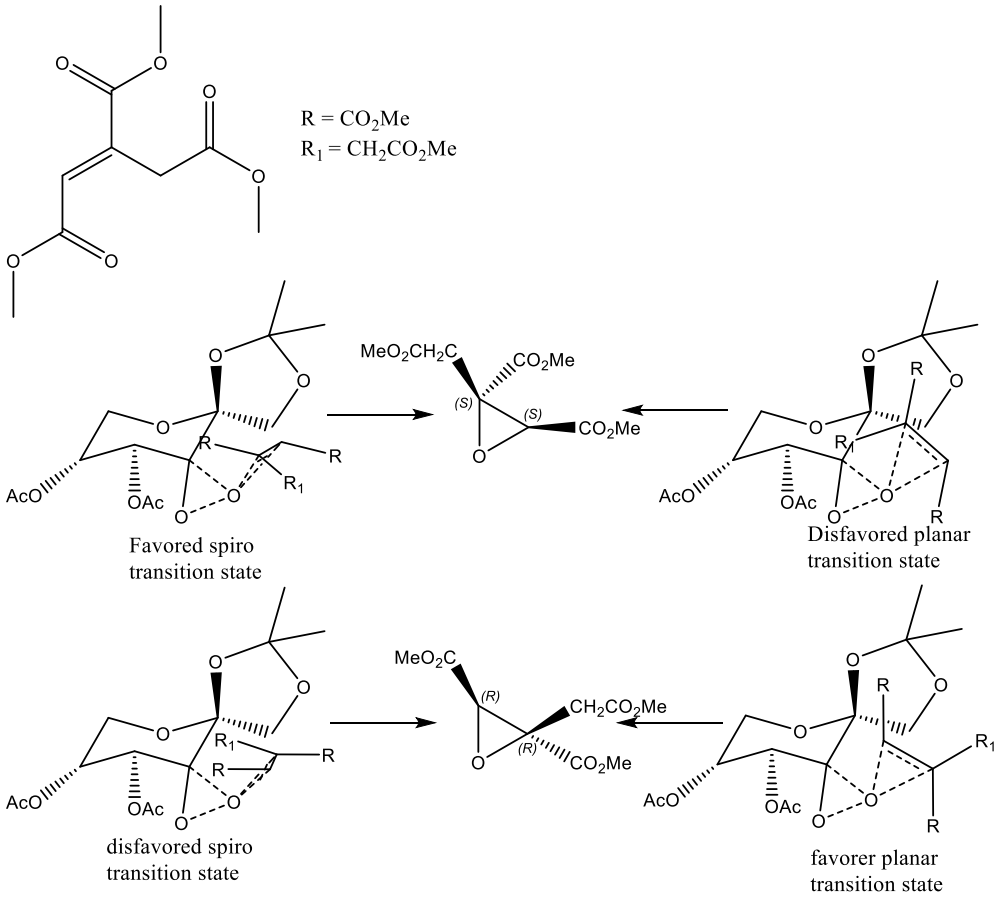


Figure 14. Transition States with Trimethyl *Z*-Aconitate. The transition state models were utilized to determine the stereochemical outcome of the epoxide product with trimethyl *Z*-aconitate and the Shi ligand for α,β-unsaturated esters.

1.4 Central Hypothesis and Objectives

Bacillus subtilis is a model organism and the entire genome has been sequenced.[1] However, there is an incomplete understanding of the metabolic pathways that are encoded within the genome. A *long-term goal* of the Reddick research group is to gain a more complete understanding of the *mmg* operon. The *overall objective* of this thesis is to develop the syntheses of 2-methylisocitrate and 2-methylcitrate. The *central*

hypotheses of these objectives are that MmgF cleaves one of the enantiomers of 2-methylcitrate to produce succinate and pyruvate, and MmgD 2-methylcitrate synthase produces the heterochiral ((2*S*-3*R*) or (2*R*,3*S*))-*2*-methylcitrate. The proposed syntheses will allow new investigations furthering the understanding of the mother cell metabolic (*mmg*) operon. Previous graduate students either were unable to finish the synthesis of 2-methylisocitrate or experienced reproducibility problems with this synthesis. Progress towards the long-term goal of the Reddick research group and the accomplishment of this Master's project's overall objective will be realized through specific objectives.

1.4.1 2-Methylisocitrate Lyase Objective

To develop the syntheses of (2*RS*, 3*RS*) and (2*RS*, 3*SR*)-2-methylisocitrate using modified literature methods for the purpose of determining the substrate of the MmgF enzyme of *B. subtilis*. In this specific objective we will utilize synthetic organic chemistry to prepare racemic (2*RS*, 3*RS*) and (2*RS*, 3*SR*)-2-methylisocitrate. The synthetic products will be used to determine the stereochemical preferences of MmgF in future studies.

1.4.2 2-Methylcitrate Synthase Objective

To develop a stereoselective synthesis of all four stereoisomers ((2*R*, 3*S*), (2*S*, 3*R*), (2*R*, 3*R*) and (2*S*, 3*S*)) of 2-methylcitrate in an enantiopure fashion. The Shi asymmetric epoxidation specific for α,β-unsaturated esters will be utilized to achieve an enantiopure epoxide product. The four stereoisomers of 2-methylcitrate made from enantiopure epoxides will be used as standards of the products of MmgD (methylcitrate synthase). This will ultimately allow the unambiguous assignment of the stereoisomers

produced by 2-methylcitrate synthases from various species, and will help settle ambiguities between our work with the *B. subtili*s and *E. coli* systems and those that were reported in the literature.

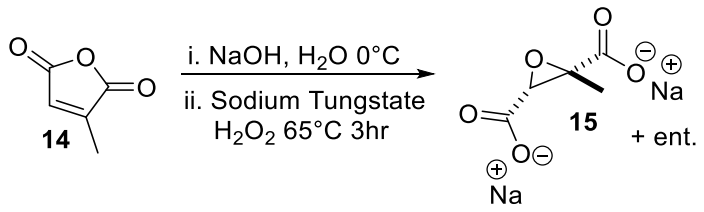
CHAPTER II

EXPERIMENTAL

2.1 Syntheses of (2*RS*, 3*RS*)-2-Methylisocitric Acid

2.1.1 *Cis*-epoxymethylsuccinate Disodium Salt 15

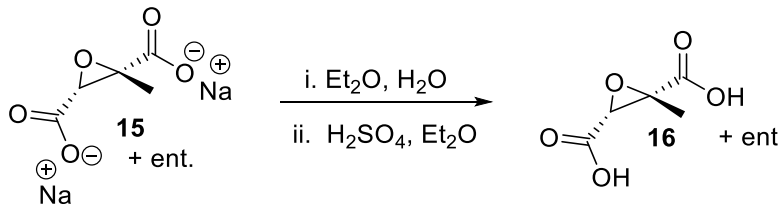
Scheme 6. Reaction Conditions to Obtain **15**



Citraconic anhydride **14** (9.56g, 1 eq) was suspended in water (30 mL) at an ambient temperature and the temperature of the mixture was decreased to 0 ℃. Sodium hydroxide (5.07 g, 1.49 eq) was dissolved in water (7.5 mL) then added slowly to the cooled citraconic anhydride suspension. Sodium tungstate (0.8517 g, 0.05 eq) dihydrate was added to the reaction followed by the dropwise addition of 33% hydrogen peroxide (10 mL, 1.03 eq). The temperature of the reaction was monitored and maintained at 65 ℃ first by cooling then by heating. The reaction was stirred for 3 hours before it was concentrated to half volume by reduced pressure. The desired compound was recrystallized with acetone (12.59 g, 87 % yield). ^{1}H NMR (D_2O, 500 MHz) δ 1.36 (s, 3H) 3.22 (s, 1H).

