5-2022

Synthesis of a Functionalized trans-Cyclooctene (TCO) for Selective Protein Isolation After Bioorthogonal Labeling with Tetrazine

Thaddeus Lawrence Puzdrakiewicz
University of Tennessee, Knoxville, tpuzdrak@vols.utk.edu

Dillon P. McBee
University of Tennessee, Knoxville, dmcbee1@vols.utk.edu

Joshua A. Baccile
University of Tennessee, Knoxville, jbaccile@utk.edu

Synthesis of a Functionalized *trans*-Cyclooctene (TCO) for Selective Protein Isolation After Bioorthogonal Labeling with Tetrazine

Thaddeus Puzdrakiewicz
University of Tennessee – Knoxville
Advisor: Joshua Baccile, PhD

Department of Chemistry
1420 Circle Drive, Knoxville, TN, 37996-1600
Baccile Lab – Buehler 433

Chancellor's Honors Program
Capstone Thesis
May 2022

Table of Contents

1.0 – Abstract

This project aims to demonstrate a cost-effective synthesis of a functionalized *trans*-cyclooctene (TCO), a common bioorthogonal click chemistry tool. The intended product of this synthesis, *trans*-cyclooct-4-enol (TCO-5-OH), will be further modified at the alcohol substituent, eventually becoming a molecular probe, selectively binding proteins with tetrazine-labeled amino acid derivatives. The most significant drawback of TCO-tetrazine click chemistry is the price of TCO; TCO-5-OH can be purchased from Sigma-Aldrich for $186/10mg of for $686/50mg. For the application explained above, TCO-5-OH is just the starting material for a two-step probe synthesis, so a conservative estimate would indicate at least a few hundred milligrams are needed. Standard literature procedures for the reaction that produces this highly strained ring are either low-yielding, expensive, or procedurally tricky.[8] This synthesis is intended to provide the starting material for the probe at a significantly lower cost, roughly $350, excluding the departmental photoreactor, nearly 80 years old. This was attempted through a three-step synthesis. First, 1,5-cyclooctadiene (**1**) was epoxidized by meta-chloroperoxybenzoic acid. The resulting epoxide (**2**) was reduced by $LiAlH_4$. Lastly, an isomerization was performed by irradiating the reaction flask with light (254nm) while actively removing the *trans* isomer (**4**) and returning the *cis* isomer (**3**) to the flask for further irradiation, shifting the equilibrium of the reaction towards the unfavorable, highly strained ring. ^1H NMR spectroscopy was used to analyze each reaction's product(s). This project aims to demonstrate a cost-effective synthesis of *trans*-cyclooct-4-enol using procedures backed by published literature[5,7] while substituting various photochemical materials for cheaper alternatives of lesser qualities. This was partially accomplished since the final product was made, but the cumulative yield was lower than anticipated, 3%. However, minor modifications to the third flow reaction would likely improve this number significantly.

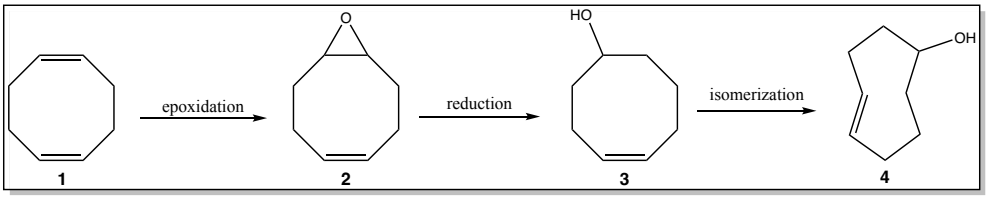


Figure 1: Overview of synthetic plan

2.0 – Background and Introduction

2.1 – Bioorthogonal Click Chemistry

Click chemistry is a classification of rapid, highly selective reactions between simple organic molecules.[1] These molecules can be chemically modified in a wide variety of ways; the researcher can essentially customize each molecule of the click pairing, imposing the desired characteristics for their specific application. Click reactions are so rapid and selective that they can occur at high rates in living organisms, an uncontrolled system with diverse molecules, much different from the controlled reaction flask in which most artificial reactions occur.[1] This robustness gives click reagents a multitude of potential biological applications. However, the reactivities of the products of a reaction must also be considered when attempting to gauge biocompatibility. Manipulations of an organism's biochemistry by these products would significantly limit the usefulness of even the quickest and most efficient reactions.

