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Bacillus subtilis is the gold standard organism for Gram-positive bacteria, just as *Escherichia coli* is it for Gram-negative bacteria. Its genome has been fully sequenced, yet not all of its genes have been characterized. One of these genes, *yqiQ,* is thought to function in the methylcitric acid cycle as a methylisocitrate lyase, homologous to its *E. coli* counterpart, *prpB*. YqiQ would separate the appropriate enantiomer of the *threo* 2-methylisocitrate pair into succinate and pyruvate. These enantiomorphs, however, are not available commercially, and must be synthesized before characterization of *yqiQ* is possible. Current literature is lacking in a complete start-to-finish synthesis of *threo* 2-methylisocitrate, and is discrete regarding the individual reactions required for its synthesis. This thesis explores steps taken towards collecting, merging and improving upon these individual reactions.

STUDIES ON THE SYNTHESIS OF *THREO* 2-METHYLISOCITRATE

by

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Master of Science

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Approved by

_____
Committee Chair

To my beautiful parents, without whom—and without whose boundless love, guidance, encouragement and support—this thesis may very well have remained unfinished.

APPROVAL PAGE

This thesis written by NICOLAS CHRISTOPHER COFFEY has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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Date of Final Oral Examination

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CHAPTER I

BACKGROUND AND OVERVIEW

I.A. Background

Insofar as *Escherichia coli* is the primary model organism for Gram-negative bacteria, *Bacillus subtilis* plays this role for Gram-positive bacteria. Its 4,214,810 base pairs have been completely sequenced (Kunst, 1997), and while less than 25% of its protein-coding genes have been characterized (Vagner, 1998), it is still the most fully characterized Gram-positive bacterium (Kunst, 1997).

B. subtilis is soil-dwelling and exists primarily in a vegetative state—it grows if nutrients are abundant and if surrounding bacterial density is not high; it does not if conditions are less "agreeable" but not stressful. For example, a reduction in glucose—the preferred carbon source—would not immediately send the organism into sporulation mode. It would first try a number of alternatives to improve its situation. These include but are not limited to: flagellar locomotion to locate new sources of nutrition, including DNA to be endocytosed and catabolized; and secretion of antibiotics to eliminate competition (Stephens, 1998).

When stressful situations do arise, *B. subtilis* will ultimately undergo sporulation, a process by which it enters a state of biochemical hibernation in order to "ride it out" until favorable conditions return. This is its last-ditch effort for survival. Many metabolic pathways are associated with this process, one of which involves six enzymes coded for

by six open reading frames (ORFs) within the metabolic mother cell genes (*mmg*) operon. It has been shown that the first three ORFs—*mmgA*, *mmgB*, *mmgC*—code for the fatty acid metabolizing enzymes acetoacetyl-CoA thiolase (Reddick, 2008), 3-hydroxybutyryl-CoA dehydrogenase (Vegunta, 2011), and acyl-CoA dehydrogenase (Smith, 2009), respectively. The *mmgD* gene encodes a citrate/methylcitrate synthase (Acharya, 2009); it was recently characterized (Hage, 2014). It also has been shown by the Reddick research group that MmgE exhibits 2-methylcitrate dehydratase activity, producing 2-methylaconitate (Hardesty, 2012), and that CitB, a citric acid cycle enzyme in *B. subtilis* that works in both cycles, converts 2-methylaconitate to 2-methylisocitrate (Sirkisoon, 2014).

The sixth and last ORF in this operon, *yqiQ*, is directly related to the subject of this thesis. Its homology to *prpB* in *E. coli* suggests that it encodes a 2-methylisocitrate lyase (Booth, 2011; Brock, 2001). Booth showed activity in the reverse sense; i.e., feeding succinate and pyruvate to YqiQ yields 2-methylisocitrate. This thesis deals with the synthesis of *trans*-2-methylisocitrate, which future research will use to characterize *yqiQ*. Please see Figure 1 (p. 2) for an overview of the methylcitric acid cycle, followed by Figure 2 (p. 2) for just the proposed reaction of YqiQ.

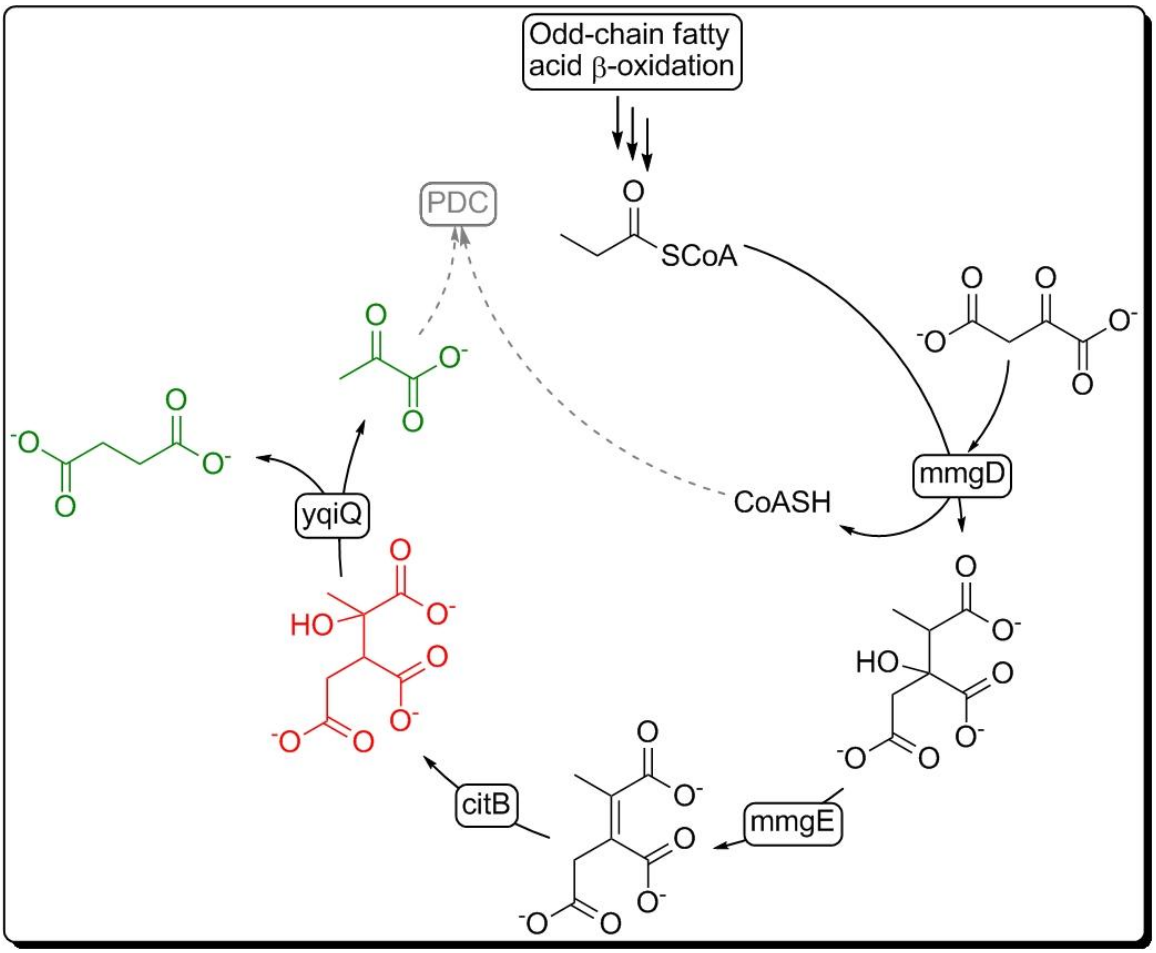

Figure 1. Overview of the Methylcitric Acid Cycle. Substrate of YqiQ—2-methylisocitrate—in red; products of YqiQ—succinate and pyruvate—in green. (PDC = Pyruvate dehydrogenase complex.)

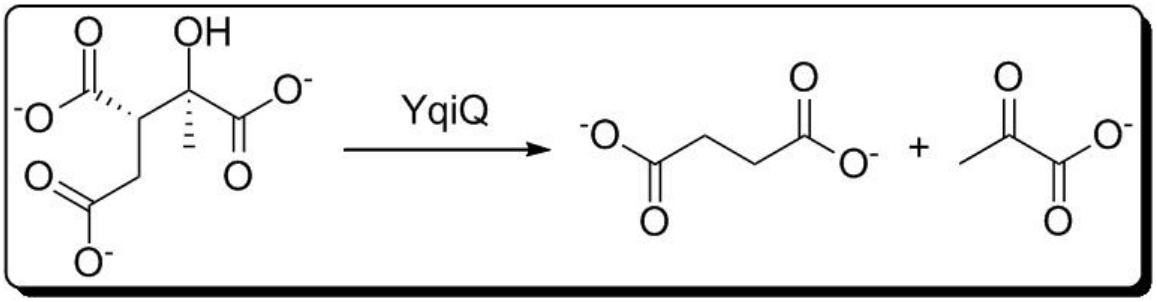

Figure 2. Proposed Reaction of YqiQ. The enzyme converts 2-methylisocitrate to succinate and pyruvate. This example shows one of two enantiomers of *threo* 2-methylisocitrate; specifically, (2*R*,3*S*)-2-methylisocitrate.

I.B. Overview

Bacillus subtilis is a Gram-positive bacterium whose genome was fully sequenced. Both of these attributes help to make *B. subtilis* a model organism: it can be grown on antibacterial media to prevent contamination, and already-characterized genes from other bacteria can be used to predict which analogous genes in *B. subtilis* control what.

When nutrients (and oxygen) are plentiful, *B. subtilis* exists as a vegetative organism, growing and multiplying. Though it was shown that *B. subtilis* can survive without oxygen (Nakano, 1998), other stressful situations—nutrient depletion and extreme temperature changes, for example—will lead the organism to produce dormant spores, able to tolerate an extreme environment indefinitely until conditions improve.

The metabolic pathways of this sporulation are not, however, fully understood yet. For the sake of knowledge qua knowledge notwithstanding, a complete understanding of sporulation and its associated metabolic pathways would help to optimize the industrial use of *B. subtilis*. The *yqiQ* gene, along with its sibling genes *mmgABCDE*, collectively form the *mmg* operon. Sequence homology would suggest that these genes are involved in fatty acid metabolism (Bryan, 1996) and in steps of the methylcitric acid cycle (Brock, 2001), both of which could be important chemical routes to antibiotics and surfactants, for example (Reddick, 2008). The substrate of YqiQ, 2-methylisocitrate, is the subject of this thesis.

CHAPTER II

OBJECTIVES AND SIGNIFICANCE

II.A. Objectives

A long-term goal of the Reddick research group is to characterize fully the *mmg* operon of *B. subtilis*. The overall objective of this thesis research is to synthesize *threo* 2-methylisocitrate, the substrate for YqiQ. A complementary objective of the research group is to understand the role of *yqiQ* with respect not only to the *mmg* operon, but also to the entire set of metabolic pathways involved in sporulation. It is hypothesized that YqiQ functions analogously to PrpB from *E. coli*; that is, as a 2-methylisocitrate lyase that yields succinate and pyruvate. The rationale that underlies the research of this thesis is that 2-methylisocitrate is commercially unavailable.

Increased knowledge of sporulation-related metabolic pathways (fatty acid degradation and the methylcitric acid cycle) would afford a more thorough understanding of the "contingency plans" present in these organisms, which is important to the medical and industrial sectors, some of whose products—antibiotics and surfactants, respectively—stem directly from *B. subtilis*. Progress towards the long-term goal of the Reddick laboratory and of this Master's thesis was realized through the following specific objective: **To develop an improved synthesis of *threo* 2-methylisocitrate.** Our approach would have us convert mesaconic acid, through several innovative steps, to *threo* 2-methylisocitrate.

II.B. Significance

Synthesis of 2-methylisocitrate is necessary to determine whether *yqiQ* codes for a methylisocitrate lyase. The molecule is not available commercially, and while the enzyme, YqiQ, has been shown to generate 2-methylisocitrate when fed pyruvate and succinate (Booth, 2011), it was with yields too low to purify effectively to use in the reverse reaction.

The *mmg* operon comprises three genes that deal with fatty acid metabolism, two genes that have been confirmed to deal with the methylcitric acid cycle, and one gene, *yqiQ*, that is purported to play a role in the methylcitric acid cycle. These two pathways—fatty acid metabolism and the methylcitric acid cycle—are crucial for energy harvest in a nutrient-depleted environment. Learning how exactly *B. subtilis* performs these tasks would not only enrich the scientific knowledge base (e.g., by providing more data for gene annotation), but could offer insight into how other, less well-studied sporulating bacteria behave, which in turn would lead scientists closer to a comprehensive, holistic understanding of the Gram-positive prokaryotic cell. In addition, more specific antibacterial drugs could be designed as a result of a broadened understanding of both the stressors that affect bacteria, and of the survival techniques that these stressors elicit.

CHAPTER III

EXPERIMENTAL SECTION

III.A. Methodology to Yield Pure Dimethyl *trans*-Epoxymethylsuccinate

Dozens of attempts were made to synthesize the *threo* enantiomers of 2-methylisocitrate. Routine strict replication of a published method (Brock, 2001) for this synthesis consistently yielded poor or no results, often prior to the final reaction. Through a combination of creativity, trial and error, and literature searches for homologous reactions and their methods, successful steps were taken towards the synthesis of *threo* 2-methylisocitrate.

Detailed below are six nonconsecutive attempts, yet overall in chronological order, whose results each provided diagnostic information to help refine a working synthesis. Please see Chapter IV for this information and analysis thereof.

III.A.1. Attempt 1

III.A.1.a. Reaction 1: *trans*-epoxymethylsuccinate disodium salt

To a mixture of mesaconic acid (11.0 g, 84.5_5 mmol) and water (30 mL) stirring at 0 °C was added sodium hydroxide (4.998 g, 124.9_6 mmol) dissolved in water (10 mL). Sodium tungstate (4.646 g, 15.81_1 mmol) was added next and upon dissolution, hydrogen peroxide 30% (10 mL, 97.9_1 mmol) was added. The solution was transferred to an oil bath at 65 °C and stirred for 90 minutes. The solvent was evaporated by vacuum to half volume and then transferred drop-wise into an excess of acetone. The acetone was

decanted and the oily precipitate was transferred drop-wise to a fresh excess of acetone. This was repeated once more. The precipitate was heated, then put on ice to attempt recrystallization. After a failed recrystallization, the solvent and any acetone were evaporated by vacuum to yield 16.07 g (84.55_1 mmol, 100%) theoretical titular compound. NMR parameters were as follows: ^{1}H-NMR (500 MHz, D_2O): δ = 1.28 (3 H, s, CH_3), 3.30 (1 H, s, OC*H*).

III.A.1.b. Reaction 2: *trans*-epoxymethylsuccinic acid

Diethyl ether (400 mL) was added to a solution of *trans*-epoxymethylsuccinate disodium salt (15.71 g, 82.65_7 mmol) in no more water than needed to dissolve it, to which was added a solution of sulfuric acid (9.30 mL, $174._5$ mmol) in ether (50 mL). This solution was stirred at room temperature for 3 hours. The solvent was decanted, and fresh ether (100 mL) was twice added to the residue, swirled, and decanted into the original volume of ether. These combined volumes of ether were dried with anhydrous magnesium sulfate and evaporated by vacuum to yield 7.55 g (51.6_7 mmol, 63%) titular compound as crystals. NMR parameters were as follows: ^{1}H-NMR (500 MHz, D_2O): δ = 1.37 (3 H, s, CH_3), 3.66 (1 H, s, OC*H*).

III.A.1.c. Reaction 3: dimethyl *trans*-epoxymethylsuccinate

Dry methanol (100 mL) and thionyl chloride (3.13 mL, 43.0_7 mmol) were combined with stirring at 0 °C. *Trans*-epoxymethylsuccinic acid (6.01 g, 41.1_3 mmol) was added, and the solution was stirred at room temperature for 48 hours. The solvent was evaporated by vacuum, and the residue was redissolved in ether (100 mL) and washed with 1 M $NaHCO_3$ (100 mL). The organic layer was dried with anhydrous

magnesium sulfate and evaporated by vacuum to yield 6.38 g (36.6_3 mmol, 89%) crude titular compound as an oil. The crude oil was purified via flash chromatography using a 70:30 chloroform:petroleum ether solvent system to yield 4.45 g (25.5_5 mmol, 62%) purified titular compound. NMR parameters for the crude compound were as follows: ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.60, 3.78, 3.82, 3.83. Integration (~25:4:4:1, respectively) did not match the expected 3:3:3:1 pattern. NMR parameters for the purified compound, used in the subsequent reaction, were more complex and were as follows: ^{1}H-NMR (500 MHz, D_2O): δ = 1.39, 1.41, 1.65, 3.60, 3.61, 3.62, 3.64, 3.67, 3.68, 3.83, 4.77, 4.98.

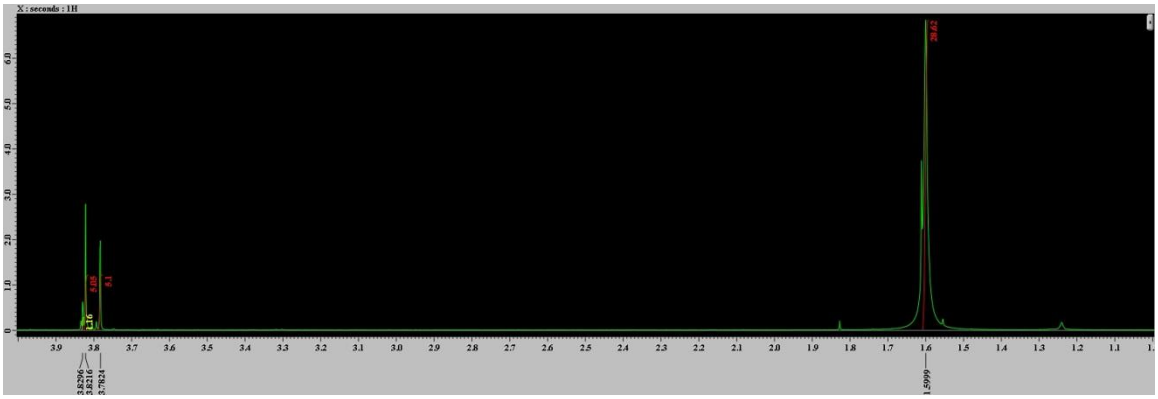

Figure 3. Attempt 1, Reaction 3: Dimethyl *trans*-Epoxymethylsuccinate, Crude, ^{1}H-NMR. NMR parameters were as follows: ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.60, 3.78, 3.82, 3.83. PPM range: 1.0-4.0.