2.1.2 *Cis*-epoxymethylsuccinic Acid 16

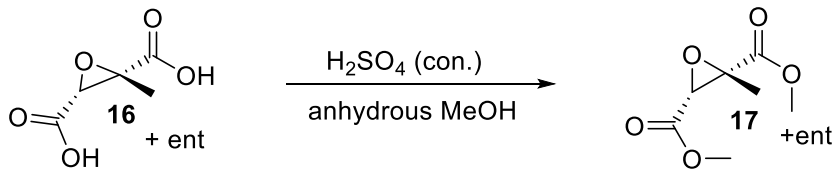
Scheme 7. Reaction Conditions to Obtain **16**



The disodium salt **15** (11.11 g, 1 eq) was dissolved in 300 mL of diethyl ether and 5 mL water. Concentrated sulfuric acid (6 mL, 1.72 eq) was dissolved in 70 mL of diethyl ether then added to the reaction and allowed to stir at room temperature overnight. The solvent was removed with reduced pressure and three triturations were performed on the resulting residue with diethyl ether (3 x 20 mL). The combined extracts were dried over anhydrous magnesium sulfate then the solvent was removed with reduced pressure (7.94 g, 83 % yield) ^{1}H NMR (D_2O, 500 MHz) δ 1.43 (s, 3H) 3.57 (s, 1H).

2.1.3 Dimethyl *cis*-epoxymethylsuccinate 17

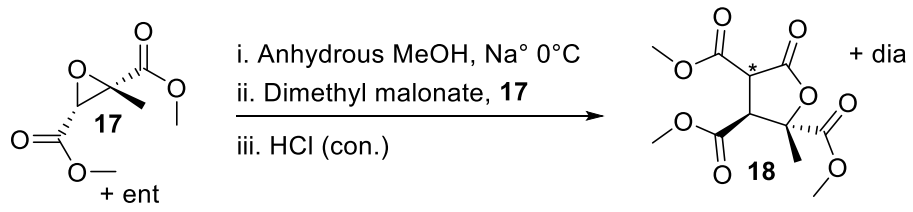
Scheme 8. Reaction Conditions to Obtain **17**



Cis-epoxymethylsuccinic acid **16** (5.05 g, 1 eq) was dissolved in 22 mL of freshly distilled methanol (from Mg/I_2) at room temperature under an argon environment. Concentrated sulfuric acid (0.277 mL, 0.15 eq) was dissolved in dry methanol then added to the reaction. The reaction was allowed to stir for three days at ambient temperature then the solvent was removed with reduced pressure. The resulting oil was taken up into diethyl ether (70 mL) and washed once with 1 M sodium sulfate (20 mL). The organic layer was dried with anhydrous sodium sulfate followed by solvent evaporation with reduced pressure. The crude oil was further purified with flash chromatography over silica gel with 20 % ethyl acetate in hexanes as the eluent (3.54 g, 59 % yield). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.61 (s, 3H), 3.46 (s, 1H), 3.74 (s, 3H), and 3.74 (s, 3H).

2.1.4 Trimethyl 2-methyl-5-oxotetrahydrofuran-2,3,4-tricarboxylate 18

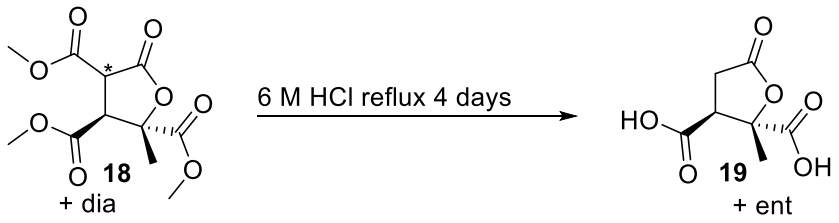
Scheme 9. Reaction Conditions to Obtain **18**



Dry methanol (10 mL) was reacted with sodium metal (0.27 g, 1 eq) at 0 °C. Distilled dimethyl malonate (1.7 mL, 1.3 eq) was added to the reaction at once. When a significant amount of white precipitate was observed dimethyl *cis*-epoxymethylsuccinate **17** (2.05 g, 1 eq) was added, and the mixture became yellow. The reaction was allowed to stir at room temperature for four days under argon atmosphere. Concentrated HCl (5 mL) was added and stirred for an additional hour and a half. The sodium chloride precipitate was removed by vacuum filtration. The solvent from the filtrate was removed under reduced pressure, then the crude oil was dissolved in diethyl ether. The organic layer was washed with water, then the aqueous layer was extracted two times with ether. The combined organic layers were dried with anhydrous magnesium sulfate. The desired product was purified with 30 % ethyl acetate in hexanes. (0.65 g, 20 % yield) ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.48 (s, 3H), 3.72 (s, 3H), 3.75 (s, 3H), 3.76 (s, 3H), 4.04 (d, 1H), and 4.14 (d, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.2, 32,0, 40.7, 49.3 52.7, 53.3, 53.7, 165.3, 168.5, 169.7, 170.6.

2.1.5 Erythro 2-methylisocitrate Lactone 19

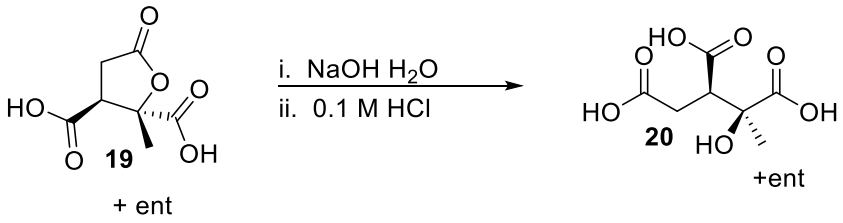
Scheme 10. Reaction Conditions to Obtain **19**



The purified trimethyl 2-methyl-5-oxotetrahydrofuran-2,3,4-tricarboxylate **18** (0.11 g) was heated at reflux for four days with 6 M HCl (6 mL). The solvent was removed by reduced pressure then trace amounts of HCl were removed by evaporation with water three times. The brown and green residue was dissolved in water and treated with activated charcoal for thirty minutes at 60℃. After removal of the carbon by vacuum filtration, the water was removed from the filtrate with reduced pressure. (0.0204 g, 27 % yield)

2.1.6 (2*SR*, 3*SR*)-2-methylisocitrate 20

Scheme 11. Reaction Conditions to Obtain **20**

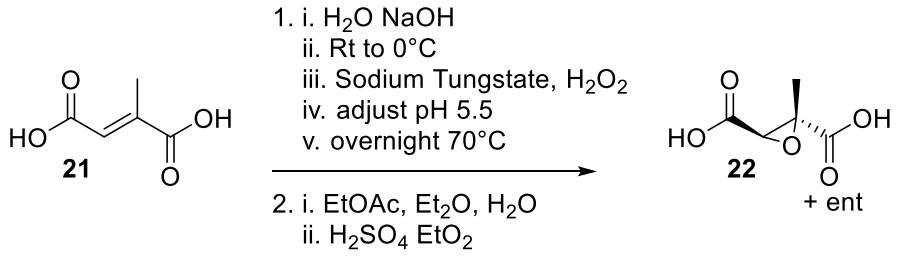


The erythro 2-methylisocitrate lactone **19** (0.05 g, 1 eq) was treated with water (0.331 mL) and sodium hydroxide (0.33 g) at 80 ℃ for 16 hours. The pH was neutralized with 0.1 M HCl then the solvent was removed with reduced pressure. The obtained solid was dissolved in water and treated with activated charcoal at 60 ℃. The mixture was filtered and the filtrate was retained and frozen. $[M\text{-}H]^-$ = 205 m/z