Figure 1: Devaraj et al.[9]

If a researcher were to use a metal-catalyzed click chemistry reaction, like Cu(II)-catalyzed azide-alkyne cycloaddition (CuAAC), in vivo, the organism would most likely die because of the low physiological compatibility of Cu(I) as well as the reactive oxidative species (ROS) that are released from this reaction.[2,3] These two components of CuAAC are known to be deadly to mammalian cells; most cells die within 60 minutes of exposure to 500uM of these reagents.[3] Instead, this researcher should choose a click chemistry pairing that yields unreactive, non-cytotoxic products.[4,5] In the early 2000s, click chemistry pairings with these traits were identified.[4,5] They are appropriately referred to as bioorthogonal click chemistry reagents.[1] They are still quick and robust under physiologic conditions, but they do not impact the biochemical activity of an organism.[1] This has given bioorthogonal click reactions an increasing level of attention over the past two decades from various fields, including biochemical sciences, material

sciences, pharmaceutical sciences, radiotherapy, drug discovery, proteomics, DNA research, molecular imaging, and more.[1]

2.2 – TCO-Tetrazine Ligation

The bioorthogonal click reaction related to this project is between *trans*-cyclooctene and tetrazine. The reaction's speed and specificity are due to its inverse-electron demand Diels Alders (IEDDA) mechanism, shown in **figure 2**, and the highly strained ring conformation of TCO.[2] The combination of these complementary attributes makes this the fastest click reaction currently identified ($k > 800\ M^{-1}s^{-1}$).[2] Further, the conjugated TCO-tetrazine product is biologically inert, as are TCO, tetrazine, and the N_2 gas released as a side product, making it an ideal tool for in vivo click chemistry.[2,3]

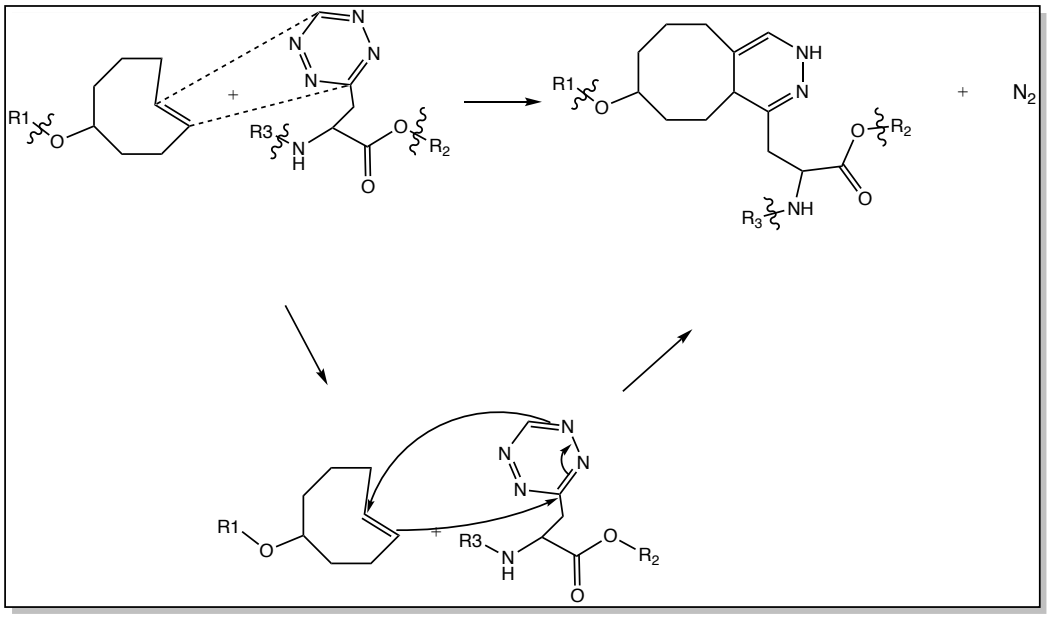


Figure 2: TCO-tetrazine ligation, an inverse-electron demand Diels Alder (IEDDA) cycloaddition