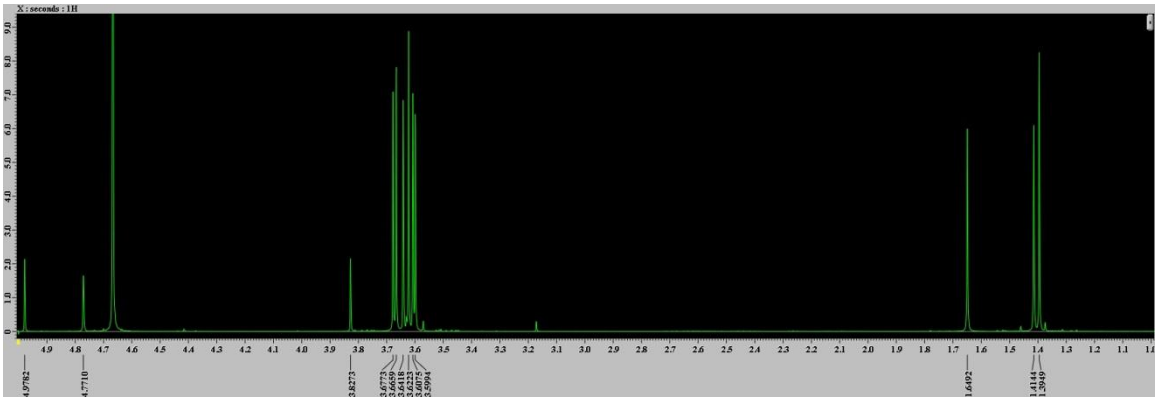

Figure 4. Attempt 1, Reaction 3: Dimethyl *trans*-Epoxymethylsuccinate, Purified, ^{1}H-NMR. Purified from Fig 1. NMR parameters were as follows: ^{1}H-NMR (500 MHz, D_2O): δ = 1.39, 1.41, 1.65, 3.60, 3.61, 3.62, 3.64, 3.67, 3.68, 3.83, 4.77, 4.98. PPM range: 1.0-5.0.

III.A.2. Attempt 2

III.A.2.a. Reaction 1: *trans*-epoxymethylsuccinate disodium salt

To a mixture of mesaconic acid (11.0 g, 84.5_5 mmol) and water (40 mL) was added a solution of sodium hydroxide (7.102 g, 177.5_5 mmol) dissolved in water (20 mL). Sodium tungstate dihydrate (5.578 g, 16.91 mmol) was added next and upon dissolution, hydrogen peroxide 30% (10.363 mL, 101.46 mmol) was added. The solution was heated to between 65 and 85 °C and stirred for 2 hours. The solvent was evaporated by vacuum to obtain 22.91 g of a white powder. Factoring out the tungstate decreased the total theoretical titular compound to 17.332 g (91.19 mmol, 108%). NMR parameters were as follows: ^{1}H-NMR (500 MHz, D_2O): δ = 1.74 (3 H, s, CH_3), 6.28 (1 H, s, OC*H*).

III.A.2.b. Reaction 2: *trans*-epoxymethylsuccinic acid

Diethyl ether (100 mL) was added to a solution of *trans*-epoxymethylsuccinate disodium salt (16.412 g, 86.35 mmol) in water (20 mL), to which was added a solution of sulfuric acid (10.586 mL, 198.6_1 mmol) in ether (40 mL). This solution was stirred at

room temperature for 3 hours. The solvent was decanted, and fresh ether (100 mL) was twice added to the residue, swirled, and decanted into the original volume of ether. These combined volumes of ether were dried with anhydrous magnesium sulfate and evaporated by vacuum to yield 9.79 g (67.0_1 mmol, 78%) theoretical titular compound as a white powder. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 2.20 (3 H, s, CH_3), 6.73 (1 H, s, C*H*).

III.A.2.c. Reaction 3: dimethyl *trans*-epoxymethylsuccinate

Dry methanol (50 mL) and thionyl chloride (11.194 mL, 154.12_3 mmol) were combined with stirring at 0 °C. *Trans*-epoxymethylsuccinic acid (9.79 g, 67.0_1 mmol), dissolved in dry methanol (50 mL), was added, and the solution was stirred at room temperature for 48 hours. The solvent was evaporated by vacuum, and the residue was redissolved in ether (100 mL) and washed with 1 M $NaHCO_3$ (100 mL). The organic layer was dried with anhydrous magnesium sulfate and evaporated by vacuum to yield 10.59 g (60.81 mmol, 91%) titular compound as an oil. NMR parameters were as follows: ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 2.27 (3 H, d, CH_3), 3.75 (3 H, s, OCH_3), 3.79 (3 H, s, OCH_3), 6.77 (1 H, q, C*H*).

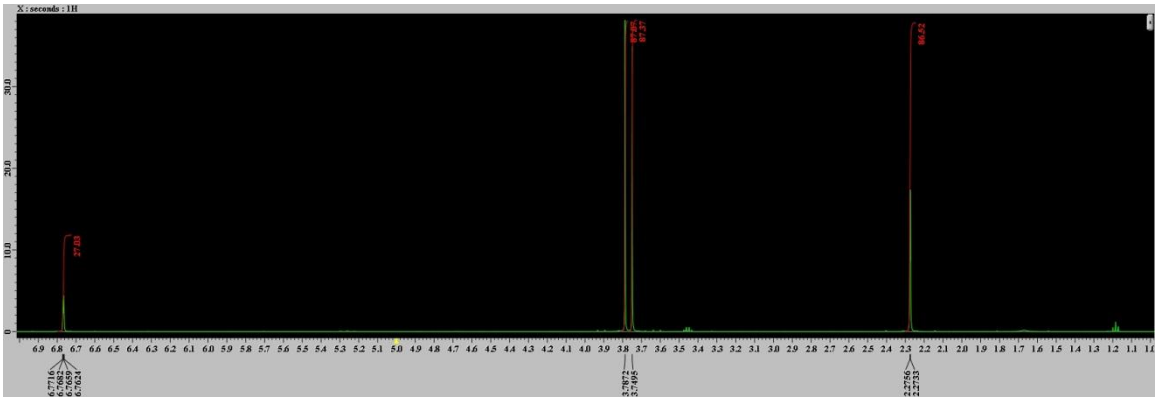

Figure 5. Attempt 2, Reaction 3: Dimethyl *trans*-Epoxymethylsuccinate, ^{1}H-NMR. NMR parameters were as follows: ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 2.27 (3 H, d, C*H*$_3$), 3.75 (3 H, s, OC*H*$_3$), 3.79 (3 H, s, OC*H*$_3$), 6.77 (1 H, q, C*H*). PPM range: 1.0-7.0.

III.A.3. Attempt 3

III.A.3.a. Reaction 1: *trans*-epoxymethylsuccinate disodium salt

To a mixture of mesaconic acid (5.017 g, 38.56_3 mmol) and water (60 mL) was added a solution of sodium hydroxide (2.283 g, 57.07_3 mmol) dissolved in water (15 mL). Sodium tungstate dihydrate (2.379 g, 7.211 mmol) was added next and upon dissolution, hydrogen peroxide 35% (3.918 mL, 44.73_3 mmol) was added. The solution was heated to between 65 and 70 °C and stirred for upwards of 2 hours. The solvent was evaporated by vacuum to obtain 9.02 g of a white powder. Factoring out the tungstate decreased the total theoretical titular compound to 6.901 g (36.31 mmol, 94%). NMR parameters were as follows: ^{1}H-NMR (500 MHz, D_2O): δ = 1.27 (3 H, s, C*H*$_3$), 3.29 (1 H, s, OC*H*).

III.A.3.b. Reaction 2: *trans*-epoxymethylsuccinic acid

Diethyl ether (169 mL) was added to a solution of *trans*-epoxymethylsuccinate disodium salt (6.901 g, 36.31 mmol) in water (7 mL), to which was added a solution of

sulfuric acid (4.088 mL, 76.68_7 mmol) in ether (20 mL). This solution was stirred at room temperature for 3 hours. The solvent was decanted, and fresh ether (100 mL) was twice added to the residue, swirled, and decanted into the original volume of ether. These combined volumes of ether were dried with anhydrous magnesium sulfate and evaporated by vacuum to yield 4.53 g (31.0_1 mmol, 85%) titular compound as a white powder. NMR parameters were as follows: ^{1}H-NMR (500 MHz, D_2O): δ = 1.38 (3 H, s, C*H*$_3$), 3.73 (1 H, s, OC*H*).

III.A.3.c. Reaction 3: dimethyl *trans*-epoxymethylsuccinate

Dry methanol (80 mL) and thionyl chloride (4.617 mL, 63.57_1 mmol) were combined with stirring at 0 °C. *Trans*-epoxymethylsuccinic acid (4.53 g, 31.0_1 mmol), dissolved in dry methanol (20 mL), was added, and the solution was stirred overnight at room temperature. The solvent was evaporated by vacuum, and the residue was redissolved in ether (100 mL) and washed with 1 M $NaHCO_3$ (100 mL). The organic layer was dried with anhydrous magnesium sulfate and evaporated by vacuum to yield 4.68 g (26.8_7 mmol, 87%) crude titular compound as an oil. An attempt at purifying the crude oil via flash chromatography was made using a 70:30 hexanes:ethyl acetate solvent system. The purified oil was subjected to ^{1}H-NMR using three separate solvents ($CDCl_3$, D_2O, CD_3OD); CD_3OD produced the least confusing results. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.51, 1.52, 1.75, 3.72, 3.73, 3.75, 3.78, 3.78, 3.83.

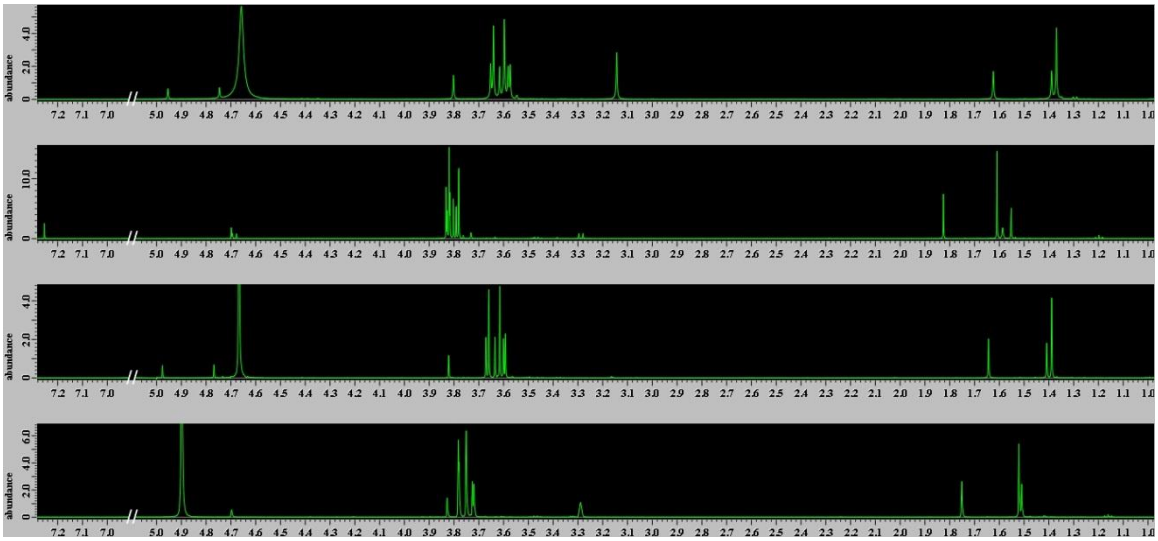

Figure 6. Attempt 3, Reaction 3: Dimethyl *trans*-Epoxymethylsuccinate, Crude vs. Purified, ^{1}H-NMR. Comparison of crude oil in D_2O (top) with purified oil in three solvents (second to fourth: $CDCl_3$, D_2O, CD_3OD). PPM range: 1.0-5.0, 7.0-7.3.

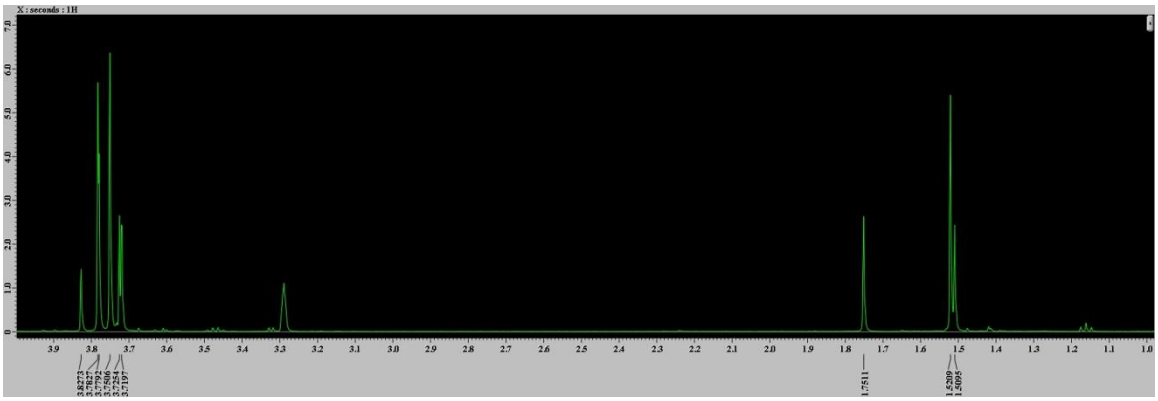

Figure 7. Attempt 3, Reaction 3: Dimethyl *trans*-Epoxymethylsuccinate, Purified Oil, ^{1}H-NMR. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.51, 1.52, 1.75, 3.72, 3.73, 3.75, 3.78, 3.78, 3.83. PPM range: 1.0-4.0.

III.A.4. Attempt 4

III.A.4.a. Reaction 1a: *trans*-epoxymethylsuccinate disodium salt

To a stirring mixture of mesaconic acid (20.0 g, 153.$_7$ mmol) and water (150 mL) was added a solution of sodium hydroxide (9.223 g, 230.6 mmol) dissolved in water (50

mL). Sodium tungstate dihydrate (5.071 g, 15.37_3 mmol) was added next and upon dissolution, hydrogen peroxide 30% (18.429 mL, 184.48 mmol) was added. The reaction was heated to and maintained at 65 °C for 3 hours, then heated to 75-80 °C for one hour. During heating, the pH was monitored and kept above 4 with as-needed drop-wise addition of 5 M NaOH (100 mL). The solvent was evaporated by vacuum to yield 22.99 g (120.9_6 mmol, 79%) titular compound. NMR parameters were as follows: ^{1}H-NMR (500 MHz, D_2O): δ = 1.26 (3 H, s, CH_3), 3.28 (1 H, s, OC*H*).

III.A.4.b. Reaction 1b: *trans*-epoxymethylsuccinate barium salt

Trans-epoxymethylsuccinate disodium salt (22.99 g, 120.9_6 mmol) was redissolved in water (80 mL). A one-molar equivalence solution of barium chloride dihydrate (~30 g) in hot water (80 mL) was added to the disodium salt solution. The barium salt immediately precipitated, and was filtered by vacuum and dried overnight to obtain 22.82 g (71.89 mmol, 59%) titular compound as a white powder. This barium salt did not dissolve in water, acetone, chloroform, methanol or DMSO—no NMR was taken.

III.A.4.c. Reaction 2: *trans*-epoxymethylsuccinic acid

In an ice bath were combined *trans*-epoxymethylsuccinate barium salt (6.00 g, 21.3_2 mmol), anhydrous magnesium sulfate (1.283 g, 10.66_1 mmol) and ether (100 mL), to which was slowly added a solution of concentrated sulfuric acid (1.136 mL, 21.32_1 mmol) in ether (50 mL). The solution was stirred for one hour at 5-10 °C, then overnight at room temperature. The resulting barium sulfate and magnesium sulfate were filtered off, and the solvent was evaporated by vacuum to approximately 20 mL, to which was added petroleum ether (70 mL). The resulting precipitate was filtered by vacuum

overnight to obtain 1.52 g (10.4 mmol, 49%) titular compound as a white powder. NMR parameters were as follows: ^{1}H-NMR (500 MHz, D_2O): δ = 1.37 (3 H, s, CH_3), 3.72 (1 H, s, OC*H*).

III.A.4.d. Reaction 3: dimethyl *trans*-epoxymethylsuccinate

III.A.4.d.1. Reaction 3.1: acidic methanol via hydrochloric acid

In a closed system of two flasks connected by cannula, hydrogen chloride gas was produced in the first by drop-wise addition of sulfuric acid onto sodium chloride (1:10 mol. eq.; in this reaction, 0.246 mL and 26.93 g, respectively). The system was set up to allow the hydrogen chloride gas to diffuse directly into dry methanol (90 mL) in the second flask (under argon gas and at 0 °C), after which was added a solution of *trans*-epoxymethylsuccinic acid (2.00 g, 13.6_9 mmol) in dry methanol (10 mL), pale/medium yellow in color. This reaction was stirred at room temperature for 48 hours. The solvent was evaporated by vacuum to yield a faint yellow oil, which was redissolved in ether (100 mL) and washed with 1 M $NaHCO_3$ (100 mL). The organic layer was dried with anhydrous magnesium sulfate and evaporated by vacuum down to a small volume. Excess petroleum ether was added and a colorless, clear oil settled at the bottom, with solid impurities. Some oil sans impurities was carefully extracted for an NMR, whose parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.52 (3 H, s, CH_3), 3.76 (3 H, s, OCH_3), 3.79 (3 H, s, OCH_3), 3.83 (1 H, s, OC*H*).

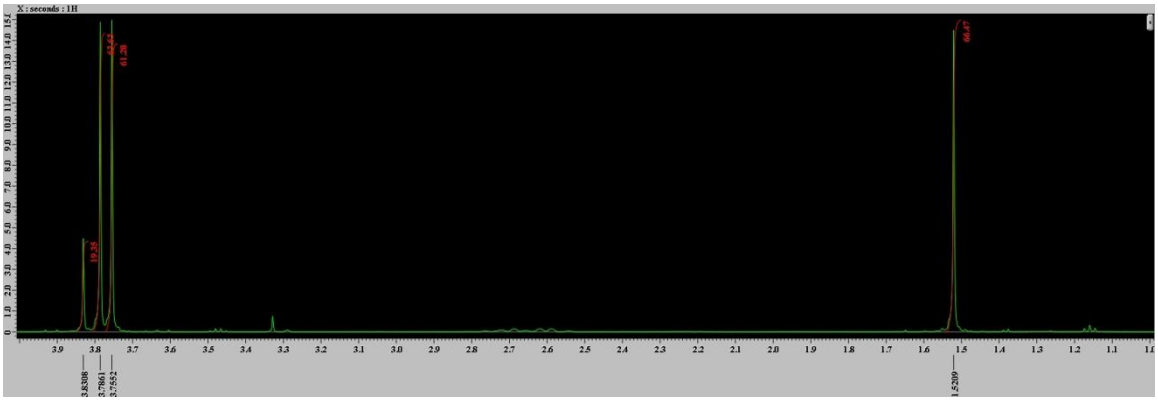

Figure 8. Attempt 4, Reaction 3.1: Dimethyl *trans*-Epoxymethylsuccinate, Colorless Oil, ^{1}H-NMR. Methyl esterification of epoxymethylsuccinic acid with acidic methanol: sulfuric acid was dripped onto sodium chloride to produce hydrogen chloride gas, which was delivered by cannula into dry methanol, to which a solution of *trans*-epoxymethylsuccinic acid in dry methanol was added. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.52 (3 H, s, CH_3), 3.76 (3 H, s, OCH_3), 3.79 (3 H, s, OCH_3), 3.83 (1 H, s, OC*H*). PPM range: 1.0-4.0.