2.2 Synthesis of (2*RS*, 3*RS*)-2-Methylisocitrate

2.2.1 *Trans*-epoxymethylsuccinic Acid 22

Scheme 12. Reaction Conditions to Obtain **22**

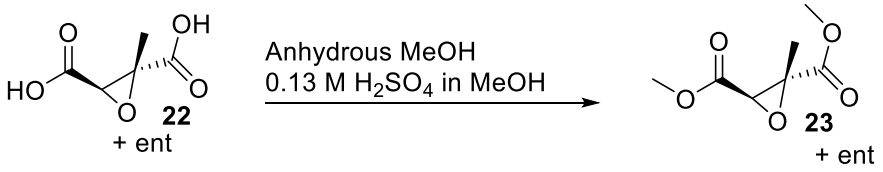


Mesaconic acid **21** (0.55 g, 1 eq) was suspended in nanopure water then treated with sodium hydroxide (0.30 g, 1.8 eq) at room temperature. Once a clear solution was observed the temperature of the reaction was decreased to 0 ℃ over a period of one hour. Sodium tungstate dihydrate (0.05 g, 0.04 eq) was added to the cooled reaction, followed by the dropwise addition of hydrogen peroxide (0.48 mL). After the dropwise addition of hydrogen peroxide was completed, the pH was immediately monitored with pH paper and adjusted to a pH of 5.5 using 6 M sodium hydroxide. The reaction was allowed to stir for one hour at 0 ℃ while the pH of the reaction was monitored and readjusted to pH 5.5. After an hour the reaction was allowed to warm to room temperature, then increased to 70 ℃, and stirred overnight at that temperature under a water-cooled condenser. After the overnight heating, the solvent was removed with reduced pressure. The obtained white solid was mixed with 5 mL ethyl acetate, 5 mL diethyl ether, and 0.5 mL water. The reaction was acidified with concentrated sulfuric acid (0.4 mL) dissolved in 3.5 mL

of diethyl ether, and allowed to stir for 2.5 hours. The organic layer was separated and dried with anhydrous sodium sulfate. The solvent was removed with reduced pressure and a white solid **22** was obtained (0.4955 g, 79 % yield). ^{1}H NMR (D_2O, 400 MHz) δ 1.33 (s, 3H) 3.70 (s, 1H). ^{13}C NMR (D_2O, 100 MHz) δ 12.3, 58.5, 170.5, and 172,7.

2.2.2 Dimethyl *trans*-epoxymethylsuccinate 23

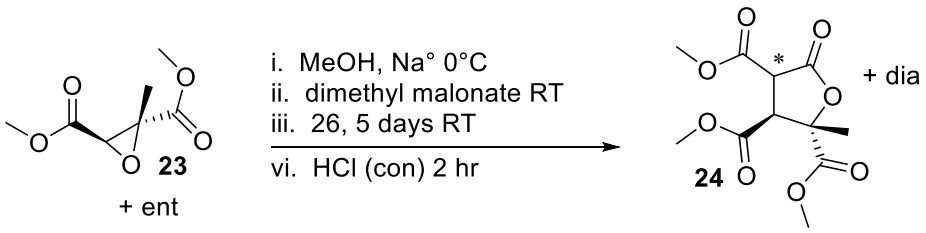
Scheme 13. Reaction Conditions to Obtain **23**



The previously synthesized *trans*-epoxymethylsuccinic acid **22** (0.51 g) was dissolved in 2.2 mL anhydrous methanol under an argon environment. Sulfuric acid 0.13 M in methanol (1.5 mL) was added and allowed to stir at room temperature for 3 days. The solvent was removed with reduced pressure then the oil was dissolved in diethyl ether. The organic layer was washed with 1 M sodium sulfate and dried over anhydrous sodium sulfate. The compound was purified over silica gel with 20 % ethyl acetate and hexanes. The solvent was evaporated with reduced pressure, then trace solvents were removed with high vacuum. A clear oil was obtained. (0.301 g, 51 % yield) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.54 (s, 3H), 3.72 (s, 3H), 3.76 (s, 3H) and 3.76 (s, 1H). ^{13}C ($CDCl_3$, 100 MHz) δ 14.1, 52.9, 52.5, 56.9, 166.7, 169.1.

2.2.3 2-methyl-5-oxo-2,3,4-tricarbomethoxytetrahyrofuran 24

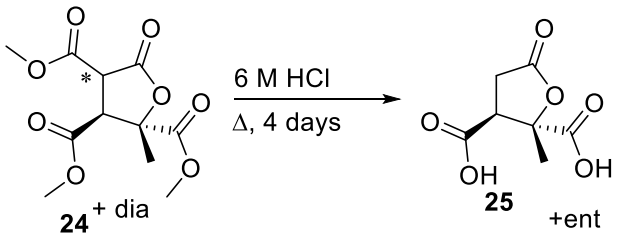
Scheme 14. Reaction Conditions to Obtain **24**



Anhydrous methanol (1.25 mL) at 0 ℃ was reacted completely with sodium metal (0.039 g, 1.2 eq.). The solution was allowed to return to room temperature and freshly distilled dimethyl malonate (0.25 mL, 1.6 eq) was added to the sodium methoxide. The reaction was allowed to stir until a white precipitate formed then the dimethyl ester **23** (0.243 g, 1 eq.) obtained from the previous step was added. The reaction was allowed to stir for five days. Concentrated hydrochloric acid (1 mL) was added to quench the reaction. Sodium chloride formed and was filtered off then the filtrate was concentrated to produce the crude product. A clean NMR spectrum has not yet been obtained to confirm the desired product **24** has been produced. Efforts to purify the compound have been made, but the mixture of compounds is co-eluting. The published literature syntheses did not purify the desired product. The crude material was carried forward in the next reaction. (0.38 g, 67 % yield) [M+H]= 275.0757 m/z

2.2.4 2-methyl-5-oxo-2,3-tricarbotetrahydrofuran 25

Scheme 15. Reaction Conditions to Obtain **25**

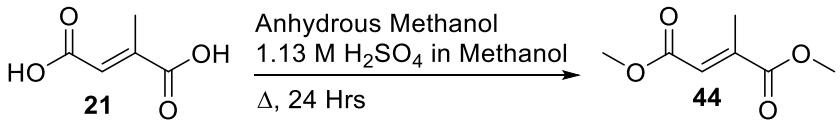


The crude produce **24** (0.2202 g, 1eq) was refluxed in 6 M HCl (4.5 mL) for 4 days followed by the removal of solvent by reduced pressure. The concentrated reaction was dissolved three time in 20 mL of water and evaporated each times to removes residual HCl. The brown oil was dissolved in 20 mL of water and treated with activated charcoal for 30 minutes at 80 °C. The reaction was filtered through a 1 cm bed of celite to remove the charcoal. The solvent was removed with reduced pressure.