In this synthesis, the functionalized TCO product will be used as a tool for selective protein isolation after bioorthogonal protein labeling with tetrazine. Tet-Phe, a phenylalanine derivative with tetrazine in the place of benzene, will be integrated into the proteomes of E. coli cells through environmental doping, summarized in **figure 3**. This integration will be procedurally random, only influenced by the natural conditions of each protein as it is synthesized. To identify any trends in Tet-Phe integration, the entire

primary structure of each protein that underwent Tet-Phe integration must be removed selectively. This will be accomplished by the TCO probe, shown in **figure 4**. After protein integration, cells will be lysed, and all non-proteins will be removed. The exposed TCOs will react with exposed tetrazines of Tet-Phe, as shown in **figure 5.** The shaded spherical structure's solid support will not dissolve upon addition. The beads will then be filtered and washed; all remaining molecules will either be unconjugated TCO probes or TCO probes conjugated to tetrazines on the amino acid derivatives which are still covalently attached to the entire primary structure of the protein, **figure 6**, as conditions that would rupture peptide bonds will have been avoided. To remove the solid phase, they will be exposed to mildly acidic conditions, causing the rink amide section of the bridge between TCO and the solid phase to break. Subsequent filtration will leave the conjugated proteins only. From here, each protein can be sequenced.

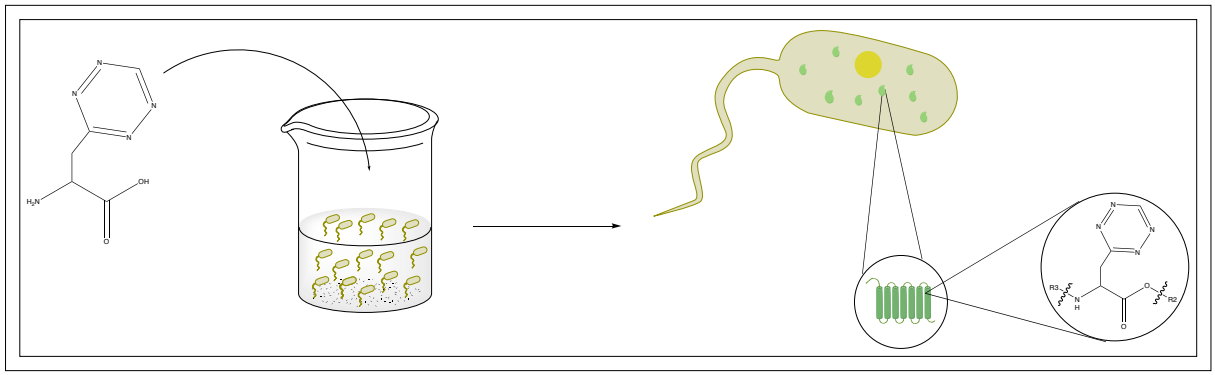


Figure 3: Simplified representation of proteomic integration of the tetrazine-phenylalanine amino acid derivative