III.A.4.d.2. Reaction 3.2: acidic methanol via sulfuric acid

Concentrated sulfuric acid (0.100 mL, 1.87_6 mmol) was added to a solution of *trans*-epoxymethylsuccinic acid (2.00 g, 13.6_9 mmol) in dry methanol (100 mL), and the reaction was stirred at room temperature for 48 hours. The solvent was evaporated by vacuum, and the remaining oil was redissolved in ether (100 mL) and washed with 1 M Na_2SO_4 (100 mL). The organic layer was dried with anhydrous magnesium sulfate and evaporated by vacuum to yield 1.69 g (9.70 mmol, 71%) titular compound as a light yellow oil. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.52 (3 H, s, CH_3), 3.76 (3 H, s, OCH_3), 3.79 (3 H, s, OCH_3), 3.83 (1 H, s, OC*H*).

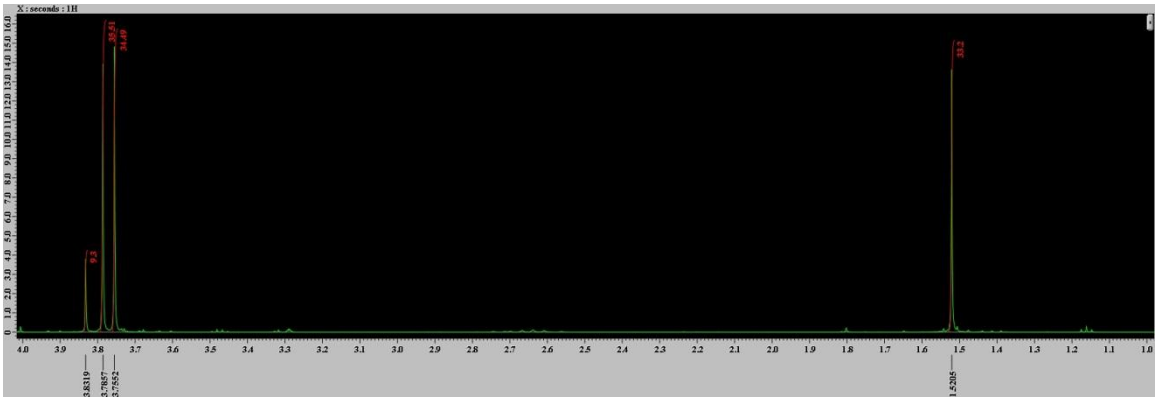

Figure 9. Attempt 4, Reaction 3.2: Dimethyl *trans*-Epoxymethylsuccinate, Colorless Oil, ^{1}H-NMR. Methyl esterification of epoxymethylsuccinic acid with acidic methanol: sulfuric acid was dripped directly into a solution of *trans*-epoxymethylsuccinic acid and dry methanol. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.52 (3 H, s, C*H*$_3$), 3.76 (3 H, s, OC*H*$_3$), 3.79 (3 H, s, OC*H*$_3$), 3.83 (1 H, s, OC*H*). PPM range: 1.0-4.0.

III.A.4.d.3. Reaction 3.3: thionyl chloride

Dry methanol (90 mL) and thionyl chloride (1.783 mL, 24.55 mmol) were combined with stirring at 0 °C. *Trans*-epoxymethylsuccinic acid (1.708 g, 11.6_9 mmol) dissolved in dry methanol (10 mL) was added, and the solution was stirred at room temperature overnight. The solvent was evaporated by vacuum, and the residue was redissolved in ether (100 mL) and washed with 1 M $NaHCO_3$ (100 mL). The organic layer was dried with anhydrous magnesium sulfate and evaporated by vacuum to yield an oil. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.52 (3 H, s, C*H*$_3$), 1.75 (s), 3.73 (d), 3.76 (3 H, s, OC*H*$_3$), 3.79 (3 H, s, OC*H*$_3$), 3.83 (1 H, s, OC*H*).

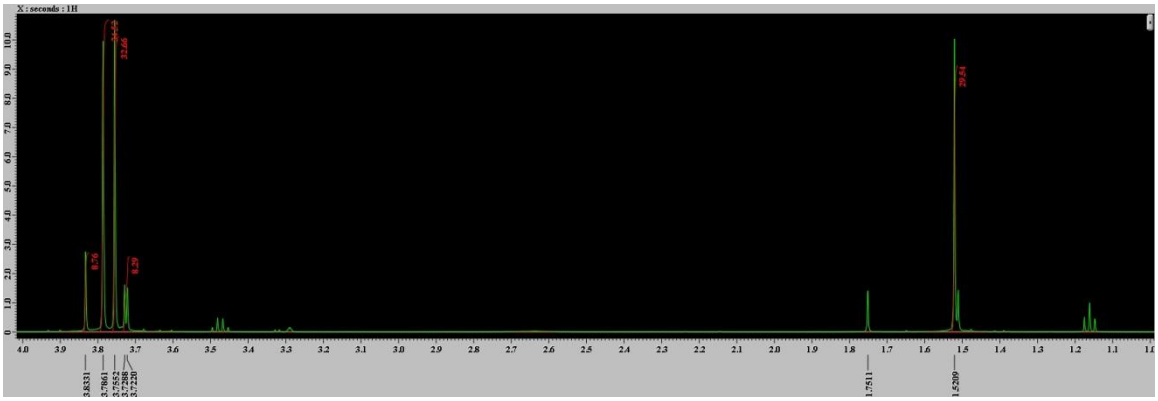

Figure 10. Attempt 4, Reaction 3.3: Dimethyl *trans*-Epoxymethylsuccinate, Oil, ^{1}H-NMR. Methyl esterification of epoxymethylsuccinic acid with chloromethane generated *in situ* by reacting dry methanol with thionyl chloride. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.52 (3 H, s, CH_3), 1.75 (s), 3.73 (d), 3.76 (3 H, s, OCH_3), 3.79 (3 H, s, OCH_3), 3.83 (1 H, s, OC*H*). PPM range: 1.0-4.0.

III.A.5. Attempt 5

III.A.5.a. Reaction 3: dimethyl *trans*-epoxymethylsuccinate

Sulfuric acid (0.100 mL, 1.87_6 mmol) dissolved in dry methanol (80 mL) was added to *trans*-epoxymethylsuccinic acid (2.61 g, 17.8_6 mmol) dissolved in dry methanol (20 mL). The solution was stirred at room temperature for 48 hours. The solvent was evaporated by vacuum, and the residue was redissolved in ether (100 mL) and washed with 1 M Na_2SO_4 (100 mL). The organic layer was dried with anhydrous magnesium sulfate and evaporated by vacuum to yield 2.61 g (15.0 mmol, 84%) titular compound. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.52 (3 H, s, CH_3), 3.75 (3 H, s, OCH_3), 3.78 (3 H, s, OCH_3), 3.83 (1 H, s, OC*H*).

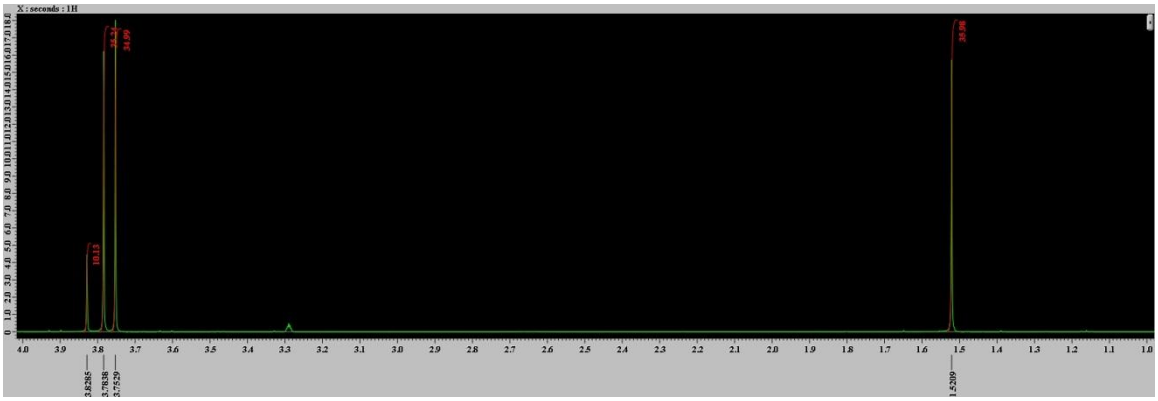

Figure 11. Attempt 5, Reaction 3: Dimethyl *trans*-Epoxymethylsuccinate, ^{1}H-NMR. Repeat of methyl esterification of *trans*-epoxymethylsuccinic acid with acidic methanol via sulfuric acid to confirm this reaction as the best of three acidification possibilities (see Attempt 4, reactions 3.1, 3.2, 3.3). NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.52 (3 H, s, CH_3), 3.75 (3 H, s, OCH_3), 3.78 (3 H, s, OCH_3), 3.83 (1 H, s, OC*H*). PPM range: 1.0-4.0.

III.A.6. Attempt 6

III.A.6.a. Reaction 1: *trans*-epoxymethylsuccinate disodium salt

Mesaconic acid (10.00 g, 76.86_5 mmol) was added to sodium hydroxide (6.149 g, 153.7_3 mmol) dissolved in water (150 mL) at 0 °C. Sodium tungstate dihydrate (2.535 g, 7.686_5 mmol) was added next and, upon dissolution, hydrogen peroxide 30% (9.213 mL, 92.23_8 mmol) was added. The solution was heated to 65 °C and stirred for 3 hours while pH was maintained above 4 with as-needed drop-wise addition of 5 M NaOH (100 mL). The solvent was evaporated by vacuum to yield the titular compound as a white powder. No mass was recorded; reaction was treated as an uninterrupted one-pot reaction. Theoretical yield (14.609 g, 76.865 mmol) was used as the starting value in Reaction 2.

III.A.6.b. Reaction 2: *trans*-epoxymethylsuccinic acid

Trans-epoxymethylsuccinate disodium salt (14.609 g, 76.865 mmol) was resuspended in ether (100 mL), to which was added drop-wise a solution of sulfuric acid

(4.10 mL, 76.8_6 mmol) in ether (~25 mL). The new solution was stirred overnight at room temperature. A small amount of solvent was evaporated by vacuum to run an NMR of the remaining residue. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 2.19 (3 H, s, CH_3), 6.72 (1 H, s, C*H*).

III.A.6.c. Reactions 1(bis)a & 1b: *trans*-epoxymethylsuccinate disodium salt → barium salt

To a stirring suspension of "mesaconic acid" (see § IV.A.6.) from Reaction 2 above (7.70 g, 59.1_8 mmol) in water (100 mL) at 0 °C was added a solution of sodium hydroxide (3.314 g, 82.86 mmol) in water (50 mL). Sodium tungstate dihydrate (1.952 g, 5.918_6 mmol) was added and, upon dissolution, hydrogen peroxide 30% (7.095 mL, 71.02_3 mmol) was added. The solution was heated to 65 °C and stirred for 3 hours while pH was maintained above 4 with as-needed drop-wise addition of 5 M NaOH (100 mL). The solvent was evaporated by vacuum to ~50 mL, to which was added an equimolar solution of barium chloride dihydrate (14.459 g) in hot water (100 mL). The precipitate was filtered off by vacuum and dried overnight to yield 14.10 g (50.10 mmol, 85%) *trans*-epoxymethylsuccinate barium salt.

III.A.6.d. Reaction 2(bis): *trans*-epoxymethylsuccinic acid

To a stirring mixture of *trans*-epoxymethylsuccinate barium salt (14.10 g, 50.10 mmol) and anhydrous magnesium sulfate (3.015 g, 25.05 mmol) in ether (100 mL) was added drop-wise a solution of sulfuric acid (2.671 mL, 50.10 mmol) in ether (~22.5 mL). This new solution was stirred at room temperature for 24 hours. The resulting barium sulfate and magnesium sulfate were filtered off and the solvent was

evaporated by vacuum to a small volume. At least three volumes of petroleum ether were added, and the precipitate was filtered by vacuum and dried overnight to yield 4.34 g (29.7_1 mmol, 59%) titular compound. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.53 (3 H, s, C*H*$_3$), 3.74 (1 H, s, OC*H*); ^{13}C-NMR (500 MHz, CD_3OD): δ = 11.92, 56.89, 57.41, 168.56, 170.90.

III.A.6.e. Reaction 3: dimethyl *trans*-epoxymethylsuccinate

Under argon gas and at 0 °C, a solution of sulfuric acid (0.171 mL, 3.21 mmol) in dry methanol (100 mL) was added drop-wise to a stirring solution of *trans*-epoxymethylsuccinic acid (4.69 g, 32.1 mmol) in dry methanol (100 mL). The combined solution was stirred at room temperature for 48 hours. The solvent was evaporated by vacuum, and the remaining residue was redissolved in ether (100 mL) and washed with 1 M Na_2SO_4 (100 mL). The organic layer was dried with anhydrous magnesium sulfate and evaporated by vacuum to yield 4.84 g (27.7_9 mmol, 87%) titular compound as a colorless oil. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.52 (3 H, s, C*H*$_3$), 3.75 (3 H, s, OC*H*$_3$), 3.78 (3 H, s, OC*H*$_3$), 3.83 (1 H, s, OC*H*); ^{13}C-NMR (500 MHz, CD_3OD): δ = 11.84, 51.87, 52.26, 56.77, 57.75, 167.12, 169.32.

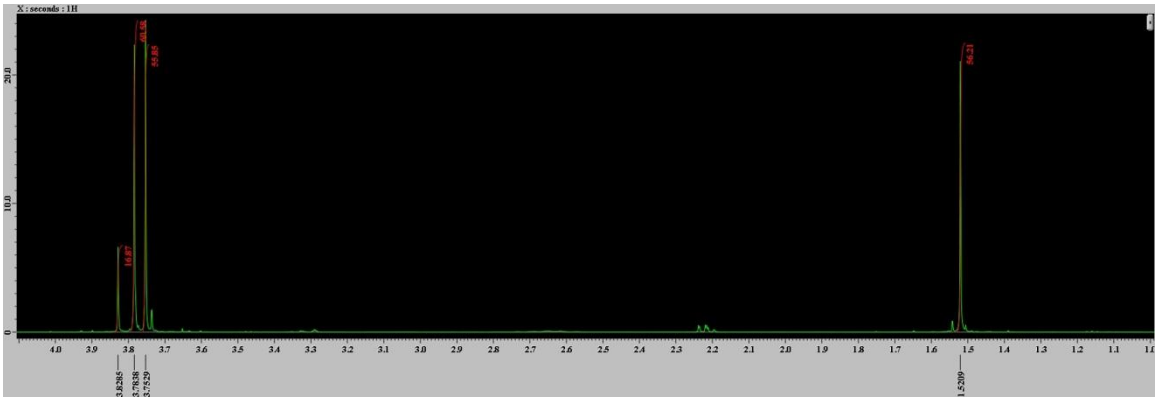

Figure 12. Attempt 6, Reaction 3: Dimethyl *trans*-Epoxymethylsuccinate, Colorless Oil, ^{1}H-NMR. Starting from mesaconic acid and proceeding through to the methyl esterification via sulfuric acid, confirmed in attempt 5, reaction 3 as the best means by which to do so. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.52 (3 H, s, C*H*$_3$), 3.75 (3 H, s, OC*H*$_3$), 3.78 (3 H, s, OC*H*$_3$), 3.83 (1 H, s, OC*H*). PPM range: 1.0-4.1.

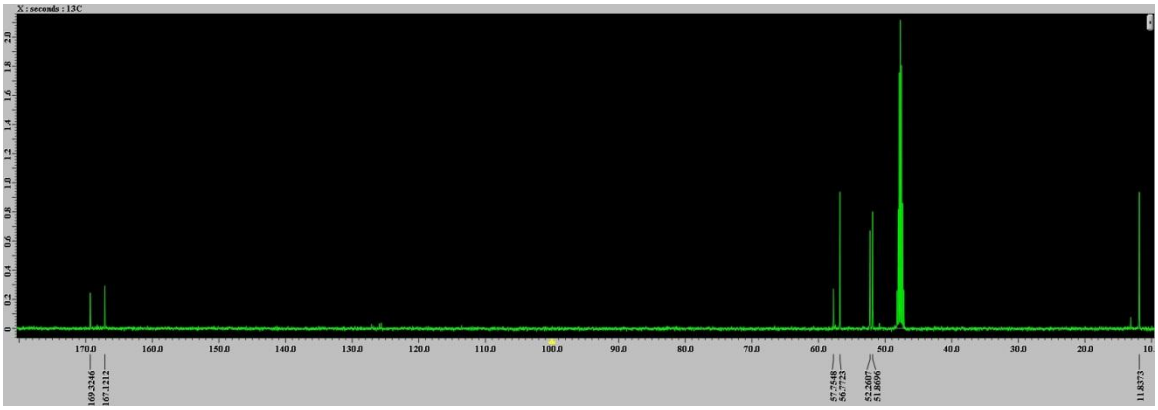

Figure 13. Attempt 6, Reaction 3: Dimethyl *trans*-Epoxymethylsuccinate, Colorless Oil, ^{13}C-NMR. Starting from mesaconic acid and proceeding through to the methyl esterification via sulfuric acid, confirmed in attempt 5, reaction 3 as the best means by which to do so. NMR parameters were as follows: ^{13}C-NMR (500 MHz, CD_3OD): δ = 11.84, 51.87, 52.26, 56.77, 57.75, 167.12, 169.32. PPM range: 10-180.

All of the preceding results led to a novel combination of reactions to synthesize dimethyl *trans*-epoxymethylsuccinate from mesaconic acid. Multiple replications have consistently yielded highly pure results. Following each reaction are reaction schemata.