2.3 Other Epoxidation Methods

2.3.1 Dimethyl Mesaconate 44

Scheme 16. Reaction Conditions to Obtain **44**

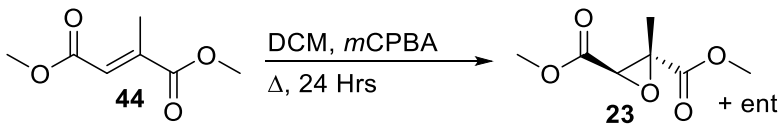


Mesaconic acid **21** (0.1008 g) was dissolved in 1 mL of dry methanol, then 0.9 mL of 0.13 M sulfuric acid in methanol was added. The reaction refluxed for 18 hours

under argon, then the solvent was removed with reduced pressure. The pale yellow oil was dissolved in diethyl ether and washed with 1 M sodium sulfate. The organic layer was dried over anhydrous sodium sulfate followed by the removal of solvent by reduced pressure. The desired compound was purified over silica gel with 10 % ethyl acetate in hexanes. (0.053 g, 43 % yield) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.11 (s, 3H), 3.59 (s, 3H), 3.63 (s, 3H), and 6.59 (s, 1H). ^{13}C NMR ($CDCl_3$. 100 MHz) δ 13.9, 51.4, 52.3, 126.2, 165.9, and 167.2.

2.3.2 Dimethyl *trans*-epoxymethylsuccinate 23 Conditions B

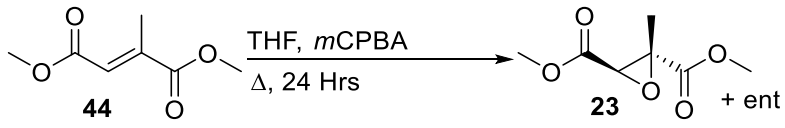
Scheme 17. Reaction Conditions to Obtain **23** from **44** with DCM



*meta-C*hloroperoxybenzoic acid (0.9548 g) was dissolved in dichloromethane (12.5 mL). Dimethyl mesaconate **44** (0.59 g) was added, and the reaction was refluxed for 24 hours. The solvent was removed with reduced pressure and the white solid was dissolved in saturated sodium carbonate and extracted 2 times with diethyl ether (30 mL). The solvent was again removed with reduced pressure and the product was purified over silica gel with 10 % ethyl acetate and hexanes. The desired product was not obtained.

2.3.3 Dimethyl *trans*-epoxymethylsuccinate 23 Conditions C

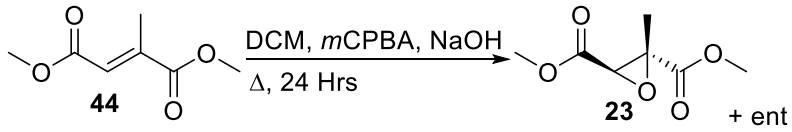
Scheme 18. Reactions Conditions to Obtain **23** from **44** with THF



meta-Chloroperoxybenzoic acid (0.15 g) was dissolved in 1 mL of tetrahydrofuran. To the reaction dimethyl mesaconate **44** (0.17 g) was added and refluxed for 24 hours. The solvent was removed with reduced pressure and the white solid was dissolved in saturated sodium carbonate and extracted with diethyl ether (2 x 15 mL). The solvent was again removed with reduced pressure and purified over silica gel with 10 % ethyl acetate and hexanes. The desired product was not obtained.

2.3.4 Dimethyl *trans*-epoxymethylsuccinate 23 Conditions D

Scheme 19. Reaction Conditions to Obtain **23** with a Nucleophilic Epoxidation

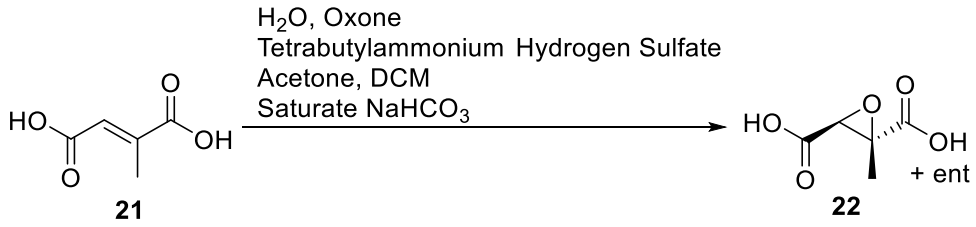


meta-Chloroperoxybenzoic acid (0.4923 g) was dissolved in dichloromethane (4.4 mL) and reacted with sodium hydroxide (0.1374 g). To the reaction dimethyl mesaconate (0.21 g) was added and refluxed for 24 hours. The solvent was removed with reduced pressure followed and the obtained white solid dissolved in saturated sodium

carbonate and extracted 3 times with diethyl ether (25 mL). The solvent was again removed with reduced pressure and purified over silica gel with 5 % ethyl acetate and hexanes. The desired product was not obtained.

2.3.5 *Trans*-epoxymethylsuccinic Acid 22 with Oxone

Scheme 20. Reaction Conditions to Obtain **22** with Curci's Method



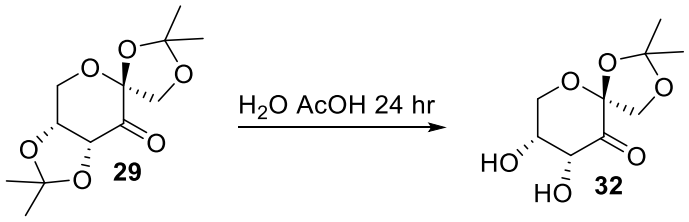
Mesaconic acid **21** (0.1597 g, 1 eq) was dissolved in dichloromethane (5 mL) then tetrabutylammonium hydrogen sulfate (0.1595 g, 3.8 eq.) and acetone (0.5 mL, 5.5 eq) were added followed by the addition of saturated sodium bicarbonate (8.3 mL). The reaction temperature was reduced to 0 ° C. Oxone (1.4653 g, 7.8 eq) was dissolved in 6 mL of water then slowly added to the reaction over 15 minutes then allowed to stir for an additional two hours. The reaction was allowed to stir for another 18 hours at room temperature. The solvent was removed with reduced pressure. The solid was dissolved in diethyl ether (5 mL), ethyl acetate (5 mL), and water (0.5 mL). Sulfuric acid (0.4 mL) was dissolved in 3.5 mL of diethyl ether then added to the reaction. The reaction stirred was at room temperature for two hours. The organic layer was separated and dried over anhydrous magnesium sulfate. The solvent was removed with reduced pressure. ^{1}H

NMR (D_2O, 400 MHz) δ 1.36 and 3.70. ^{13}C NMR (D_2O, 100 MHz) δ 12.3, 54.5, 170.5, and 172.7.

2.4 Modification of the Shi Catalyst for Asymmetric Epoxidations

2.4.1 Shi Catalyst Diol 32 with Acetic Acid

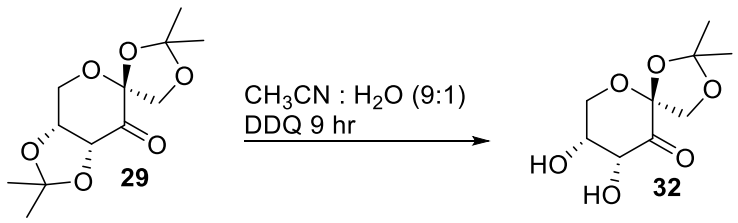
Scheme 21. Reaction Conditions to Obtain **32** with Acetic Acid



A solution of acetic acid (80 mL) and water (20 mL) was added at once to the Shi diketal catalyst **29** (0.06 g). The reaction was allowed to stir for 24 hours at room temperature followed by the removal of the solvent at room temperature. The crude mixture was dissolved in methylene chloride (30 mL), dried with anhydrous magnesium sulfate, and filtered. The filtrate was then evaporated to afford a white solid. ^{1}H NMR ($CDCl_3$, 400MHz) δ 1.33(s, 3H), 1.53 (s, 3H), 3.28 (m, 0.3 H), 3.97 (DD, J = 12, 4 Hz, 1H), 3.99 (d, J = 8 Hz, 1H), 4.32 (d, J = 12 Hz, 1H), 4.39 (m, 1H), 4.67 (d, J = 12 Hz, 1H), and 4.72 (d, J = 4 Hz, 1H) ^{13}C ($CDCl_3$, 100 MHz) δ 26.24, 26.39, 63.32, 69.59, 73.61, 74.20, 104.36, 133.64, and 199.03.