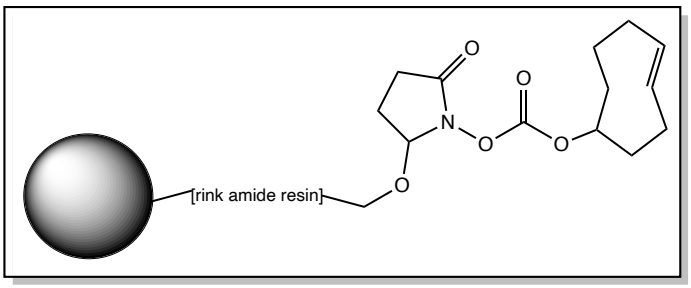


Figure 4: Molecular structure of TCO probe

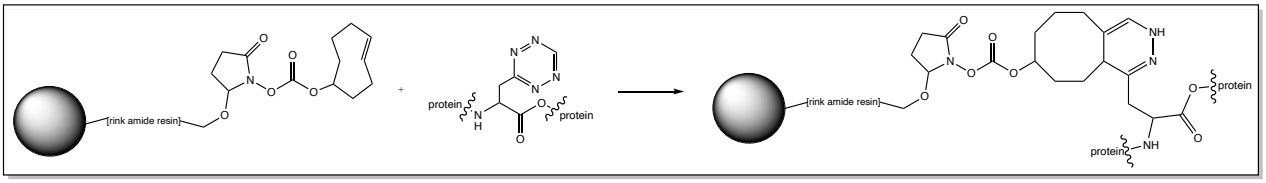


Figure 5: TCO – Tet-Phe ligation

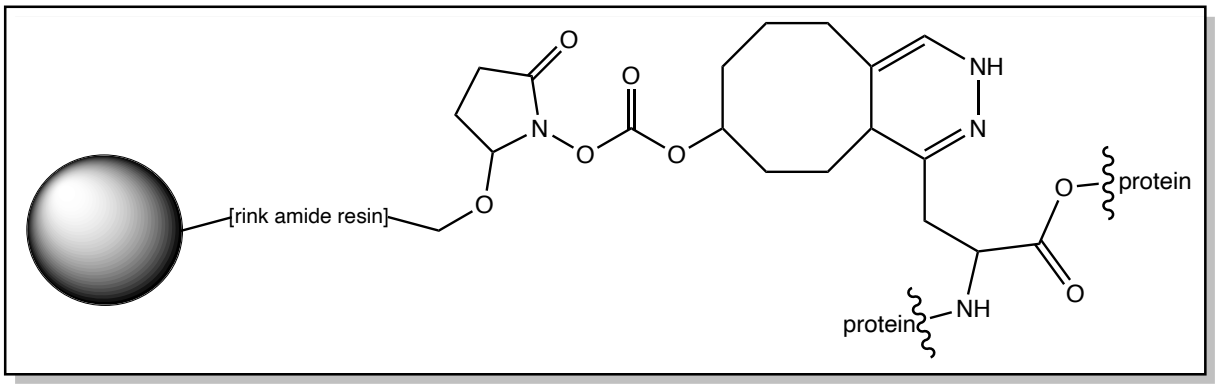


Figure 6: Structure of insoluble TCO probe after ligation with a tetrazine group integrated into the primary structure of a protein

2.3 – Difficult, Expensive, and Inefficient Synthesis of TCO

In contrast to the robustness of the above click reaction, TCO and its derivatives have notoriously difficult syntheses that require expensive materials while proceeding at low yields.[5] For the most part, these difficulties and expenses arise at the last step of the synthesis, the isomerization of the double bond from the highly favored *cis* isomer to the highly strained and unfavored *trans* isomer. Materials for this photoisomerization, listed in the supplementary information section of Fox et al.[6], cost roughly $5,000.

These synthetic barriers account for its high price; *trans*-cyclooct-4-enol is currently selling at $186/10mg or $686/50mg from Sigma-Aldrich. Further manipulation of the functionalized TCO would inevitably result in the loss of precious compound. Even at the most minor scales, each run would consist of hundreds of dollars-worth of compound, so a cost-effective means of producing the desired TCO derivative is necessary. This synthesis attempts to provide such a means by following the procedures outlined in Fox et al.[5,7] while substituting photochemical lab materials,

saving money by sacrificing quality, and producing a TCO derivative of equivalent yield and purity those produced with typical photochemical materials. Because TCO-tetrazine ligation is such a common click chemistry pairing, and bioorthogonal click chemistry is so prevalent throughout a wide array of fields within the scientific community, many researchers wishing to work with TCO will not have pre-existing access to a quartz reaction flask, fluid metering pump, and a contemporary photoreactor. This project aims to demonstrate a cost-effective synthesis of *trans*-cyclooct-4-enol using procedures backed by published literature while substituting various photochemical materials for cheaper alternatives.

3.0 – Synthesis Overview

The synthesis depicted below gives the desired TCO derivative. It proceeds through three individual reactions: epoxidation, reduction, and photoisomerization.