III.B. Final Reactions to Yield Dimethyl *trans*-Epoxymethylsuccinate

III.B.1. Reaction 1: *trans*-epoxymethylsuccinate barium salt

Mesaconic acid (20.00 g, 153.7_3 mmol) was added to nano-pure water (400 mL) already stirring in a multi-necked round-bottom flask at 0 °C. Sodium hydroxide pellets (9.223 g, 230.6 mmol) were dissolved in water (50 mL) and poured into the flask. Once all of the mesaconic acid had dissolved, sodium tungstate dihydrate (5.071 g, 15.37_3 mmol) was added. After it had dissolved, hydrogen peroxide 30% (18.429 mL, 184.48 mmol) was added, and the solution was stirred at 0 °C for a further 10-15 minutes. The flask was transferred to a sand bath to heat the reaction to 65-70 °C, and the solution was stirred for 3 hours. Immediately following the transfer, an electronic pH probe was used to maintain the pH above 4 by dropwise addition of a 5 M NaOH solution (100 mL) as needed. (No more than one hour's worth of monitoring was needed; i.e., the pH stopped decreasing as the reaction proceeded.) After 3 hours, the temperature was reduced to approximately 40 °C, and the remaining portion of NaOH was added. (It was noted that the solution was pale yellow in color and smelled strongly of Play-Doh.) After 10 more minutes of stirring, the solvent was evaporated by vacuum at 40 °C until no more than roughly 70-100 mL remained. (Alternatively, the solvent can be evaporated completely, leaving behind a white crystalline powder, and then 70-100 mL H_2O can be reintroduced.) To this concentrated solution of *trans*-epoxymethylsuccinate disodium salt was added barium chloride dihydrate (37.557 g, 153.73 mmol) dissolved in very hot water (150 mL). The titular compound immediately precipitated and was filtered by vacuum overnight to yield 43.45 g (136.8_8 mmol, 89%). This barium salt does not

dissolve in water, acetone, methanol, chloroform or DMSO. No NMR was collected.

NMR parameters from the disodium salt, however, were as follows: ^{1}H-NMR (500 MHz, D_2O): δ = 1.28 (3 H, s, C*H*$_3$), 3.29 (1 H, s, OC*H*).

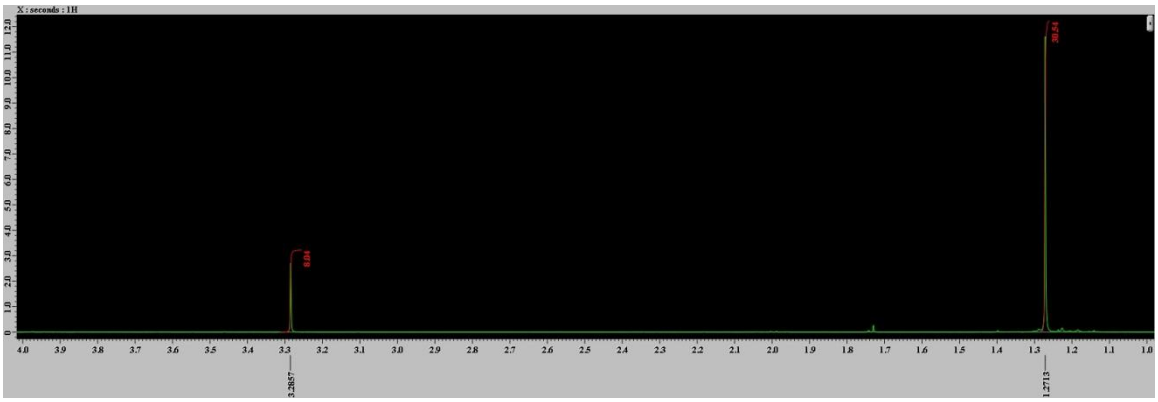

Figure 14. Current Method, Reaction 1: *trans*-Epoxymethylsuccinate Disodium Salt, White Powder, ^{1}H-NMR. Sodium tungstate dihydrate and hydrogen peroxide were added to mesaconic acid in a one-pot, pH- and temperature-controlled reaction to synthesize, ultimately, *trans*-epoxymethylsuccinate barium salt. Not being soluble in any of the available deuterated solvents, however, an NMR of its immediate precedent (the disodium salt) was taken. It should not, in theory, be any noticeably different. NMR parameters were as follows: ^{1}H-NMR (500 MHz, D_2O): δ = 1.28 (3 H, s, C*H*$_3$), 3.29 (1 H, s, OC*H*). PPM range: 1.0-4.0.

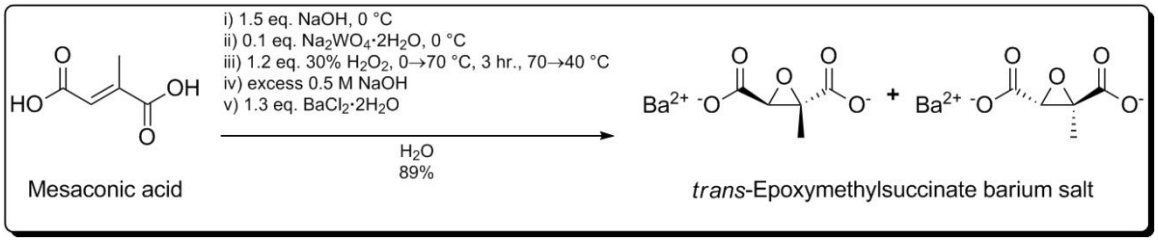

Figure 15. Overview of Reaction Steps to Create a *trans*-Epoxymethylsuccinate Barium Salt From Mesaconic Acid. Both members of the enantiomeric pair are shown here. Subsequent figures will show just one member of the pair to avoid clutter.

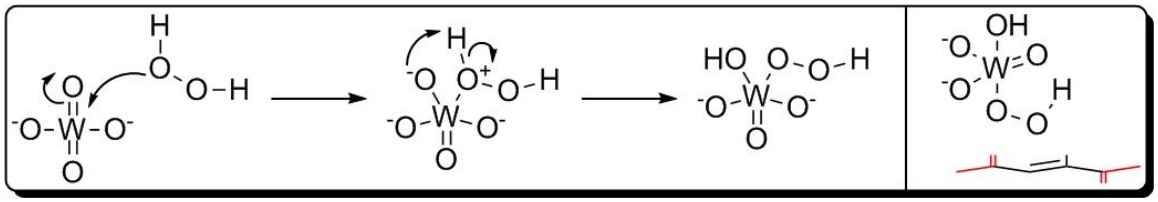

Figure 16. Mechanism by Which Hydrogen Peroxide Activates Catalytic Tungstate, and Its Position Relative to Mesaconate Prior to Epoxidation.

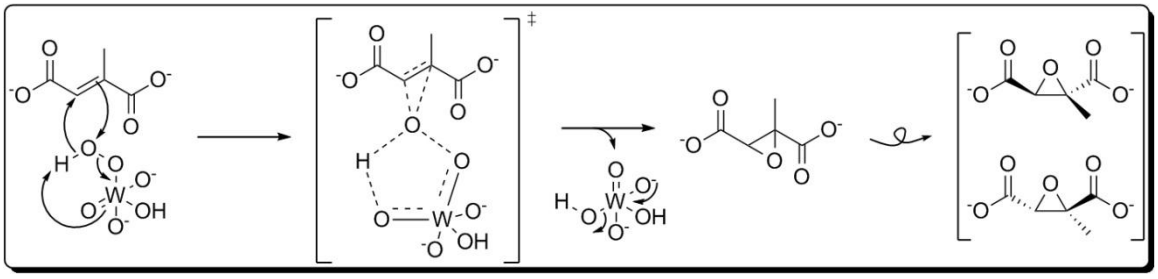

Figure 17. Mechanism for Epoxidation of Mesaconic Acid by Hydrogen Peroxide-Activated Tungstate via the "Butterfly" Mechanism (Bartlett, 1950).

III.B.2. Reaction 2: *trans*-epoxymethylsuccinic acid

Trans-epoxymethylsuccinate barium salt (43.45 g, 136.8_8 mmol) was added to anhydrous diethyl ether (400 mL) and stirred at 0 °C. Anhydrous magnesium sulfate (1.977 g, 16.42_6 mmol) was added, followed by sulfuric acid (14.59 mL, 273.7_6 mmol) dissolved in ether (70 mL). The solution was removed from the ice bath and allowed to stir overnight at room temperature. The resulting barium sulfate was filtered off and kept, and the filtrate was dried with anhydrous magnesium sulfate. The solvent was then evaporated by vacuum until a small amount remained (some titular compound may be seen to start crashing out), to which were added 5 volumes of petroleum ether. The flask was swirled vigorously by hand to precipitate the titular compound, which was then filtered and dried overnight. At the same time, the barium sulfate was redissolved in fresh

ether and stirred overnight to obtain more titular compound, and the filtration/evaporation/collection procedures were repeated. Both amounts of di-acid were combined for a total yield of 16.19 g (110.8_2 mmol, 81%). NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.53 (3 H, s, C*H*$_3$), 3.74 (1 H, s, OC*H*); ^{13}C-NMR (500 MHz, CD_3OD): δ = 11.89, 56.84, 57.34, 168.44, 170.78.

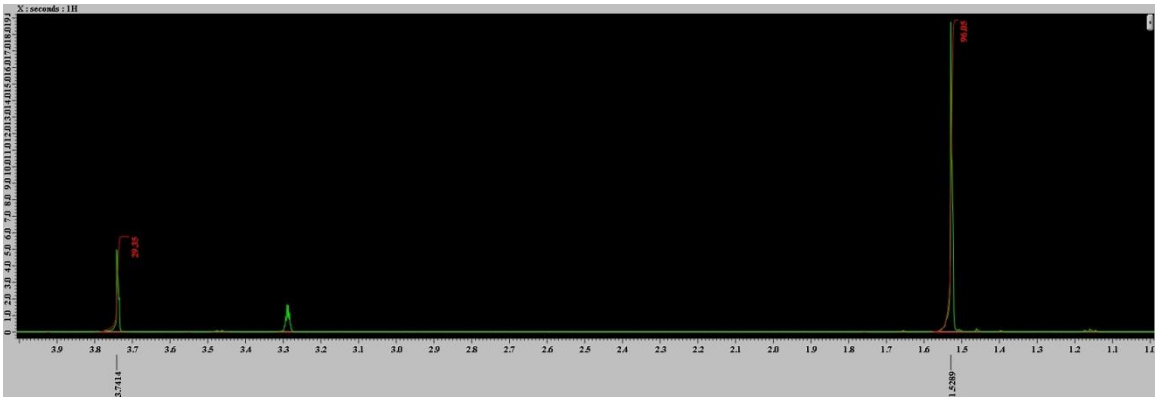

Figure 18. Current Method, Reaction 2: *trans*-Epoxymethylsuccinic Acid, White Powder, ^{1}H-NMR. Simple acidification via sulfuric acid of *trans*-epoxymethylsuccinate barium salt. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.53 (3 H, s, C*H*$_3$), 3.74 (1 H, s, OC*H*). PPM range: 1.0-4.0.

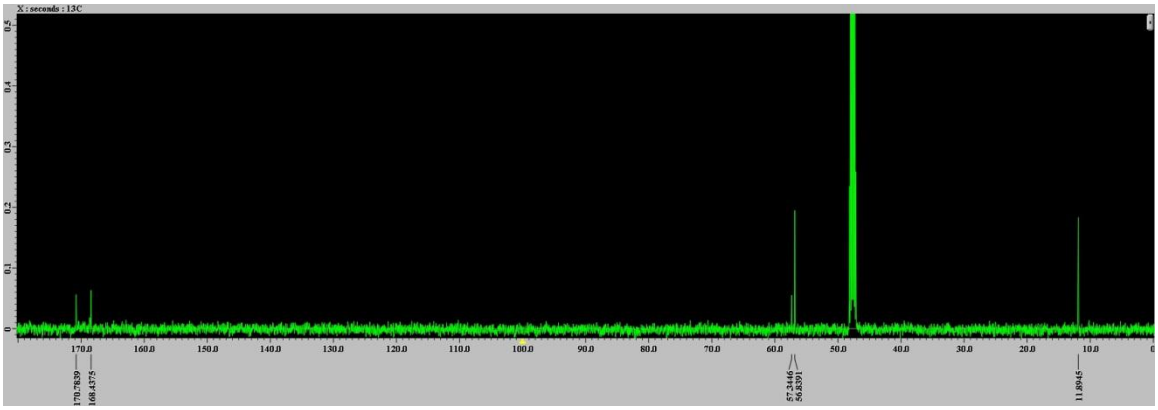

Figure 19. Current Method, Reaction 2: *trans*-Epoxymethylsuccinic Acid, White Powder, ^{13}C-NMR. Simple acidification via sulfuric acid of *trans*-epoxymethylsuccinate barium salt. NMR parameters were as follows: ^{13}C-NMR (500 MHz, CD_3OD): δ = 11.89, 56.84, 57.34, 168.44, 170.78. PPM range: 0-180.

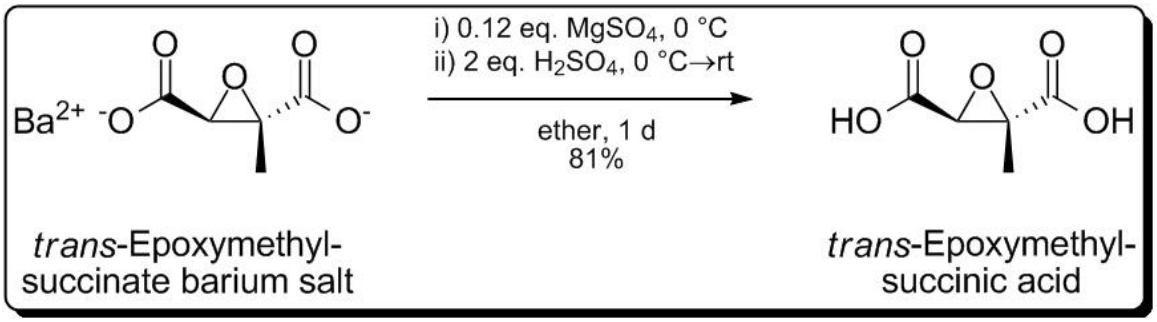

Figure 20. Overview of Reaction Steps to Convert *trans*-Epoxymethylsuccinate Barium Salt to *trans*-Epoxymethylsuccinic Acid.

III.B.3. Reaction 3: dimethyl *trans*-epoxymethylsuccinate

Trans-epoxymethylsuccinic acid (16.05 g, 109.8_6 mmol) was dissolved in dry methanol (70 mL), to which was added a solution of sulfuric acid (0.878 mL, 16.4_8 mmol) dissolved in dry methanol (50 mL). The combined solutions were stirred at room temperature for 2 days. The solvent was evaporated by vacuum, and the resulting residue was redissolved in ether (100 mL). A liquid-liquid extraction was performed with 1 M Na_2SO_4 (100 mL). The organic layer was dried with anhydrous magnesium sulfate and evaporated by vacuum. A second round of vacuum evaporation was performed under higher vacuum to yield a total of 15.44 g (88.65_8 mmol, 81 %) titular compound. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.51 (3 H, s, C*H*$_3$), 3.75 (3 H, s, OC*H*$_3$), 3.78 (3 H, s, OC*H*$_3$), 3.82 (1 H, s, OC*H*); ^{13}C-NMR (500 MHz, CD_3OD): δ = 13.14, 53.14, 53.54, 58.05, 59.03, 168.36, 170.57.

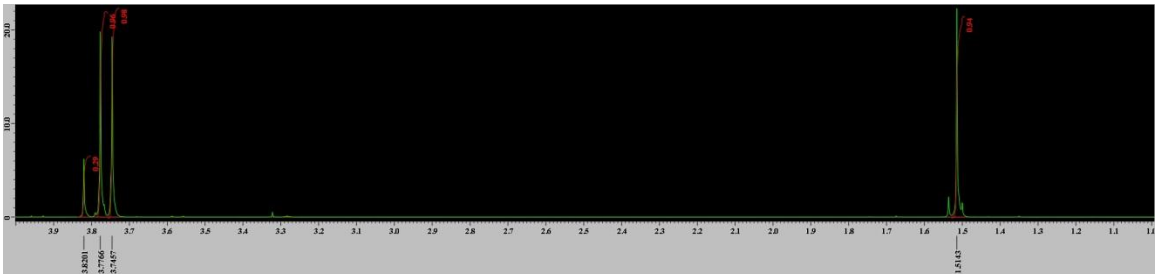

Figure 21. Current Method, Reaction 3: Dimethyl *trans*-Epoxymethylsuccinate, Colorless Oil, ^{1}H-NMR. Addition of *trans*-epoxymethylsuccinic acid to acidified methanol. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.51 (3 H, s, C*H*$_3$), 3.75 (3 H, s, OC*H*$_3$), 3.78 (3 H, s, OC*H*$_3$), 3.82 (1 H, s, OC*H*). PPM range: 1.0-4.0.

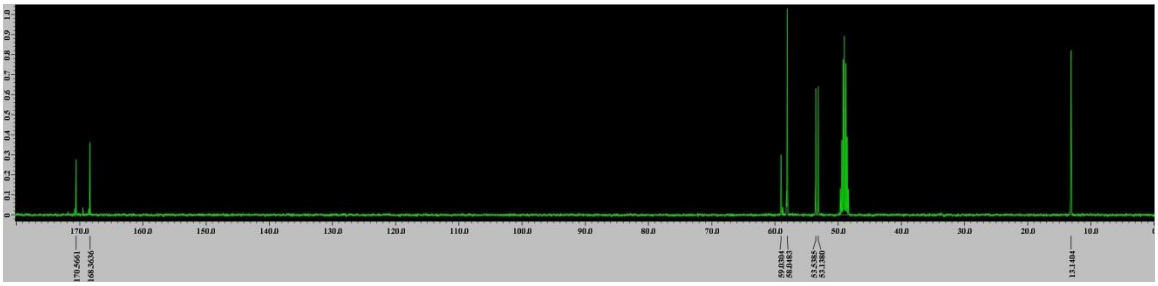

Figure 22. Current Method, Reaction 3: Dimethyl *trans*-Epoxymethylsuccinate, Colorless Oil, ^{13}C-NMR. Addition of *trans*-epoxymethylsuccinic acid to acidified methanol. NMR parameters were as follows: ^{13}C-NMR (500 MHz, CD_3OD): δ = 13.14, 53.14, 53.54, 58.05, 59.03, 168.36, 170.57. PPM range: 1-180.