2.4.2 Shi Catalyst Diol 32 with DDQ

Scheme 22. Reaction Conditions to Obtain **32** with DDQ

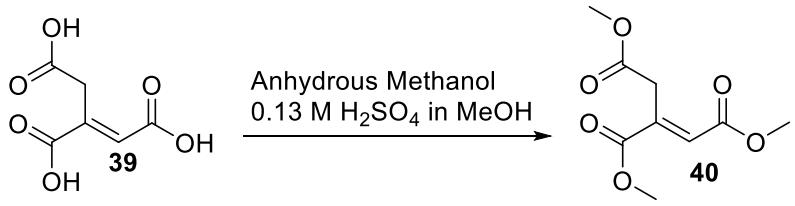


The Shi diketal catalyst **29** (0.0674 g) was dissolved in acetonitrile (0.81 mL) and water (0.09 mL) then DDQ was added. The reaction was allowed to stir for nine hours at room temperature. The reaction was concentrated with reduced pressure at room temperature. The thick red oil was then dissolved in ethyl acetate and dried with anhydrous sodium sulfate, filtered, and the solvent was removed with reduced pressure. The crude product was purified with flash chromatography of silica gel with 10 % methanol in methylene chloride. The desired product was not obtained according to the NMR results.

2.5 Synthesis of Substrates for Asymmetric Epoxidation

2.5.1 Trimethyl-*E*-2-methylaconitate 40

Scheme 23. Reaction Conditions to Obtain **40**



E-aconitic acid **39** (0.7 g, 1 eq.) was dissolved in anhydrous methanol (5 mL) then sulfuric acid (0.05 mL) as added. The reaction was refluxed under argon for 23 hours. The solvent was removed by reduced pressure then dissolved in water and extracted with ethyl acetate (20 mL) three times. The combined organic layers were washed with brine and dried over anhydrous sodium sulfate. The solvent was removed with reduced pressure and purified over silica gel with 30 % ethyl acetate and petroleum ether. (0.026 g, 3 % yield) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.53 (s, 3H), 3.61 (s, 3H), 3.66 (s, 3H), 3.80 (s, 2H), and 6.78 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 32.7, 51.9, 52.0, 52.7, 128.8, 139.8, 165.6, 166.2, 170.1.

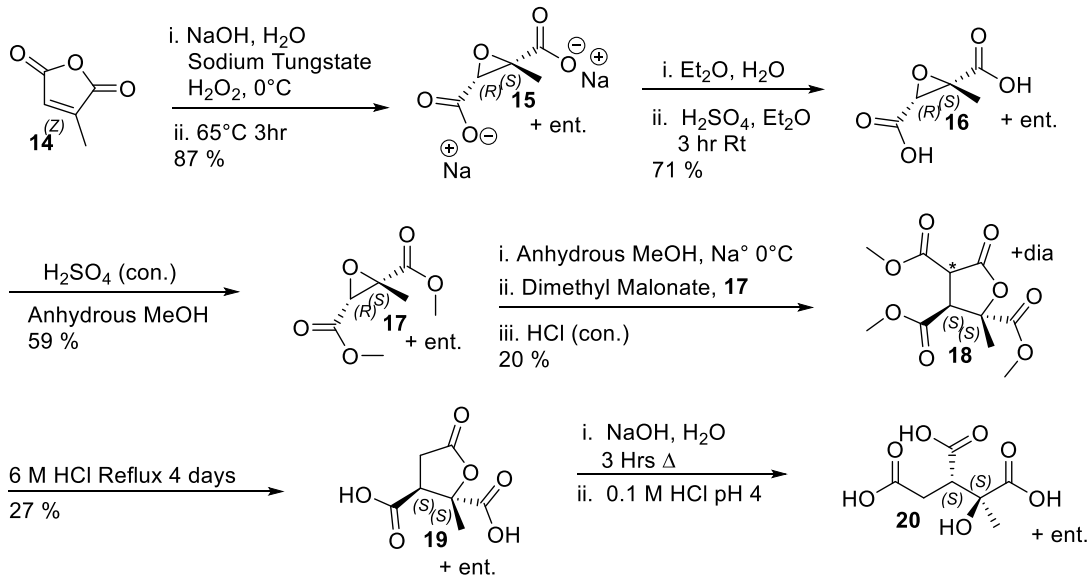
CHAPTER III

RESULTS AND DISCUSSION

3.1 Analysis of ((*2RS,* 3*RS*) and (*2RS,* 3*SR*))-2-Methylisocitrate

3.1.1 Analysis of (2*RS,* 3*RS*)-2-Methylisocitrate

Scheme 24. Modified Literature Procedures for the Citraconic Anhydride Pathway



The published literature procedures by Brock et al. had to be modified greatly in order for the reactions to be reproducible for our lab. The esterification required two reagents to be distilled prior to the reaction. A previous graduate student decided to utilize a Fischer esterification[36] instead because only methanol would need to be distilled. The lactonization was altered by increasing the equivalents of sodium metal used to

deprotonate the dimethyl malonate. In the procedure by Brock et al. it was stated that 1 equivalent of sodium was used, but in the procedure only 0.1 equivalents were reported. The second to last step was altered by increasing the reaction time from three days to four. The final step of the synthesis was not published even though 2-methylisocitrate was used in the enzymology section of the paper. Another paper was consulted to synthesize the desired product; however, the procedure was altered to include 0.1 M HCl instead of Amberlite and the pH was decreased to 4 instead of 7. This change was made because the pK_a values of carboxylic acid are approximately 4.5. Scheme 25 outlines the reactions conditions used to obtain (2*RS*, 3*RS*)-2-methylisocitrate.

The final product was analyzed with mass spectrometry. The calculated mass of the neutral (2*RS.* 3*RS*)-2-methylisocitrate is 206.0427 amu. The mass spectrum analysis was ran in negative mode therefore the mass of a proton must be subtracted, bringing the mass to 205.0354 amu. The observed mass from the analysis was 205.04 amu. This is within the acceptable limits, therefore the desired product was synthesized.

Figure 15 shows the base peak chromatogram obtained from mass spectrometry analysis. In the chromatogram two 205.04 amu peaks were observed. This was due to the epimerization of one of the stereocenters due to all the acid/base chemistry used in the synthesis. The major product peak is the (2*RS*, 3*RS*)-2-methylisocitrate set of enantiomers, the minor peak is the (2*RS*, 3*SR*)-2-methylisocitrate enantiomers.