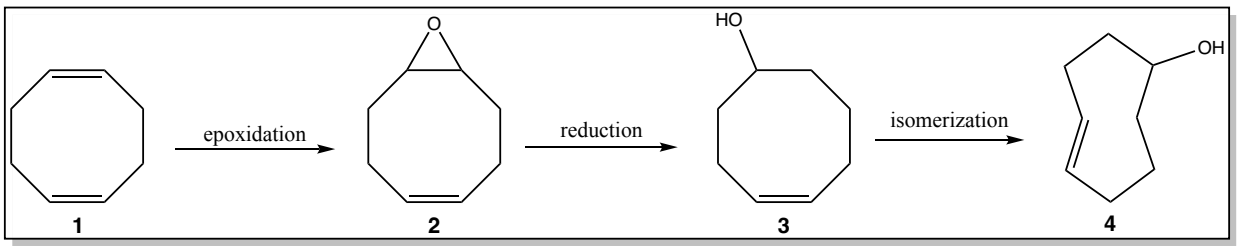
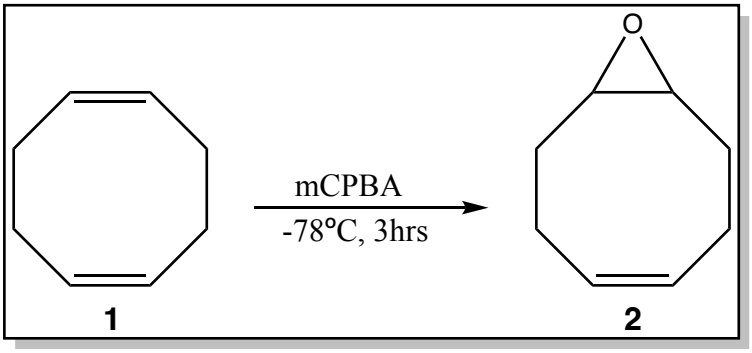


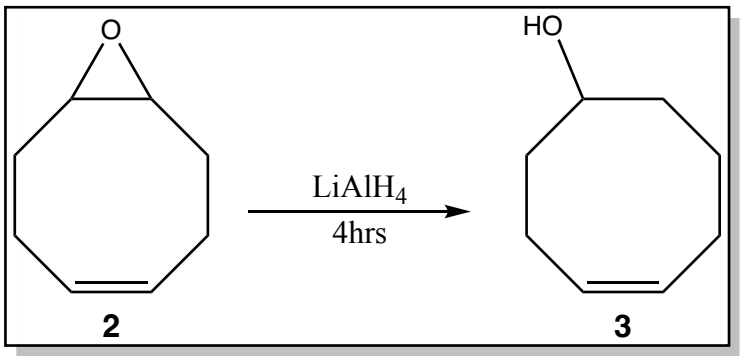
Figure 7: Outline of overall synthesis, ending with a functionalized TCO in the form of *trans*-cyclooct-4-enol (TCO-5-OH)

Figure 8: Epoxidation of 1,5-cyclooctadiene (**1**) to 1,2-epoxycyclooct-5-ene (**2**)



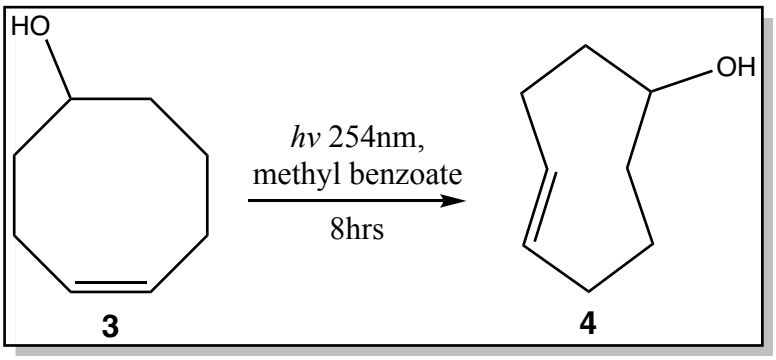
The epoxidation shown above in **figure 8** forms the heteroatomic cyclooctene, **2**. The reaction must be conducted at very low temperatures to minimize the formation of the di-epoxide. One double bond must be preserved, as it gives TCO the high electrophilicity needed for the IEDDA cycloaddition with tetrazine, a molecule with a low electron density. Two manipulations favored mono-epoxidation: using excess COD, **1**, and submerging the reaction mixture in an acetone-dry ice bath, a bath at -78°C.