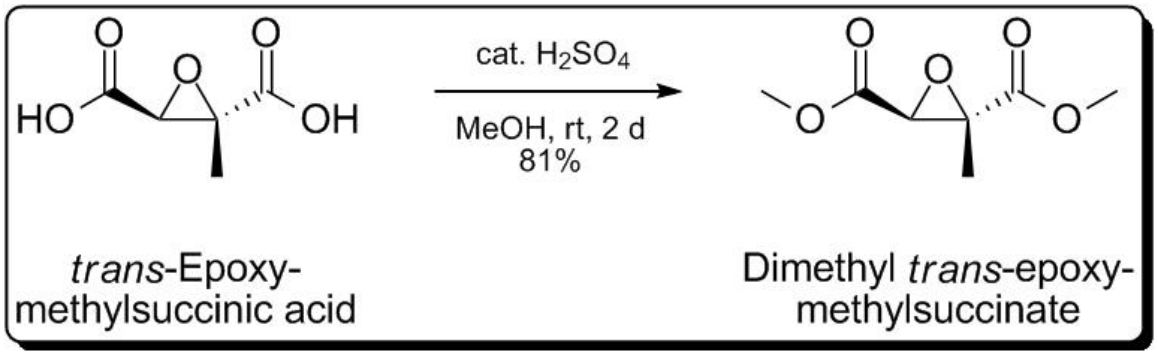

Figure 23. Overview of Reaction Steps to Convert *trans*-Epoxymethylsuccinic Acid to Dimethyl *trans*-Epoxymethylsuccinate.

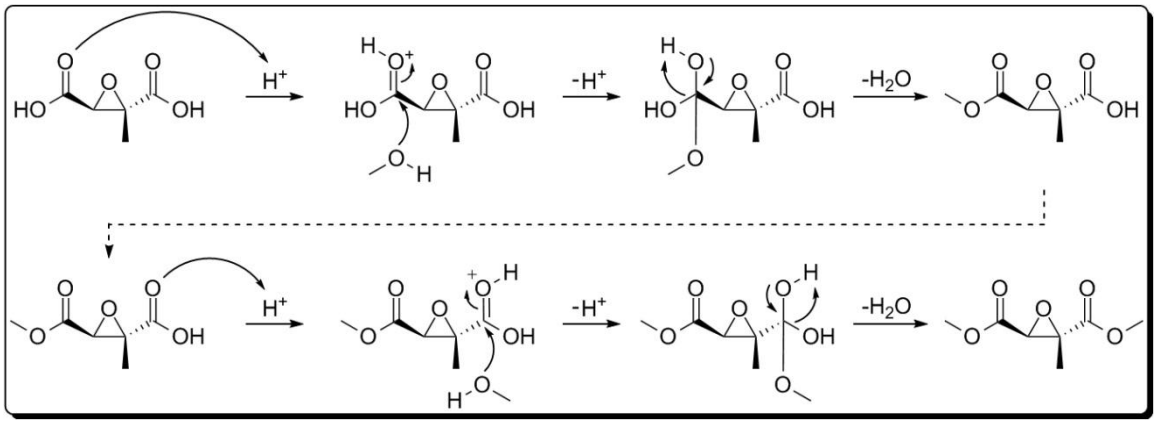

Figure 24. Fisher Esterification of *trans*-Epoxymethylsuccinic Acid in Methanol to Yield Dimethyl *trans*-Epoxymethylsuccinate.

III.C. Lactonization

Once the methodology for synthesizing pure dimethyl *trans*-epoxymethylsuccinate had been formulated and perfected, work began to obtain successful lactonization—ultimately, β,γ-dicarboxylate-γ-methyl-γ-butyrolactone (more succinctly, β,γ-dicarboxylate-γ-valerolactone, but the explicit stating of "γ-methyl" (and, accordingly, butyro-) is for continuity re 2-methylisocitrate, the target substance), by way of 2-methyl-5-oxo-2,3,4-tricarbomethoxytetrahydrofuran.

Below are two schemata, one general and one detailed, for the above-mentioned series of reactions.

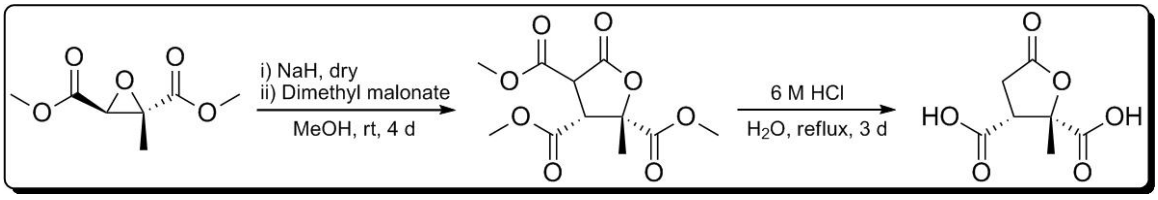

Figure 25. Overview of Reaction Steps to Convert Dimethyl *trans*-Epoxymethylsuccinate, via 2-Methyl-5-oxo-2,3,4-tricarbomethoxytetrahydrofuran, to β,γ-Dicarboxylate-γ-methyl-γ-butyrolactone.

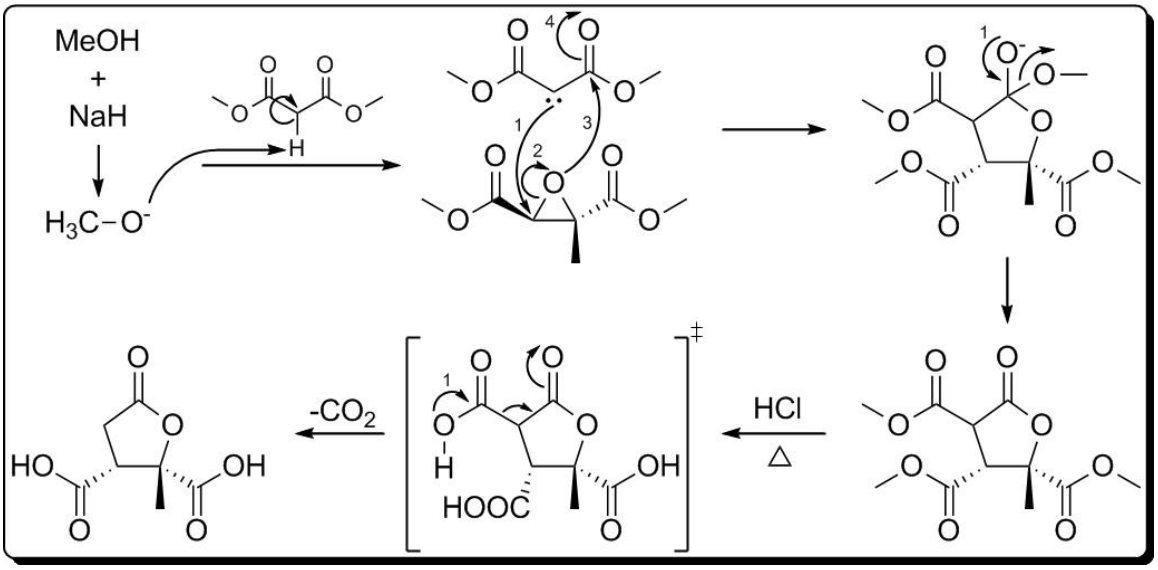

Figure 26. Detailed Mechanisms of Reactions Proceeding From *trans*-Epoxymethylsuccinate, via 2-Methyl-5-oxo-2,3,4-tricarbomethoxytetrahydrofuran, to β,γ-Dicarboxylate-γ-methyl-γ-butyrolactone. Sodium hydride deprotonates methanol to release hydrogen gas and form the alkoxide salt, which deprotonates dimethyl malonate. The electrons of the negatively charged carbon attack the less sterically hindered carbon of the epoxide, freeing the now negatively charged oxygen to attack the partially positive carbonyl carbon of the malonate and form a five-membered lactone. After workup, hydrochloric acid serves to protonate the methylester moieties of the lactone, the α moiety of which autodecarboxylates with heat. Loss of CO_2 results in the target lactone.

III.C.1. Solid sodium metal vs. dry sodium hydride

It was considered that the source of sodium might be affecting the lactonization reaction, to the extent that a source other than solid sodium might lead to a cleaner, more informative NMR spectrum of crude—perhaps even purified—2-methyl-5-oxo-2,3,4-tricarbomethoxytetrahydrofuran. Dry sodium hydride was chosen as an alternative. A reaction was set up within an NMR tube to allow for progressive scans of the reaction.

III.C.1.a. Reaction 4-NaH: 2-methyl-5-oxo-2,3,4-tricarbomethoxytetrahydrofuran

In an NMR tube, dimethyl *trans*-epoxymethylsuccinate (0.02316 g, 0.133 mmol) was added to deuterated methanol (0.650 mL), and an NMR ("0a") was taken. Dimethyl malonate (0.0152 mL, 0.133 mmol) was added, followed ten minutes later by a second

NMR ("0b"). Dry sodium hydride (0.0032 g, 0.13_3 mmol) was added, and the first ("1") of four hourly scans was taken. The fifth ("5") scan was taken 24 hours after the first. An additional mole equivalent each of dimethyl malonate (0.0152 mL) and sodium hydride (0.0032 g) were added to the solution, and a scan was run then ("+M.E.") and again two days later ("+M.E., 2d"). NMR parameters for each of the preceding reactions were as follows: ^{1}H-NMR (500 MHz, CD_3OD){

[0a]: δ = 1.52 (3 H, s, CH_3), 3.75 (3 H, s, OCH_3), 3.78 (3 H, s, OCH_3), 3.83 (1 H, s, OC*H*).

[0b]: δ = 1.52 (3 H, s, CH_3), 3.42 (2 H, s, CH_2), 3.70 (6 H, s, OCH_3), 3.75 (3 H, s, OCH_3), 3.78 (3 H, s, OCH_3), 3.83 (1 H, s, OC*H*).

[1]: δ = 1.48, 1.52, 3.60, 3.83.

[5]: δ = 1.48, 1.52, 1.53, 3.60, 3.83.

[+M.E.]: δ = 1.49, 1.51, 1.53, 1.54, 3.64, 3.84.

[+M.E., 2d]: δ = 1.49, 1.51, 1.53, 1.54, 3.58, 3.64.

}; ^{13}C-NMR (500 MHz, CD_3OD){

[0a]: δ = 11.83, 51.81, 52.21, 56.75, 57.74, 167.07, 169.28.

[0b]: δ = 11.83, 40.31, 51.56, 51.82, 52.21, 56.75, 57.74, 167.07, 167.51, 169.28.

[1]: δ = 11.88, 12.42, 13.10, 56.77, 56.84, 57.75, 59.78, 60.23, 167.10, 168.66, 169.33, 171.09, 171.75, 174.27.

[5]: δ = 11.83, 12.42, 13.10, 56.77, 56.84, 57.75, 59.77, 60.23, 167.10, 168.66, 169.33, 171.09, 171.76, 174.27.

[+M.E.]: δ = 11.85, 12.43, 13.06, 13.66, 56.81, 56.96, 57.16, 57.83, 59.88, 60.44, 167.14, 168.61, 169.42, 171.11, 171.71, 174.28.

[+M.E., 2d]: δ = 12.43, 13.06, 13.66, 25.34, 56.95, 57.15, 59.88, 60.44, 168.61, 171.71, 173.06, 174.28.

}.

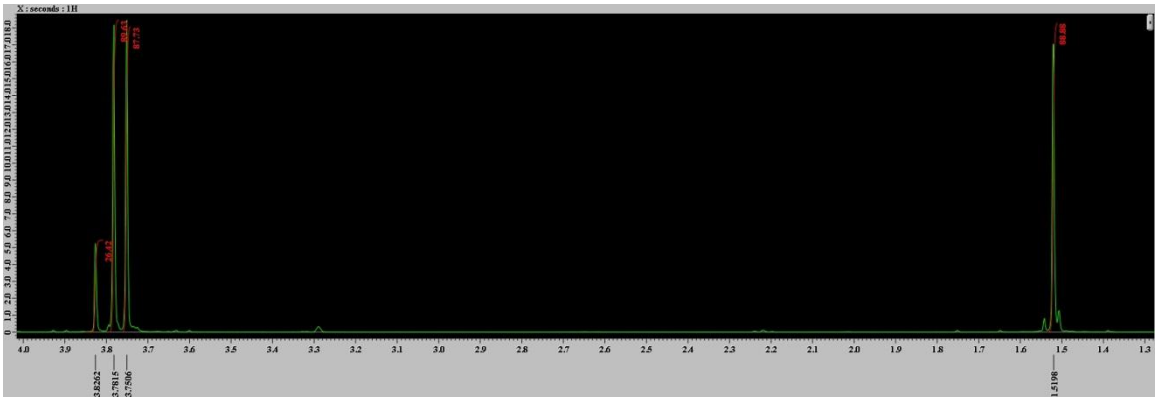

Figure 27. Sodium Source Comparison, NaH, Reaction 4(0a), ^{1}H-NMR. Dimethyl *trans*-epoxymethylsuccinate in methanol. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.52 (3 H, s, C*H*$_3$), 3.75 (3 H, s, OC*H*$_3$), 3.78 (3 H, s, OC*H*$_3$), 3.83 (1 H, s, OC*H*). PPM range: 1.3-4.0.

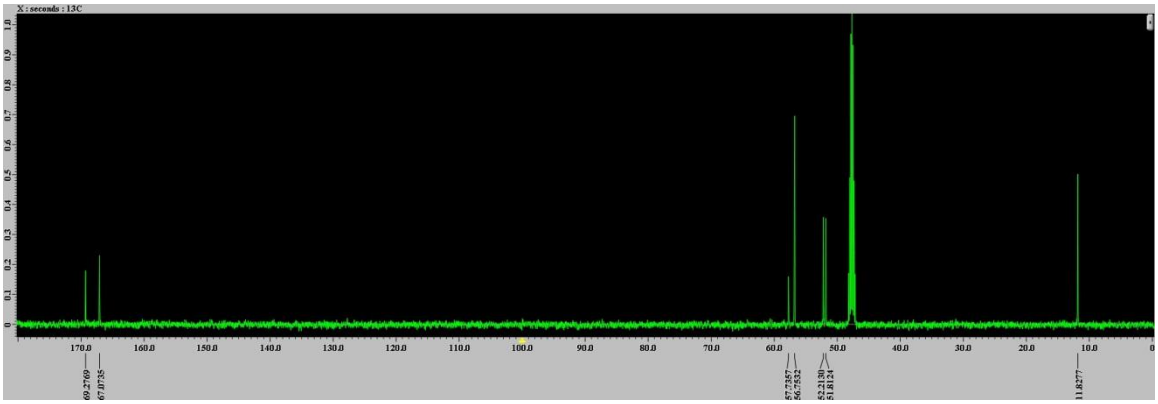

Figure 28. Sodium Source Comparison, NaH, Reaction 4(0a), ^{13}C-NMR. Dimethyl *trans*-epoxymethylsuccinate in methanol. NMR parameters were as follows: ^{13}C-NMR (500 MHz, CD_3OD): δ = 11.83, 51.81, 52.21, 56.75, 57.74, 167.07, 169.28. PPM range: 0-180.

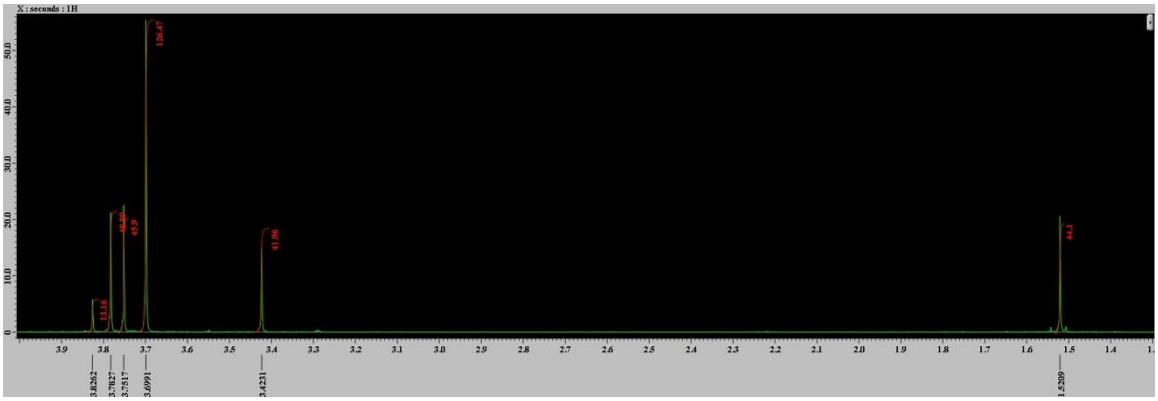

Figure 29. Sodium Source Comparison, NaH, Reaction 4(0b), ^{1}H-NMR. Dimethyl *trans*-epoxymethylsuccinate in methanol, plus dimethyl malonate. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.52 (3 H, s, CH_3), 3.42 (2 H, s, CH_2), 3.70 (6 H, s, OCH_3), 3.75 (3 H, s, OCH_3), 3.78 (3 H, s, OCH_3), 3.83 (1 H, s, OC*H*). PPM range: 1.3-4.0.

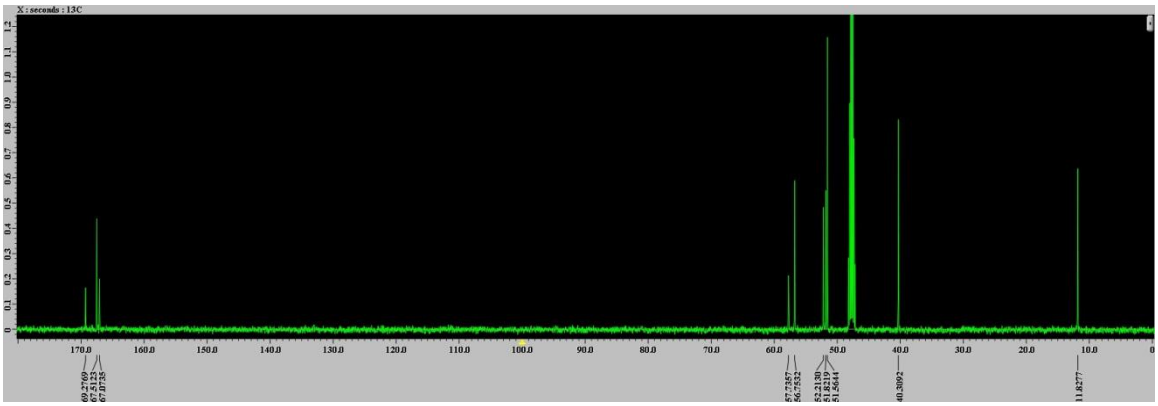

Figure 30. Sodium Source Comparison, NaH, Reaction 4(0b), ^{13}C-NMR. Dimethyl *trans*-epoxymethylsuccinate in methanol, plus dimethyl malonate. NMR parameters were as follows: ^{13}C-NMR (500 MHz, CD_3OD): δ = 11.83, 40.31, 51.56, 51.82, 52.21, 56.75, 57.74, 167.07, 167.51, 169.28. PPM range: 0-180.