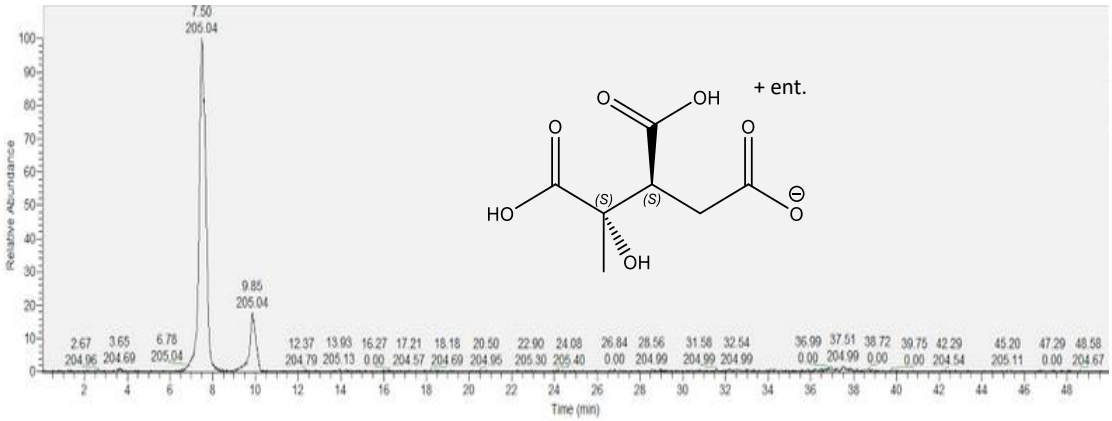


Figure 15. Base Peak Chromatogram of (2*SR*, 3*SR*)-2-Methylisocitrate. The base peak chromatogram obtained from mass spectrometry analysis shows two products with the same weight were obtained. The major product are the (2*RS*, 3*RS*)-2-methylisocitrate enantiomers, the minor peak obtained due to epimerization are the (2*RS*, 3*SR*)-2-methylisocitrate.

3.1.2 Analysis of the (*2RS*, 3*SR*)-2-Methylisocitrate

The stated reaction conditions from the published synthesis required great modifications in order to obtained the desired intermediates in the synthesis of (2*RS*, 3*SR*)-2-methylisocirate. The first reaction in the linear synthesis was a tungstate epoxidation. The epoxide is an important intermediate, because it will eventually become the alcohol group on carbon 3. If the reaction was conducted with the stated conditions from the literature reference the desired epoxide was not obtained, therefore many steps were taken to make the epoxidation more reliable. The modifications were attempted as outlined below.

First, the procedure was modified by removing the three hour stir at room temperature with ethyl acetate, diethyl ether, water and concentrated sulfuric acid dissolved in diethyl ether. Instead the reaction was lowered to a pH of 2 with 0.1 M HCl

to try and extract the epoxy product into the organic layer. The desired product was not obtained.

The second modification of the original procedure involved increasing the equivalents of the base to three to deprotonate both carboxylic acid groups and the hydrogen peroxide. The desired product was not obtained.

The third modification increasing the equivalents of the hydrogen peroxide to both two and three equivalents. The equivalents of the sodium tungstate catalyst and the hydrogen peroxide were also adjusted to determine if more catalyst or oxidant were needed to push the reaction forward *via* le Chatelier's principle. The desired product was not obtained.

Next the starting material was recrystallized to insure that no contaminants were stopping the epoxidations from occurring. The desired product was not obtained.

Then pH of the reaction was monitored to ensure that it stayed around pH 5.5.[37] The pH was changed at different time intervals including immediately after the hydrogen peroxide was added, and other time intervals such as 5, 10, and 30 minutes. The pH was also changed after the one hour cold stir of the mesaconic acid, sodium hydroxide, hydrogen peroxide, and the tungstate catalyst was completed, and immediately before the reaction was increased to 70 ℃. The desired product was obtained only when the pH was changed as soon as the hydrogen peroxide was added.

Once the tungstate and hydrogen peroxides are introduced to the reaction the pH would drop between 4 and 4.5. At such low pH's the tungstate catalyst tends to take on a polymeric form;[37] however, when the pH is increased to 5.5 the catalyst spreads

homologously throughout the reaction.[37] This allows the catalyst to take on the bidentate form with the substrate and the epoxidation will occur.

The final product in the linear synthesis has not been obtained yet, but the intermediate **24** has been confirmed by mass spectrometry analysis, this was due to the inability of the intermediate to be identified by NMR. The obtained NMR spectrum contained several unexpected peaks due to the inability to purify the desired compound and the presence of eight stereoisomers consisting of four major diastereomers and four minor diastereomers. This is due to the diastereomers obtained from the opening of the epoxide with dimethyl malonate. The epoxide is opened on the least sterically hindered side. The opening of the epoxide allows for rotation around the 3C and 4C bond. Even though there are eight stereoisomers present in the reaction, the amount of stereoisomers will be reduced in the next step due to the loss of the temporary stereocenter.

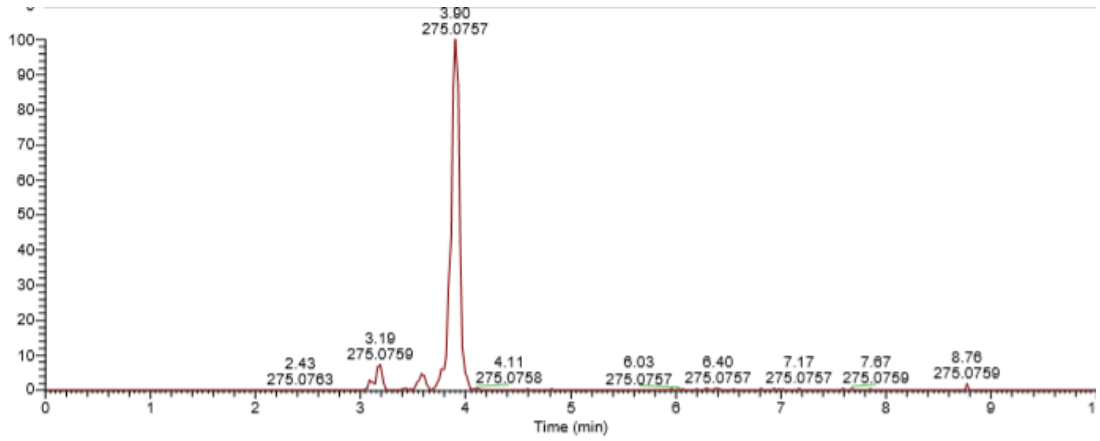
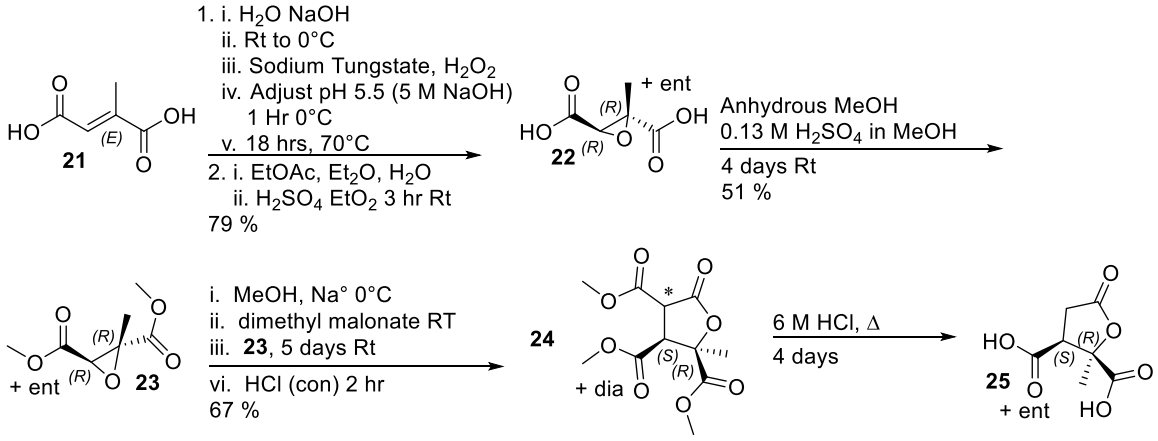


Figure 16. Base Peak Chromatogram of 2-methyl-5-oxo-2,3,4-tricarbomethoxytetrahydofuran. The base peak chromatogram obtained from mass spectrum analysis shows two product peaks. This is due to three stereocenters formed during the reaction. There is due to diastereomer formation and 8 stereoisomers being present.