Figure 9: Reduction of 1,2-epoxycyclooct-5-ene (**2**) to *cis*-cyclooct-4-enol (**3**)



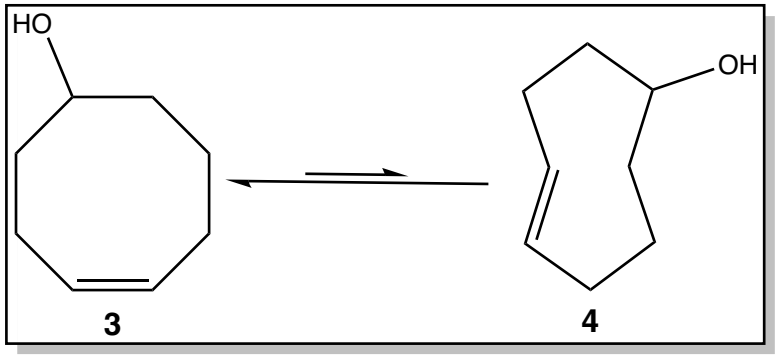
The reduction shown above in **figure 9** forms the alcohol, **3**, from which the molecular bridge to the insoluble resin will be built. $LiAlH_4$, a powerful reducing agent, was used to accomplish this since it cannot reduce carbon-carbon double bonds. This allows for the complete preservation of the singular double bond without sacrificing the yield of the reduced product. Additionally, one $LiAlH_4$ molecule can reduce an epoxide to an alcohol four times, ensuring maximum product formation without a long reaction time.

Figure 10: Isomerization of *cis*-cyclooct-4-enol (**3**) to *trans*-cyclooct-4-enol (**4**)



The isomerization shown above in **figure 10** forms the *trans*-isomer, **4**, finally giving the electrophilic, functionalized, highly strained ring that will react with tetrazine-tagged proteins *in vivo*. Methyl benzoate is added to the reaction flask as a singlet sensitizer, effectively absorbing light greater than 254nm. This allows the cis-starting material to absorb the high-frequency, 254nm light emitted from the bulbs of the photoreactor.

Figure 11a: Equilibrium between *cis*-cyclooct-4-enol (**3**) and *trans*-cyclooct-4-enol (**4**)



The isomerization step of the synthesis reflects the expensiveness of TCO-5-OH. Because the equilibrium is so heavily favorited towards the *cis*-isomer, shown above in **figure 11a**, any successful isomerization must include active removal of the newly formed *trans*-isomers if any significant quantity is to be created. This is achieved by passing the reaction mixture through a column of $AgNO_3$ impregnated SiO_2 gel. Ag^{+2} cations form a metal complex with the electrons of *trans*-isomers but not with *cis*-isomers, as shown below in **figure 11b**. If the reaction solution is continuously pumped from the flask, through the column, and back into the flask, yields of up to 77% may be obtained.[7] This method of equilibrium shifting is attempted in this synthesis.

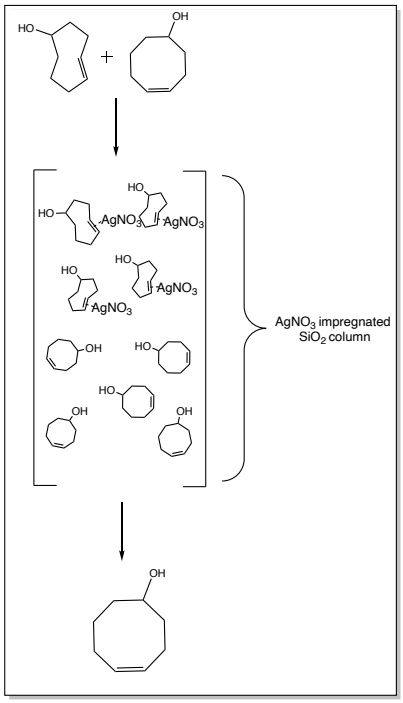


Figure 11b: Representation of the separation of CCO-5-OH and TCO-5-OH by $AgNO_3$