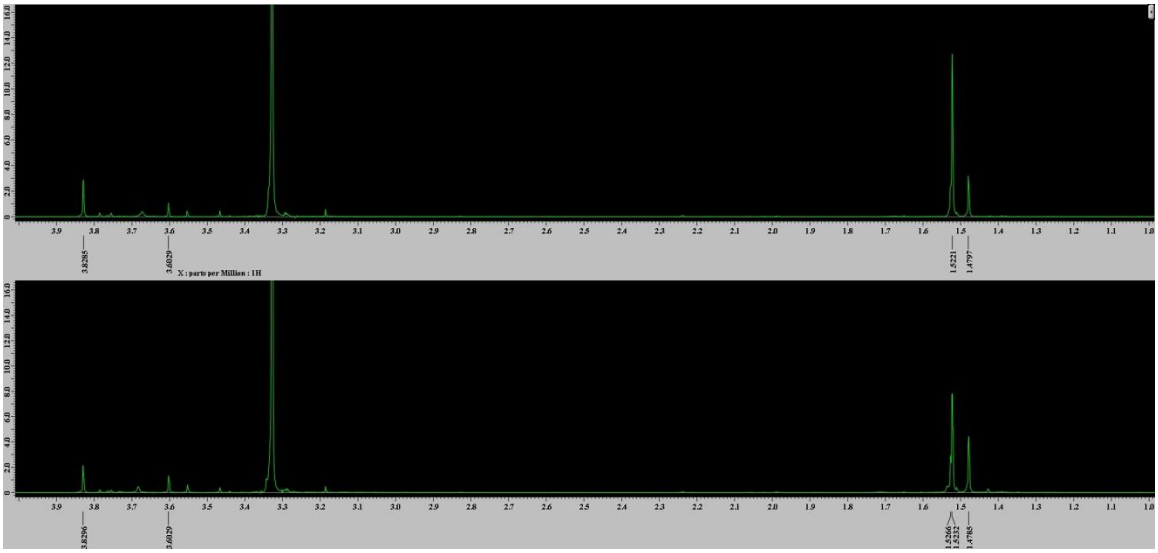

Figure 31. Sodium Source Comparison, NaH, Reactions 4(1) & 4(5), ^{1}H-NMR. Dimethyl *trans*-epoxymethylsuccinate in methanol, plus dimethyl malonate and NaH: immediately (top), after 24 hours (bottom). NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.48, 1.52, 3.60, 3.83 (top); δ = 1.48, 1.52, 1.53, 3.60, 3.83 (bottom). PPM range: 1.0-4.0.

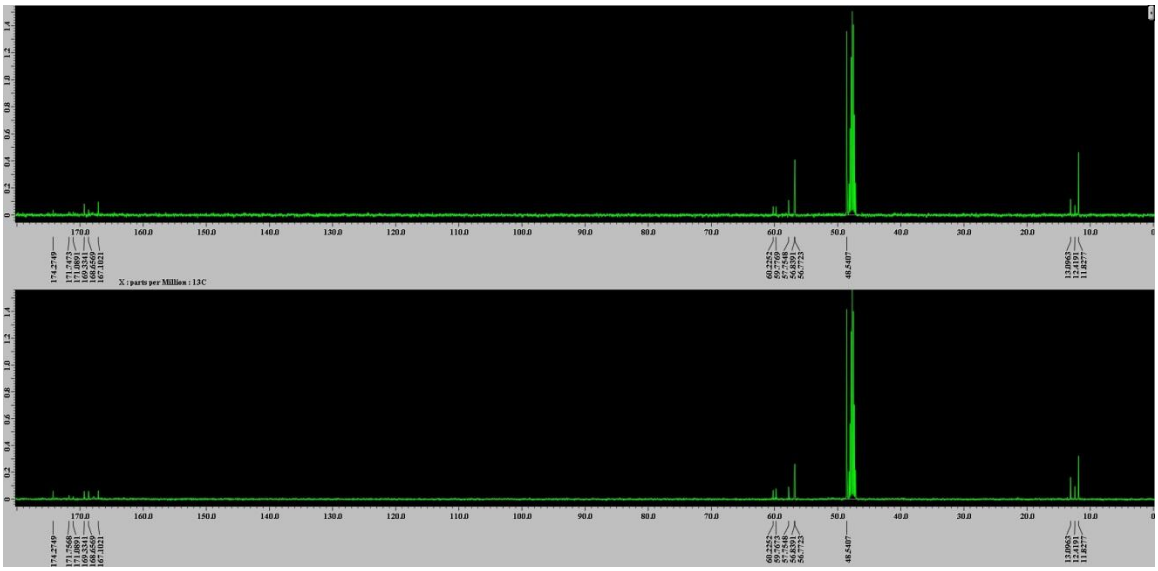

Figure 32. Sodium Source Comparison, NaH, Reactions 4(1) & 4(5), ^{13}C-NMR. Dimethyl *trans*-epoxymethylsuccinate in methanol, plus dimethyl malonate and NaH: immediately (top), after 24 hours (bottom). NMR parameters were as follows: ^{13}C-NMR (500 MHz, CD_3OD): δ = 11.83, 12.42, 13.10, 56.77, 56.84, 57.75, 59.78, 60.23, 167.10, 168.66, 169.33, 171.09, 171.75, 174.27 (top); δ = 11.83, 12.42, 13.10, 56.77, 56.84, 57.75, 59.77, 60.23, 167.10, 168.66, 169.33, 171.09, 171.76, 174.27 (bottom). PPM range: 0-180.

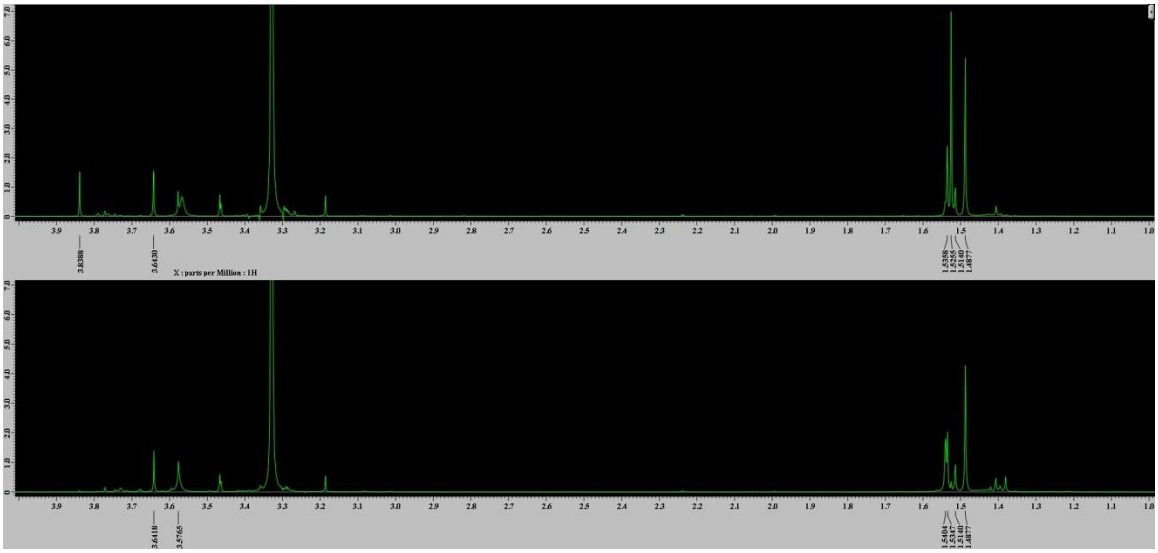

Figure 33. Sodium Source Comparison, NaH, Reactions 4(+M.E.) & 4(+M.E., 2d), ^{1}H-NMR. Dimethyl *trans*-epoxymethylsuccinate in methanol, plus dimethyl malonate and NaH, with one extra mole equivalent of each: immediately (top), and after 48 hours (bottom). NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.49, 1.51, 1.53, 1.54, 3.64, 3.84 (top); δ = 1.49, 1.51, 1.53, 1.54, 3.58, 3.64 (bottom). PPM range: 1.0-4.0.

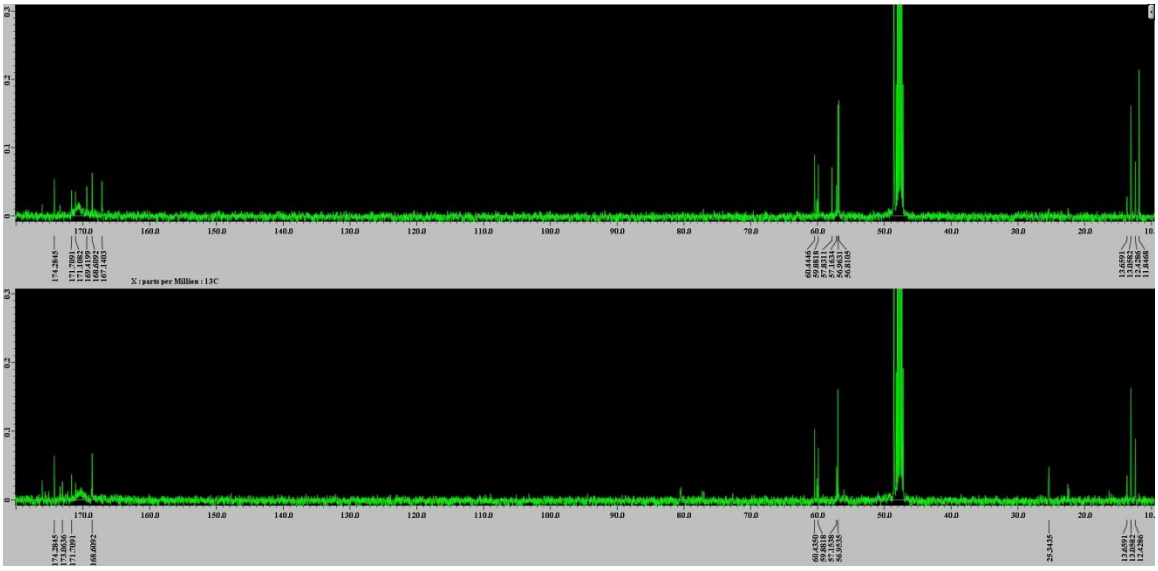

Figure 34. Sodium Source Comparison, NaH, Reactions 4(+M.E.) & 4(+M.E., 2d), ^{13}C-NMR. Dimethyl *trans*-epoxymethylsuccinate in methanol, plus dimethyl malonate and NaH, with one extra mole equivalent of each: immediately (top), and after 48 hours (bottom). NMR parameters were as follows: ^{13}C-NMR (500 MHz, CD_3OD): δ = 11.85, 12.43, 13.06, 13.66, 56.81, 56.96, 57.16, 57.83, 59.88, 60.44, 167.14, 168.61, 169.42, 171.11, 171.71, 174.28 (top); δ = 12.43, 13.06, 13.66, 25.34, 56.95, 57.15, 59.88, 60.44, 168.61, 171.71, 173.06, 174.28 (bottom). PPM range: 10-180.

A reaction with solid sodium metal was set up in an NMR tube to compare with the sodium hydride results.

III.C.1.b. Reaction 4-$Na_{(s)}$: 2-methyl-5-oxo-2,3,4-tricarbomethoxytetrahydrofuran

Sodium metal (0.00313 g, 0.136 mmol) was added to deuterated methanol (0.650 mL) in an NMR tube. Dimethyl malonate (0.0156 mL, 0.136 mmol) was then added and, after a period of waiting as before, an NMR ("1") was taken. Dimethyl *trans*-epoxymethylsuccinate (0.02368 g, 0.136 mmol) was added next, and another NMR ("2") was taken. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD){

[1]: No peaks.

[2]: δ = 1.48, 1.52, 3.58, 3.83.

}; ^{13}C-NMR (500 MHz, CD_3OD){

[1]: No peaks.

[2]: δ = 11.83, 13.10, 57.75, 167.10, 169.32.

}

III.C.2. Does addition order of reactants matter?

In addition to yielding spectroscopically visible results, sodium hydride was found to be easier to work with physically, and was used in an experiment to determine whether the order in which reactants were added to the deuterated methanol would affect the reaction. The addition order had been changed in the first NaH reaction in order to track changes in dimethyl *trans*-epoxymethylsuccinate via NMR from the start. The reaction below revisits the typical solvent→sodium→malonate order.

III.C.2.a. Reaction 4-NaH: 2-methyl-5-oxo-2,3,4-tricarbomethoxytetrahydrofuran

Dry sodium hydride (0.0045 g, 0.18_9 mmol) was added to deuterated methanol (0.650 mL) in an NMR tube. Dimethyl malonate (0.020 mL, 0.17_5 mmol) was added and, after a period of waiting as before, an NMR ("1") was taken. Dimethyl *trans*-epoxymethylsuccinate (0.0254 g, 0.145_8 mmol) was added, and another NMR ("2") was taken. NMR parameters were as follows: ^{1}H-NMR (400 MHz, CD_3OD){

[1]: δ = 3.60 (s).

[2]: δ = {1.49 (s), 3.61 (s), 3.80 (s)} with a {3:3:1} integration ratio.

}; ^{13}C-NMR (400 MHz, CD_3OD){

[1]: No peaks.

[2]: δ = Questionable: 12.28, 27.24, 169.10, 169.20, 171.18.

}.

The reaction was stirred overnight, and a new NMR was taken the next day. The parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.48 (s), 1.52 (s), 3.60 (s), 3.83 (s); ^{13}C-NMR (500 MHz, CD_3OD): δ = 11.83, 12.42, 13.10, 56.77, 57.76, 59.78, 60.23, 167.10, 169.33.

III.C.3. Is the dimethyl *trans*-epoxymethylsuccinate still an epoxide?

A possible source of the complex NMR spectra could have been that the dimethyl *trans*-epoxymethylsuccinate starting product for Reaction 4 was not actually dimethyl *trans*-epoxymethylsuccinate. If somehow the epoxide ring had opened up to yield a diol, then the starting product would have been dimethyl 2,3-dihydroxy-2-methylsuccinate.

There are published data for this product; namely, NMR parameters, which are as follows: "^{1}H-NMR (200 MHz, $CDCl_3$): δ = 1.54 (3 H, s), 3.24 (1 H, bs), 3.45 (1 H, bs), 3.76 (3 H, s), 3.82 (3 H, s), 4.37 (1 H, s); ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 22.4, 52.5, 52.9, 75.5, 76.5, 171.7, 174.6" (Gogoi, 2004). An NMR spectrum was taken of the potentially-not dimethyl *trans*-epoxymethylsuccinate using the same solvent; parameters were as follows: ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.56 (3 H, s, CH_3), 3.74 (3 H, s, OCH_3), 3.78 (3 H, s, OCH_3), 3.78 (1 H, s, OC*H*); ^{13}C-NMR (400 MHz, $CDCl_3$): δ = 12.78, 52.67, 53.06, 56.91, 58.00, 166.72, 169.07. It was concluded that the epoxide ring had not opened to yield a diol because of the lack of hydroxyl hydrogen peaks.

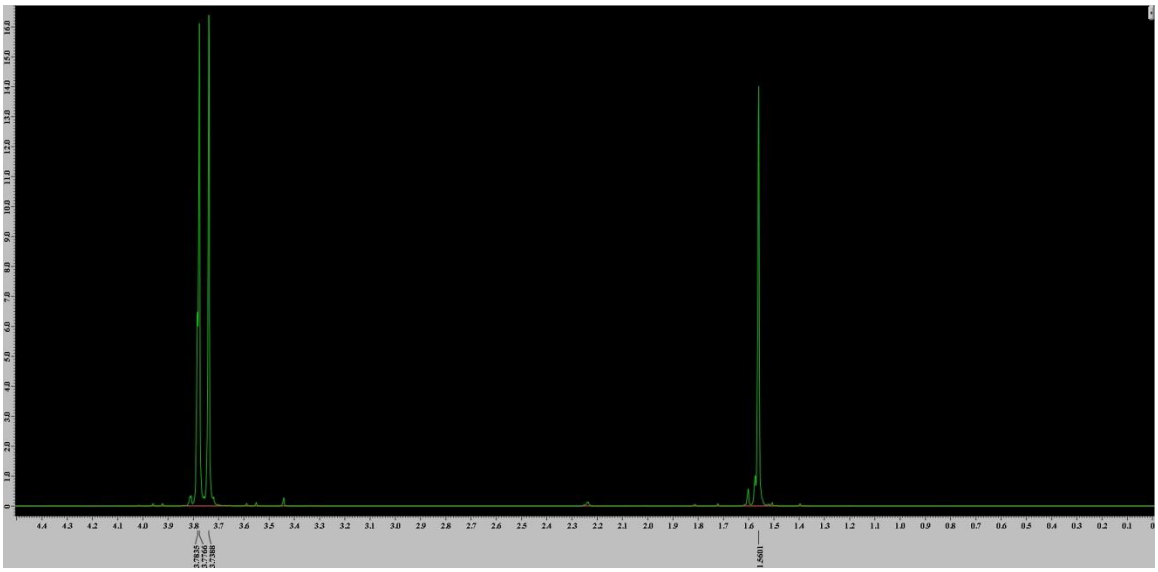

Figure 35. Epoxide-Diol Comparison (Dimethyl *trans*-Epoxymethylsuccinate), ^{1}H-NMR. Testing for presence of diol (dimethyl 2,3-dihydroxy-2-methylsuccinate) to see if epoxide ring might not have opened up. NMR parameters were as follows: ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.56 (3 H, s, CH_3), 3.74 (3 H, s, OCH_3), 3.78 (3 H, s, OCH_3), 3.78 (1 H, s, OC*H*). Hydroxyl peaks present in the diol—"3.24 (1 H, bs), 3.45 (1 H, bs)" (Gogoi, 2004)—were lacking here; the epoxide ring had not opened up. PPM range: 0.0-4.5.