The exact mass of 2-methyl-5-oxo-2,3,4-tricarbomethoxytetrahyrofuran is 274.0767 amu. Since the analysis was run in positive mode the weight of a proton must be added to the molar mass bringing the calculated mass to 275.0767 amu. The observed mass was 275.0757 amu which has a mass error of 3.6 ppm. The acceptable mass error is 5 ppm, therefore the lactone was synthesized. Figure 16 shows the base peak chromatogram obtained from mass spectrometry analysis. The obtained spectrum shows two 275.0757 amu peaks The linear reaction sequence with modified literature conditions is shown in Scheme 25.

Scheme 25. Modified Literature Procedures for the Mesaconic Pathway



3.1.3 Other Epoxidation Methods

In an effort to side step the tungstate epoxidation to form *trans*-2-methylisocitrate **22** other methods were explored such as *m*CPBA with various solvents and the addition of a base, but the epoxide was never formed. Another method tried utilized the dioxirane ring formed with Oxone and acetone. The desired epoxide was obtained with this

method, but the *trans*-epoxy acid was unable to be separated from the starting material as seen in Figure 17. The methyl group of the starting material appears at 1.99 ppm and a vinyl proton is observed at 6.63 ppm. The *trans*-epoxy acid peak appear at 1.39 and 3.74 ppm. Integration of the peaks indicated there was approximately 60 % conversion of the starting material to the desired epoxide.

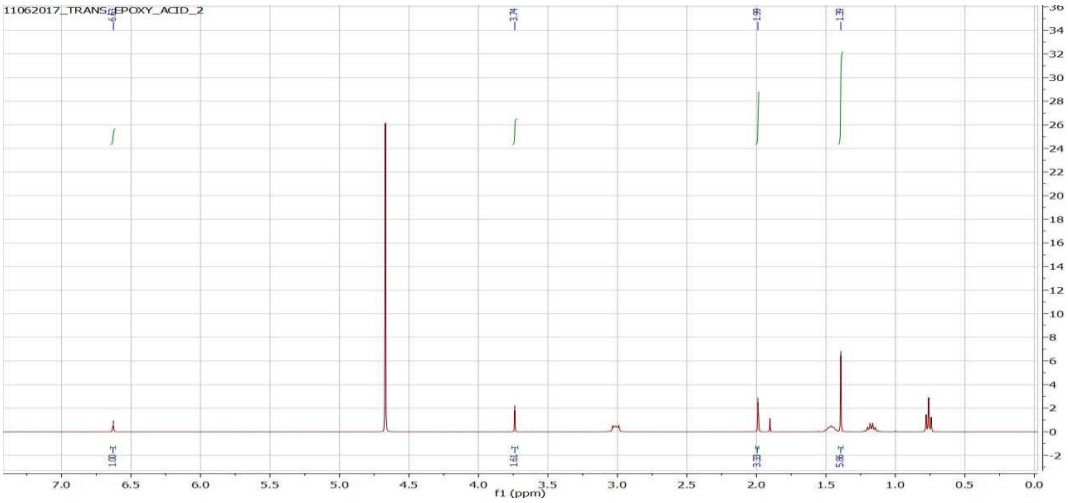


Figure 17. ^{1}H NMR Analysis of Oxone Reaction. The obtained ^{1}H NMR (D_2O, 400 MHz) shows both starting material and the desired epoxide present. The mesaconic acid peaks appear at 3.63 and 1.99 ppm. *Trans*-epoxymethylsuccinic acid peak are located at 1.39 and 3.74 ppm.

3.2 Enantiopure Synthesis of 2-Methylcitrate

3.2.1 Modification of the Shi Ligand

The modification to the commercially available Shi chiral ketone ligand has proven to be more difficult than expected. The vicinal diol **32** is unstable; therefore, the purification of **32** has not been successful. When the solvent was removed with reduced pressure and heated the molecule degrades and vinyl protons are present in the NMR

spectrum even though there are no vinyl protons present in **32.** Also if flash column chromatography was used for purification the desired product **32** would degrade.

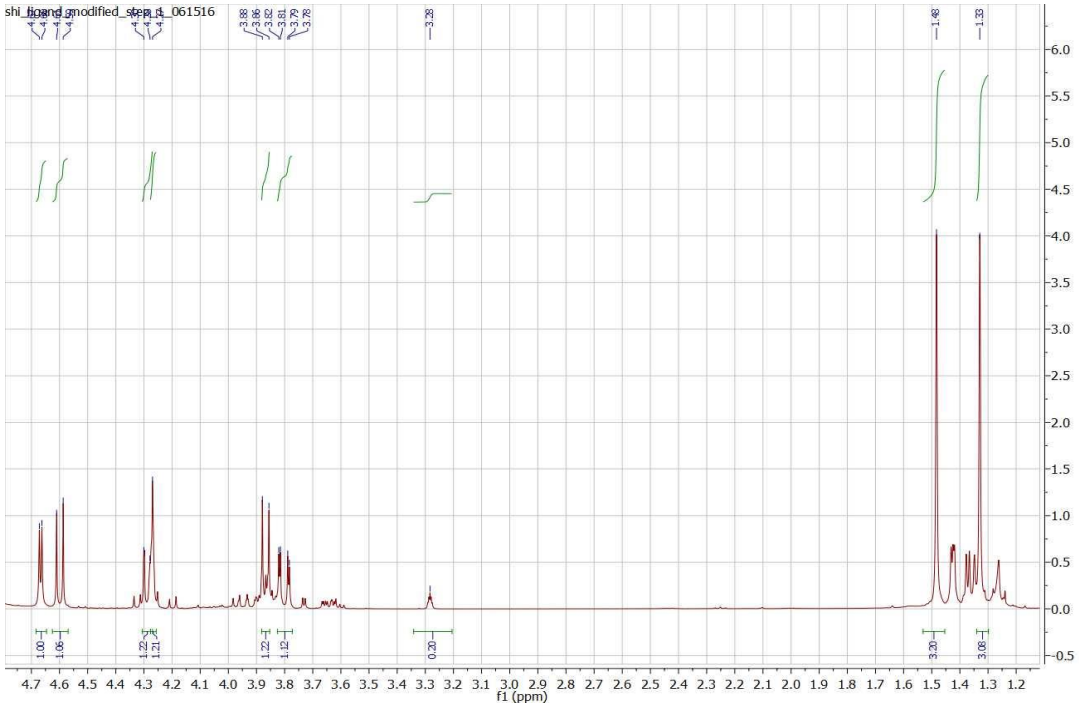


Figure 18. ^{1}H NMR of Modified Shi Ligand. The Shi ligand was analyzed with ^{1}H NMR. When compared to the reference peaks and J-coupling constants it was determined that the desired product was synthesized.