4 .0 – Experimental Details

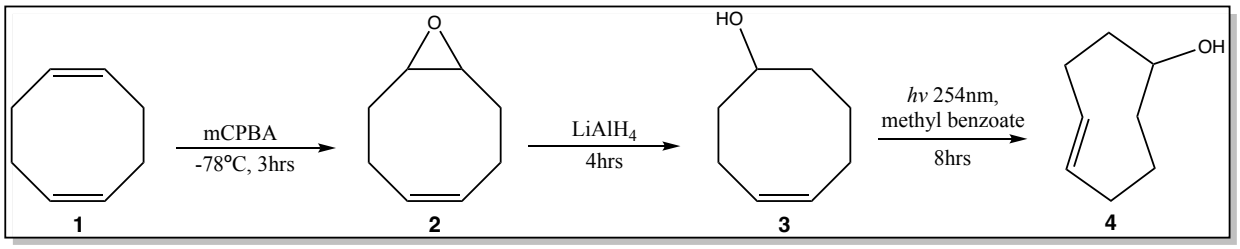


Figure 12: Detailed outline of overall synthesis

4.1 – Epoxidation Procedure

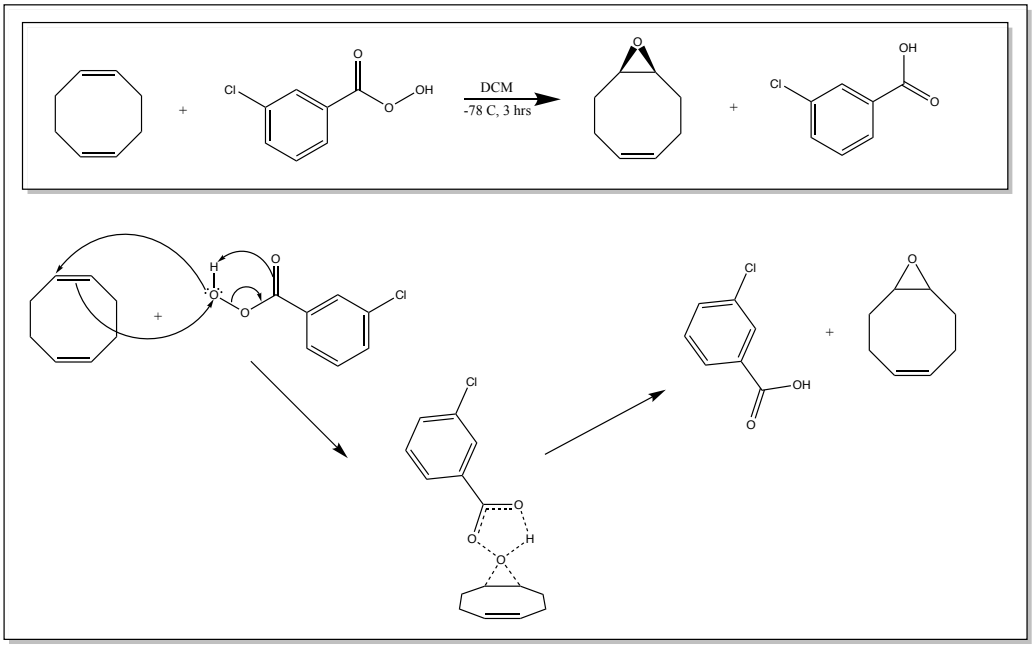


Figure 13: Detailed representation of electron movement involved in this epoxidation

Epoxidation of 1,5-cyclooctadiene was the first reaction of this synthesis. The procedure outlined in Fox et al.[5] was followed. To a dry 50mL round bottom flask equipped with a small stir bar, 0.87g (8.2mmol) 1,5-cyclooctadiene was added along with 5mL DCM. The flask was then capped with a rubber stopper, which punctured with a needle through which Argon flowed. A second needle was then pushed through the stopper to act as a vent. After a few minutes, this needle was removed, and the flask remained under positive pressure from this inert gas. An insulated cup was then filled with a mixture of acetone and dry ice, and the round bottom was placed in this bath for