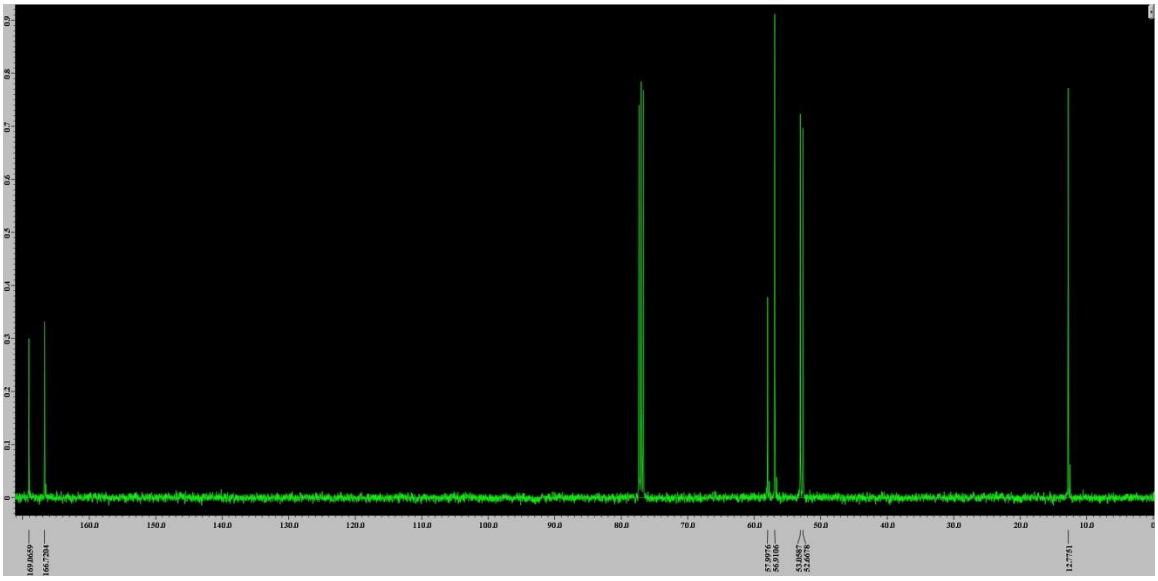

Figure 36. Epoxide-Diol Comparison (Dimethyl *trans*-Epoxymethylsuccinate), ^{13}C-NMR. Testing for presence of diol (dimethyl 2,3-dihydroxy-2-methylsuccinate) to see if epoxide ring might not have opened up. NMR parameters were as follows: ^{13}C-NMR (400 MHz, $CDCl_3$): δ = 12.78, 52.67, 53.06, 56.91, 58.00, 166.72, 169.07. There was negligible difference between the two ^{13}C-NMR spectra, as expected. PPM range: 0-170.

III.C.4. Proof of concept

As a test to see that lactonization had occurred, diethyl malonate was substituted for dimethyl malonate. The theory was that an ethyl moiety would be present in the triester lactone, which would show up as a distinct set of triplet-quadruplet peaks, not dissimilar to those of diethyl ether, in a proton NMR. Below are comparative reactions (dimethyl malonate vs diethyl malonate), and respective reaction methods and NMR spectra.

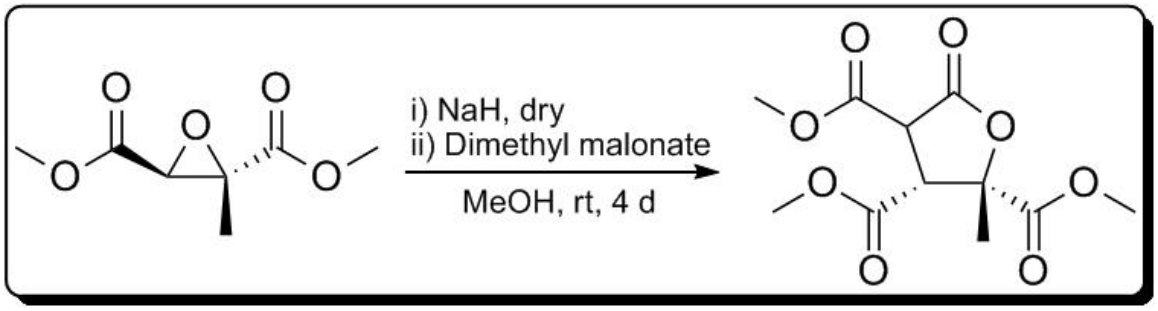

Figure 37. Overview of Reaction Steps to Convert Dimethyl *trans*-Epoxymethylsuccinate to 2-Methyl-5-oxo-2,3,4-tricarbomethoxytetrahydrofuran. Sodium hydride deprotonates methanol to release hydrogen gas and to form sodium methoxide, which deprotonates dimethyl malonate. The electrons of the negatively charged carbon attack the dimethyl *trans*-epoxymethylsuccinate at the less-sterically-hindered epoxide carbon, breaking open the epoxide and freeing the negatively-charged oxygen to attack the nearer carbonyl carbon of the malonate, which ultimately loses [a] methanol [moiety].

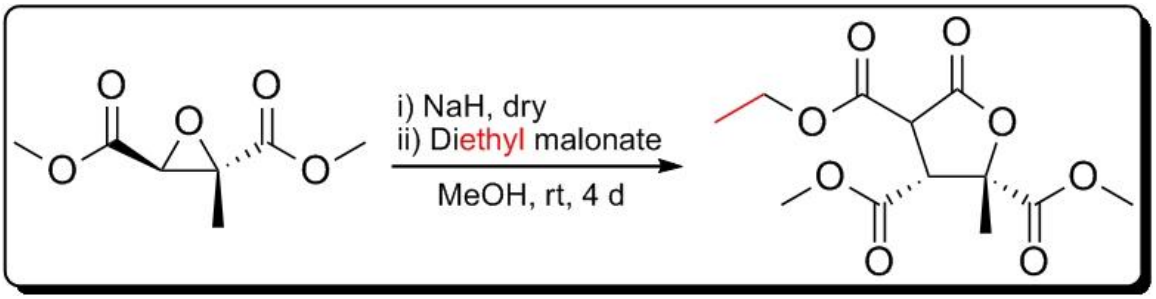

Figure 38. Overview of Reaction Steps for the Proof-of-Concept Reaction to Convert Dimethyl *trans*-Epoxymethylsuccinate to 2-Methyl-5-oxo-2,3-dicarbomethoxy-4-carboethoxytetrahydrofuran. Sodium hydride deprotonates methanol to release hydrogen gas and to form sodium methoxide, which deprotonates diethyl malonate. The electrons of the negatively charged carbon attack the dimethyl *trans*-epoxymethylsuccinate at the less-sterically-hindered epoxide carbon, breaking open the epoxide and freeing the negatively-charged oxygen to attack the nearer carbonyl carbon of the malonate, which ultimately loses [an] ethanol [moiety].

III.C.4.a. Reaction 4a: 2-methyl-5-oxo-2,3,4-tricarbomethoxytetrahydrofuran

Sodium metal (0.686 g, 29.8_5 mmol) was dissolved in dry methanol (~60 mL) at 0 °C, after which dimethyl malonate (3.42 mL, 29.8_5 mmol) was added. Upon the appearance of white particulates in the solution, dimethyl *trans*-epoxymethylsuccinate (4.00 g, 22.9_6 mmol) was added, and the reaction was stirred at room temperature for 4 days. The solvent was evaporated by vacuum and an NMR of the residue was taken.

NMR parameters (and major peaks) were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.06 (t), 1.31 (s), 1.89 (s), 3.57 (s), 3.65 (s), 3.95 (q).

III.C.4.b. Reaction 4-Et: 2-methyl-5-oxo-2,3-dicarbomethoxy-4-carboethoxytetrahydrofuran

Sodium metal (0.137 g, 5.97_2 mmol) was dissolved in dry methanol (~36 mL) at 0 °C, after which diethyl malonate (0.911 mL, 5.97_2 mmol) was added. Upon the appearance of white particulates in the solution, dimethyl *trans*-epoxymethylsuccinate (0.80 g, 4.5_9 mmol) was added, and the reaction was stirred at room temperature for 4 days. The solvent was evaporated by vacuum and an NMR of the residue was taken. NMR parameters (and major peaks) were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.16 (t), 1.48 (s), 1.52 (s), 1.54 (s), 3.66 (s).

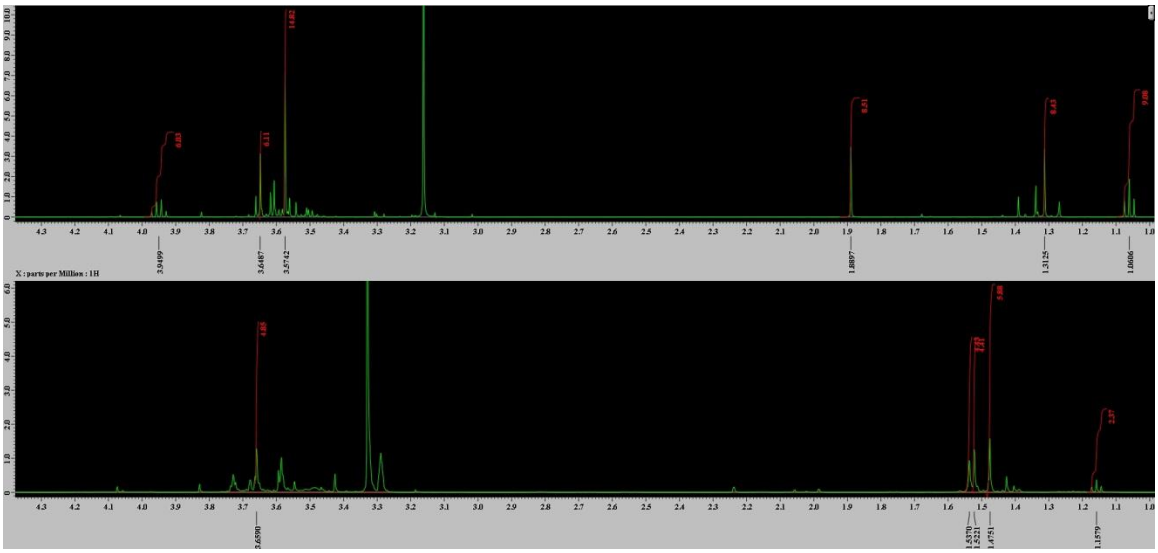

Figure 39. Proof of Concept Comparison Between Reactions of *trans*-Epoxymethylsuccinate With Dimethyl Malonate (top) and Diethyl Malonate (bottom), ^{1}H-NMR. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.06 (t), 1.31 (s), 1.89 (s), 3.57 (s), 3.65 (s), 3.95 (q) (top); δ = 1.16 (t), 1.48 (s), 1.52 (s), 1.54 (s), 3.66 (s) (bottom). PPM range: 1.0-4.4.

III.C.4.c. Reaction 4b: 4a + HCl (-NaCl)

In an effort to purify crude 4a, an attempt was made to dry-load it onto a flash chromatography column, first by redissolving it in a minimal amount of ethyl acetate and adding silica gel (1 g sample : 100 g silica). The resulting mixture was an unmanageable "bubbly, oily, gluey mess." To separate crude 4a from the silica gel, an excess of ethyl acetate was added, the solution was stirred overnight, and the silica gel was filtered off by vacuum. The ethyl acetate was then evaporated by vacuum from the filtrate, and the residue (essentially crude "4a" once again) was redissolved in excess methanol and stirred overnight. The methanol was evaporated by vacuum, and the residue was redissolved in dry methanol to mimic conditions at the end of Reaction 4a's four days of stirring. Hydrochloric acid (10 mL) was added and stirred for an hour, and the precipitated sodium chloride was filtered by vacuum. The solvent was evaporated by vacuum. NMR parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.40 (s), 1.42 (s), 1.53 (s), 1.80 (s), 3.42 (s), and a chaotic mix of peaks between 3.7 and 3.8.

III.C.4.d. Reaction 4b (cont.)

Crude 4b was redissolved in ethyl acetate to precipitate any clandestine NaCl, which was filtered by vacuum. The solvent was evaporated by vacuum, and the residue was redissolved in fresh ethyl acetate (2-3 mL). Flash chromatography was performed using a predetermined 60:40 ether:petroleum ether solvent system. Two major TLC spots were identified. Their respective fractions were combined and the solvents were evaporated by vacuum. NMR spectra were more complex but equally uninformative. Their parameters were as follows: Fraction 1: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.40 (s),

1.48 (s), 1.53 (s), 1.80 (s), 3.31 (s), 3.32 (s), 3.32 (s), 3.36 (s), and a more chaotic mix of peaks between 3.7 and 3.8; Fraction 2: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.42 (s), 1.48 (s), 1.51 (s), 1.53 (s), 3.42 (s), and a less chaotic mix of peaks between 3.7 and 3.8, with three distinct peaks at 3.70 (s), 3.73 (s) and 3.76 (s). Samples of both fractions were sent for mass spectrometric analysis, the target molecule being 2-methyl-5-oxo-2,3,4-tricarbomethoxytetrahydrofuran ($C_{11}H_{14}O_8$, 274.068870 amu). Fraction 1 was shown to contain the target molecule, with peaks at 275.0767_6 amu ($C_{11}H_{14}O_8 + H^+$) and 297.0586_4 amu ($C_{11}H_{14}O_8 + Na^+$). Subjection of this fraction to HCl and reflux to decarboxylate the lactone, however, resulted in a product that, aside from solvent, yielded no ^{1}H-NMR peaks.

CHAPTER IV

RESULTS AND DISCUSSION

IV.A. On the Six Synthesis Attempts re § III.A.

This chapter presents research notes, observations and results regarding the progression of attempts towards synthesis of pure dimethyl *trans*-epoxymethylsuccinate.

Brock et al. detail in their paper a total synthesis of *erythro* 2-methylisocitrate from citraconic anhydride (Brock, 2001). Molar equivalents derived from those reactions' calculations were applied to the same reactants to begin with, but with mesaconic acid as the starting product, towards a total synthesis of *threo* 2-methylisocitrate. (Brock showed the *threo* set of enantiomers to contain the active substrate for *prpB*, the *E. coli* homolog of *yqiQ*.)

Note: Some of the six attempts' theoretical dimethyl *trans*-epoxymethylsuccinate products were taken forward to attempt lactonization, but these results not only yielded either confusion or nothing, they also offered no insight towards improving the methods leading up to, and including, methyl esterification. Because these methods are the focus of § III.A., those subsequent reactions were omitted from that section and were instead relegated to Appendix A.

IV.A.1. Attempt 1

Reaction 1 in Brock's directions ("*Cis*-epoxymethylsuccinate disodium salt") resulted in a white solid precipitate upon drop-wise addition to acetone. Here, however,

an oil precipitated, leading to a deviation from Brock. In a previous attempt of this reaction, believing the oil to be a combination of the target compound and impurities, a recrystallization method (acetone/benzene) was performed on it after decanting all but a minute amount of the acetone. This method, used by Brock to recrystallize the decarboxylated lactone (β,γ-dicarboxylate-γ-methyl-γ-butyrolactone ("*erythro* 2-methylisocitrate lactone" in source paper)), was not successful in recrystallizing our target compound, *trans*-epoxymethylsuccinate disodium salt. In this current attempt, therefore, a bit of chemical brute force was applied in the form of high-temperature (97 °C) evaporation by vacuum to remove all traces of benzene, acetone and solvent. The result was a white powder that, while at an unbelievable 100% yield, did produce an NMR spectrum that matched expectations based on Brock.

The NMR of the crude product in Reaction 3 looked promising with respect to the number of peaks; the integrations, however, were nonsensical. TLC spotting led to the solvent system used to purify the product by column chromatography. The NMR of this "purified" product produced a more complex NMR spectrum. Impurities introduced during the column chromatography seem, in retrospect, to be the only explanation. At the time, however, it was thought that the complexity could have been due to the epoxidation's not going to completion, or to byproducts thereof.

Not even 1.5 molar equivalents of sodium hydroxide were used in Reaction 1. It was thought that perhaps full deprotonation of mesaconic acid was required (to avoid the aforementioned byproducts and to obtain complete epoxidation), which led to the increase in NaOH in Attempt 2.

IV.A.2. Attempt 2

As previously stated, the molar equivalents of sodium hydroxide were increased in Reaction 1 from just under 1.5 to just over 2. Also, the events from Attempt 1 obviated the need to use drop-wise addition into acetone; this step was removed.

Proton-NMR peaks in the 6-7 ppm range for Reaction 1 and Reaction 2 indicated the presence of vinylic protons, as in the starting product, mesaconic acid. For reasons then unknown, either epoxidation had not taken place, or some new reaction had occurred to produce a proton that mimicked the signature of a vinylic proton. It was decided to go forward with Reaction 3 to see if the vinylic peak would remain.

Prior to this attempt, it was learned by which mechanism thionyl chloride methylates the epoxymethylsuccinate carboxylic acids. One molecule of thionyl chloride reacts with one molecule of methanol (the solvent) to yield one molecule each of sulfur dioxide, hydrogen chloride, and methyl chloride. The latter is responsible for transferring its methyl group to the carboxylate, with a net loss of another molecule of hydrogen chloride. Therefore, two moles of $SOCl_2$ are needed for each mole of epoxymethylsuccinate. Brock's method used 1.05 molar equivalents; in this current attempt, that was changed to 2.3. (N.B.: Subsequent to all attempts, it was learned that the previous mechanism was incorrect. Thionyl chloride and methanol yield HCl and dimethyl sulfite. The HCl produced activates the carbonyls, and Fisher methyl esterification proceeds. A molar equivalent of $SOCl_2$ slightly greater than 1 is sufficient.)

The vinylic proton in the ^{1}H-NMR spectrum for Reaction 3 persisted, and suggested that dimethyl mesaconate was our product; i.e., epoxidation of mesaconic acid

never took place. The thought at this point was that perhaps the hydrogen peroxide used had decomposed, and a new source of H_2O_2 was planned for Attempt 3. The change in amount of NaOH was never considered a cause because of the ^{1}H-NMR spectrum, which, despite the vinylic proton, presented with only four peaks, each with the expected integrations. In other words, full deprotonation of mesaconic acid was thought to have cleared up the complex ^{1}H-NMR in Attempt 1.

IV.A.3. Attempt 3

In order to test just the change in source of H_2O_2, molar equivalence ratios from Attempt 1 were reapplied. The new H_2O_2 was a 35% solution. Vinylic peaks disappeared in ^{1}H-NMRs of Reactions 1 and 2, leading initially to the conclusion that decomposed H_2O_2 had been responsible for the lack of epoxidation—for the presence of the vinylic proton—earlier. (This is revisited in the analysis of Attempt 6.)

Very little peak elucidation resulted from purifying the crude dimethyl *trans*-epoxymethylsuccinate in Reaction 3, but the purified sample dissolved in CD_3OD produced the least confusing spectrum, as well as the spectrum that most closely resembled the Reaction 3 spectrum from Attempt 2, less the vinylic peak.

IV.A.4. Attempt 4

A more in-depth search of the literature turned up a paper that detailed the synthesis of *trans*-epoxysuccinic acid from fumaric acid (Payne, 1959). This synthesis was followed directly except where it was necessary to repurpose it to address synthetic issues encountered thus far starting with mesaconic acid.