In an effort to reduce the acidity of the silica gel used in the purification process the column was pretreated with triethylamine. However, the desired product was still not obtained. Triethylamine was then included in the eluent during purification, but the silica gel still degraded **32**.To confirm the presence of the vicinal diol **32** NMR analysis of the crude product was performed. The ^{1}H NMR in Figure 18 shows the desired product peaks. The chemical shifts and calculated J-coupling constants correspond to the

reported literature values. To further confirm the desired product ^{13}C NMR was obtained as seen in Figure 19.

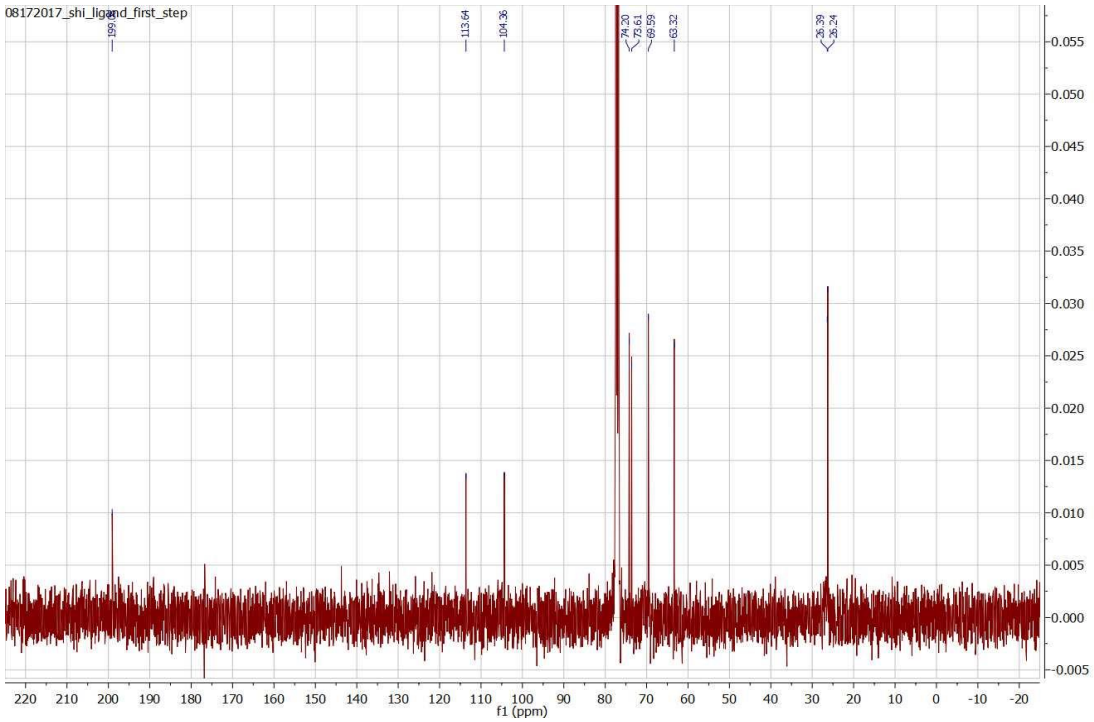


Figure 19. ^{13}C NMR of Modified Shi Ligand. The ^{13}C NMR was used to insure all the carbons were intact since the proton NMR was not clean due to an inability to purify the compound.

3.2.2 Analysis of Substrate for Asymmetric Epoxidation

The substrate for the Shi asymmetric epoxidation was synthesized with a 3 % yield due to the volatility of the trimethyl *E*-aconitate. During the evaporation of the solvent the compound was put under reduced pressure which decreased the boiling point of the solvent and the desired product. Due to the decreased boiling point the compound was removed along with the solvent. This can be overcome by using a heavier protecting

group for the carboxylic acids. Tert-butyl or benzyl group could be used instead of the methyl esters.

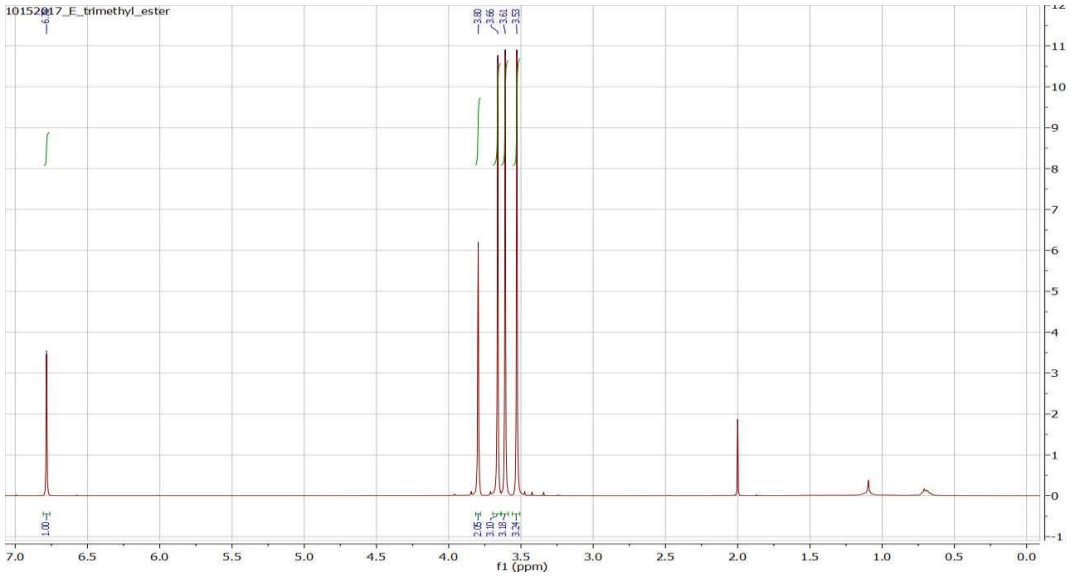


Figure 20. ^{1}H NMR of Trimethyl *E*-Aconitate. ^{1}H NMR was conducted to confirm the presence of the desired compound. The product peaks are observed at 3.53, 3.61, 3.66, 3.80, and 6.78 ppm.

The desired product was confirmed with NMR analysis shown in the Figure 20. The ^{1}H NMR showed three methyl esters at 3.53, 3.61, and 3.66 ppm, a vinyl proton at 6.78 ppm, and a methylene group 3.80 ppm. The protons of the methylene group are shifted downfield due to the electron withdrawing groups on either side leading to the de-shielding of the protons. Acetone is present in the spectrum at 2.01 ppm due to residual solvent used to clean the flash column chromatography fraction tubes.

3.3 Conclusion

Both of the projects presented here involved organic synthesis of substrates for the enzymatic study of MmgD and MmgF. The published literature procedures presented many issues that needed to be solved. Through a systematic problem-solving approach the linear syntheses of (2*RS*, 3*RS*)-2-methylisocitrate was successfully completed. The synthesis of (2*RS*, 3*SR*)-2-methylisocitrate required many modifications especially with the tungstate epoxidation. The linear syntheses presented in this work can be replicated successfully and will lead to the production of the desired products.

The synthesis of enantiopure 2-methylcitrate has been designed utilizing the asymmetric Shi epoxidation to set the stereocenters. Future work on this project should include the completion of the needed modification for the Shi ligand to successfully form an epoxide on an α,β-unsaturated ester, followed by the total synthesis of the enantiomer of the Shi ligand for α,β-unsaturated esters. With these Shi ligands all four diastereomers of 2-methylcitrate can be obtained, then used as standards to compare to the enzymatic product of MmgD.

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