New to this attempt, in Reaction 1, were the two different stirring temperatures, and the monitoring of pH. Confidence was low in the accuracy of the pH monitor because of its persistent indication of pH 4.01, regardless of elapsed reaction time or amount of additional sodium hydroxide added. Near the end of the reaction, the pH probe was changed for another, which registered 9.0. Because the ^{1}H-NMR showed expected peaks and integrations for the disodium salt, the product was taken further. The pH situation would be addressed in the next full attempt.

New as well to this attempt was an ion exchange of the *trans*-epoxymethylsuccinate salt, from sodium to barium. Note that while Payne performed both the disodium salt transfer to acetone *and* the Na^{+}-Ba^{2+} ion exchange, the acetone step was ignored in our synthesis for two reasons: 1) experience showed us that the *trans* salt precipitates into an oil not easily manageable, and 2) the ion exchange isolates the epoxymethylsuccinate salt, rendering any intermediate [acetone] step superfluous at best. The one drawback to the barium salt was its insolubility in any of the NMR solvents on hand (D_2O, $(CD_3)_2CO$, $CDCl_3$, CD_3OD, $(CD_3)_2SO$).

Regarding Reaction 2, note that calculations in this reaction [erroneously] used a molecular weight of 281.411 g/mol for the barium salt. It is, in fact, a dihydrate salt, and using the proper value of 317.441 g/mol would have increased the reported yield to 55%.

A rereading of Brock found a very brief mention of his treatment of *trans*-epoxymethylsuccinic acid to obtain dimethyl *trans*-epoxymethylsuccinate; namely, "anhydrous acidic methanol." An experiment was therefore conceived to investigate two different methods of acidifying dry methanol, and to compare them not only to each

other, but also to the use of thionyl chloride as hitherto the only method used for methyl esterification. Direct addition of sulfuric acid to methanol proved to be the most efficient method.

IV.A.5. Attempt 5

Some leftover *trans*-epoxymethylsuccinic acid was used to test the conclusion reached in Attempt 4. As predicted, the ^{1}H-NMR peaks were clean, matched the previous spectrum, and were devoid of stray peaks.

IV.A.6. Attempt 6

With methods for Reaction 2 and Reaction 3 squared away, the issue of the H_2O_2 (see Attempt 3 above) was revisited. To retest that a change in H_2O_2 was responsible for allowing the epoxidation to proceed, the original source of H_2O_2 was used, while the molar equivalence ratios from Attempt 2 were reinstated. Recall that these included more than 2 molar equivalents of NaOH.

Reaction 1 (combining Reactions 1a and 1b from Attempt 4) was preceded by one just like it (same molar equivalents) where the barium salt yield was 14%. It was believed to be due to a fault in the sodium-barium exchange reaction, either by addition of barium chloride immediately following the three hours of stirring (no evaporation by vacuum—i.e., no prior decrease in volume of disodium salt solution), or by an "overly-porous filter" (lab notebook quote). This current reaction (Attempt 6, Reaction 1) allowed for evaporation by vacuum but then proceeded directly to acidification—no barium.

A ^{1}H-NMR spectrum for Reaction 2 showed two peaks, one of which showed up in the vinylic range, indicating that the product was almost certainly mesaconic acid, having simply been de- and reprotonated; i.e., no epoxidation occurred.

The resulting conclusion was that the purpose of NaOH was solely to deprotonate mesaconic acid in order to dissolve it in water. Neither single nor double deprotonation have any intrinsic effect on epoxidation. It was not the new source of H_2O_2 that eliminated the vinyl peak in Attempt 3; rather, it was the return to the lower molar equivalence of NaOH.

Note: As with Attempt 5, incorrect molar mass values for the barium salt were used in Reactions 1(bis)a, 1b and 2(bis). It was not confirmed until later that the barium salt is in fact a dihydrate ($C_5H_4O_5Ba{\cdot}2H_2O$). The correct molar mass was used in § III.B. for the Final Reactions.

IV.B. Solid Sodium Metal vs. Dry Sodium Hydride

Proton- and ^{13}C-NMR spectra displayed expected peaks and integrations for dimethyl *trans*-epoxymethylsuccinate by itself and after addition of dimethyl malonate (Figures 27 and 28, and 29 and 30, respectively). Upon addition of sodium hydride, however, methyl ester peaks disappeared immediately from the ^{1}H-NMR spectrum, shrank dramatically in the ^{13}C-NMR spectrum, and remained so in both a day later (Figures 31 and 32). Given the reactants and products of this reaction, a ^{1}H-NMR or ^{13}C-NMR scan at any point in time should have shown at least methyl ester proton peaks or carbonyl carbon peaks, respectively. One possibility for these absences is that the heat of the exothermic reaction $NaH_{(s)} + CH_3OH_{(l)} \rightarrow Na^{+}_{(aq)} + CH_3O^{-}_{(aq)} + H_{2(g)}$ decomposed

much of the material. Unfortunately, this would not help to explain the peaks that did remain, most notably epoxide-related proton peaks at 3.83 (OC*H*) and 1.52 (CH_3), and their respective carbon peaks at 56.77 and 11.83.

This decomposition idea seems not to have been considered at the time. An attempt was made to test for any remaining dimethyl *trans*-epoxymethylsuccinate by the addition of an extra molar equivalent each of NaH and dimethyl malonate. While there were negligible spectral changes between the previous two sets of spectra, the post-extra molar equivalent spectra (Figures 33 and 34) actually showed change. Most notably, the former proton spectra showed barely any change at peaks 1.52 and 3.83, while the latter spectra showed marked reduction at 1.53 and disappearance at 3.84. This indicated that the epoxide ring, the "invisible" methyl ester carboxylates that flank it notwithstanding, was reacting in some way, almost certainly by opening up. To form the target lactone, though? That is uncertain. While expected proton peaks—two sets of doublets from the adjoining α-carbon and β-carbon protons of the lactone—eluded us, carbon-13 peaks indicated the formation of some new product (loss of 11.85, 56.81, 57.83 and 167.14 ppm; gain of ~22.5 and 25.34 ppm).

Comparing the sodium hydride NMR tube reactions with those of solid sodium metal showed several advantages to using sodium hydride. First, the material itself is easier and less messy to work with, and second, peaks were either analyzable or at least present. It is unknown why the reaction mixture of solid sodium metal and dimethyl malonate in deuterated methanol yielded no peaks, nor why the subsequent reaction mixture (after addition of dimethyl *trans*-epoxymethylsuccinate) yielded only some peaks

that pertained to the succinate—methyl ester protons and carbons were missing from their respective spectra.

Sodium hydride, therefore, was used in two reactions to test specifically whether addition order matters, which it does. The preferred order was confirmed to be the standard one: methanol→sodium→dimethyl malonate→dimethyl *trans*-epoxymethylsuccinate.

IV.C. Proof of Concept Reactions

Note that these reactions were performed before the sodium comparison reactions in the previous section; i.e., they were performed with sodium metal. This could be, but likely is not, an explanation for the inconclusiveness.

Despite no ether nor ethanol's having been used, the dimethyl malonate reaction ^{1}H-NMR spectrum displayed an ethyl moiety. The di*ethyl* malonate reaction ^{1}H-NMR spectrum displayed only the triplet (CH_2) of such a moiety. Neither spectrum showed two sets of doublets, as would be expected by the adjacent α-carbon and β-carbon protons of the lactone. The concept of the reaction mechanism was not proven because expected peaks were not observed. This remains a valid test for proof of concept; however, only once reaction conditions for lactonization have been perfected and the dimethyl malonate-based lactone is shown conclusively to have been formed should this test be revisited.

In the event that the desired product was present among impurities in the dimethyl malonate reaction ("4a"), this crude mixture was taken forward and ultimately shown indeed to contain the lactone. This confirms that we are on the right path, and that

purification is theoretically all that stands in the way of decarboxylation by reflux, followed by lactone ring opening, to obtain the desired *threo* 2-methylisocitrate.

CHAPTER V

FUTURE WORK

The overarching goal of the Reddick lab, as regards the work presented within this thesis, is to characterize fully the *yqiQ* gene from *B. subtilis*. This characterization comprises two parts: the synthesis of the enzyme's substrate, *threo* 2-methylisocitrate, and enzymatic assays to confirm the enzyme's hypothesized lysing function.

As of now, diastereomerically pure *threo* 2-methylisocitrate still eludes us. With successful contrivance of a methodology to synthesize highly pure dimethyl *trans*-epoxymethylsuccinate complete, the chief obstacle remaining is its obstinacy to lactonize with dimethyl malonate to form 2-methyl-5-oxo-2,3,4-tricarbomethoxytetrahydrofuran. Following this step, subsequent decarboxylation and ring opening should prove to be less challenging, in theory. The enantiomeric purification of *threo* 2-methylisocitrate would help to discover which member of the *threo* pair is accepted by the enzyme. Purification would also produce very clean HPLC spectra that could more concretely be matched with standards.

The second major task, once the activity of YqiQ has been confirmed with pure *threo* 2-methylisocitrate (Figure 40), is to measure the kinetics of the enzyme via several UV/Vis spectroscopy experiments.

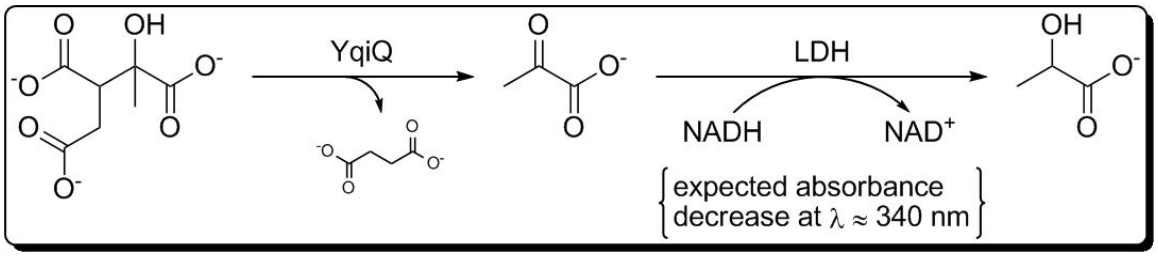

Figure 40. Schematic Detailing the Test to Measure YqiQ Activity. YqiQ activity, with the as-yet-unknown *threo* 2-methylisocitrate enantiomer as subtrate, will be assessed indirectly by the consumption of NADH. In the presence of pyruvate and NADH, lactate dehydrogenase produces lactate and NAD^+, respectively. NADH absorbs light at 340 nm. Because of the direct correlation between pyruvate and NADH conversions, a decrease in the absorbance of light at 340 nm would confirm that YqiQ is splitting 2-methylisocitrate into succinate and pyruvate.

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APPENDIX A

ADDITIONAL REACTIONS

Attempt 1.

Reaction 4: 2-methyl-5-oxo-2,3,4-tricarbomethoxytetrahydrofuran

Sodium metal (0.556 g, 24.1_8 mmol) was dissolved in dry methanol (30 mL) at 0 °C. Dimethyl malonate (2.768 mL, 24.18 mmol) was added until white particulates appeared, to which was added dimethyl *trans*-epoxymethylsuccinate (3.24 g, 18.6 mmol). The solution was stirred at room temperature for 4 days. Concentrated hydrochloric acid (10 mL) was added (a color change from deep red to bright yellow was noted), and the solution was stirred for another 90 minutes. Sodium chloride was filtered off and the solvent was evaporated by vacuum. The residue was dissolved into ether (50 mL), and a liquid-liquid extraction was performed with water (20 mL) and two extractions of ether (25 mL ea.). The organic layer collection was dried with anhydrous magnesium sulfate and evaporated by vacuum to yield 4.74 g (17.2_8 mmol, 93%) theoretical titular compound as an oil. NMR parameters were as follows: ^{1}H-NMR (500 MHz, D_2O): δ = 2.03, 3.16.

Reaction 5: β,γ-dicarboxylate-γ-methyl-γ-butyrolactone

To unpurified 2-methyl-5-oxo-2,3,4-tricarbomethoxytetrahydrofuran (1.59 g, 5.79_8 mmol) was added 6 M hydrochloric acid (20 mL). The solution was refluxed (~150 °C) for 3-4 days. The solvent was evaporated by vacuum and rinsed/evaporated three times with water (to remove traces of HCl) to yield 0.75 g (3.9_8 mmol, 69%)

theoretical titular compound. NMR parameters were as follows: ^{1}H-NMR (500 MHz, D_2O): δ = 1.33, 1.39, 1.41, 1.44, 1.57, 1.63, 2.03.

Reaction 6: *threo* 2-methylisocitric acid

Three equivalents of sodium hydroxide (0.4735 g, 11.83_8 mmol) in water (20 mL) were added to β,γ-dicarboxylate-γ-methyl-γ-butyrolactone (0.74 g, 3.9_3 mmol), and the solution was stirred at room temperature for 3 hours. The solution was acidified with concentrated hydrochloric acid (~20 mL), and evaporated by vacuum to obtain 0.97 g (4.7_7 mmol, 121%) theoretical titular compound. NMR spectra showed no significant peaks.

Attempt 3.

Reaction 4: 2-methyl-5-oxo-2,3,4-tricarbomethoxytetrahydrofuran

Sodium metal (0.521 g, 22.6_8 mmol) was dissolved in dry methanol (10 mL) at 0 °C. Dimethyl malonate (2.596 mL, 22.68_1 mmol) was added until white particulates appeared, to which was added dimethyl *trans*-epoxymethylsuccinate (3.95 g, 22.6_8 mmol) (color became bright yellow). The solution was stirred at room temperature for 4 days. Concentrated hydrochloric acid (10 mL) was added and the solution was stirred for another 90 minutes. Sodium chloride was filtered off and the solvent was evaporated by vacuum. The residue was dissolved into ether (50 mL), and a liquid-liquid extraction was performed with water (20 mL) and two extractions of ether (25 mL ea.). The organic layer collection was dried with anhydrous magnesium sulfate and evaporated by vacuum to yield 5.65 g (20.6 mmol, 91%) theoretical titular compound as a yellow oil. NMR

parameters were as follows: ^{1}H-NMR (500 MHz, CD_3OD): δ = 1.75, 1.80, 3.42, and a jumble of peaks between 3.70 and 3.79.

Reaction 5: β,γ-dicarboxylate-γ-methyl-γ-butyrolactone

To unpurified 2-methyl-5-oxo-2,3,4-tricarbomethoxytetrahydrofuran (5.65 g, 20.6 mmol) was added 6 M hydrochloric acid (20 mL). The solution was refluxed (~105 °C) for 3 days. The solvent was evaporated by vacuum and rinsed/evaporated three times with water to remove traces of HCl to yield 3.08 g (16.3_7 mmol, 79%) theoretical titular compound. NMR parameters were as follows: ^{1}H-NMR (500 MHz, D_2O): δ = 1.57 (3 H, s, C*H*$_3$), 2.86 (2 H, d, C*H*$_2$), 3.41 (1 H, t, C*H*). Two additional major peaks were present, with integrations [relative to the aforementioned C*H*] of 1.64 (1.63, s) and 1.44 (3.16, s).

Reaction 6: *threo* 2-methylisocitric acid.

A stirring solution of β,γ-dicarboxylate-γ-methyl-γ-butyrolactone (3.12 g, 16.5_8), sodium sulfate (1 g), and 10% sodium hydroxide (1.065 g) in water (50 mL) was brought to a boil, then allowed to cool to room temperature, after which a solution of 10% sulfuric acid in water (10 mL) was added. The solvent was evaporated by vacuum to obtain 2.36 g (11.4_5 mmol, 69%) theoretical titular compound as a golden oil. NMR parameters were as follows: ^{1}H-NMR (500 MHz, D_2O): δ = 0.99 (1 H?, t, C*H*?), 1.57 (3 H, s, C*H*$_3$), 2.86 (2 H, d, C*H*$_2$), 3.40 (1 H, t, C*H*).

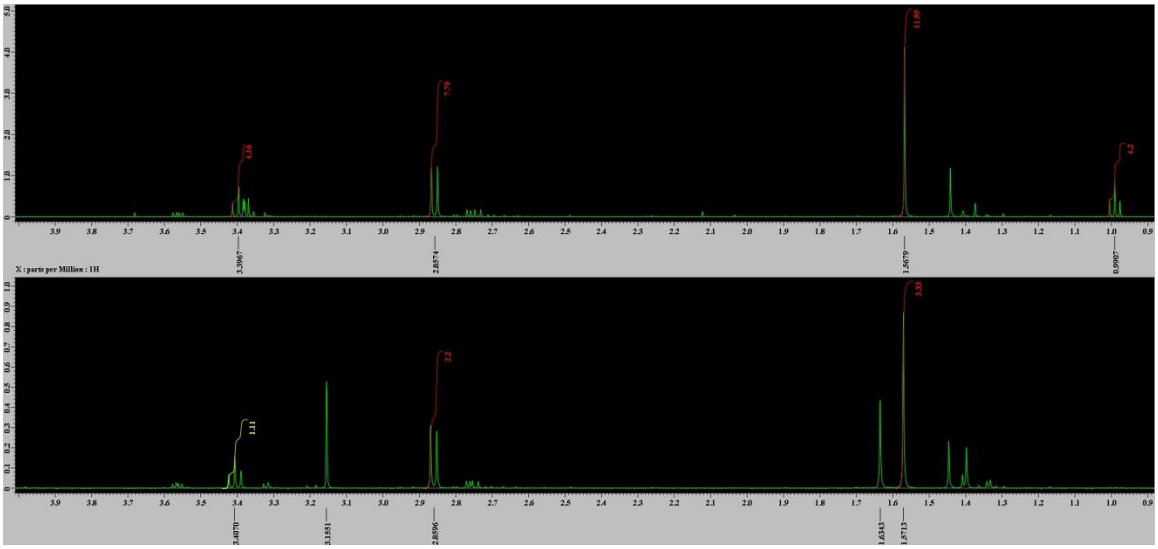

Figure 41. Attempt 3, Reactions 5 (top: β,γ-Dicarboxylate-γ-methyl-γ-butyrolactone) and 6 (bottom: *threo* 2-Methylisocitric Acid), ^{1}H-NMR. Reaction 5 (top) NMR parameters were as follows: ^{1}H-NMR (500 MHz, D_2O): δ = 1.57 (3 H, s, C*H*$_3$), 2.86 (2 H, d, C*H*$_2$), 3.41 (1 H, t, C*H*). Two additional major peaks were present, with integrations [relative to the aforementioned C*H*] of 1.64 (1.63, s) and 1.44 (3.16, s). Reaction 6 (bottom) NMR parameters were as follows: ^{1}H-NMR (500 MHz, D_2O): δ = 0.99 (1 H?, t, C*H*?), 1.57 (3 H, s, C*H*$_3$), 2.86 (2 H, d, C*H*$_2$), 3.40 (1 H, t, C*H*). PPM range: 1.0-4.0.