15 minutes under constant stirring. During these 15 minutes, 1.39g (8.06mmol) 70% mCPBA was dissolved in 20mL DCM in an Erlenmeyer flask. This solution was then injected very slowly into the round bottom to not disrupt the low temperature of the COD – DCM solution inside. The Erlenmeyer was then washed with 5mL of DCM, injected similarly into the round bottom. Aluminum foil was placed over the round bottom, further insulating the acetone-dry ice bath. The reaction was left like this for 3 hours.
Reaction progression was intermittently monitored by thin-layer chromatography. After 3 hours, TLC plates indicated that the reaction had finished. The flask contents were then transferred to a separatory funnel containing 30mL Na_2CO_3, 30mL brine solution, and 20mL DI water. The reaction flask was washed with about 10mL DCM x2; this DCM was also transferred to the separatory funnel. A biphasic extraction was then performed with three washes of 20mL DCM. The organic layers (bottom) were collected into an Erlenmeyer and dried with $MgSO_4$. After filtration, the solvent was evaporated, and the remaining yellow to clear, viscous liquid was left overnight under vacuum. The following day, ^1H NMR data was collected. This process was repeated several times as needed. A few of these trials were conducted in ice baths, i.e., at 0°C since there was no dry ice available due to supply chain issues.

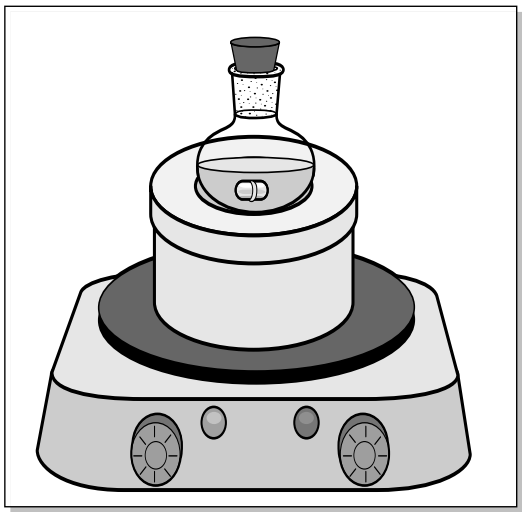


Figure 14: Epoxidation setup (not pictured: positive pressure Argon through an injected needle)

4.2 – Reduction Procedure

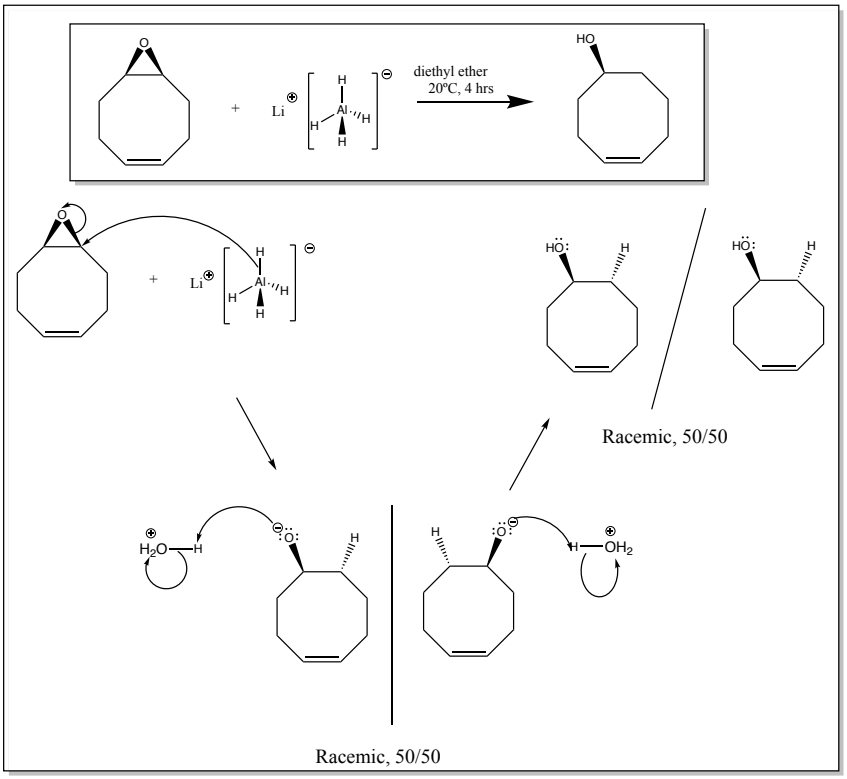


Figure 15: Detailed representation of electron movement involved in this reduction

The reduction of 1,2-epoxycycloct-5-ene was conducted according to the procedure included in Fox et al.[5] To an oven-dried 100mL round bottom flask equipped with a medium stir bar, 178mg (5.2mmol) $LiAlH_4$ was added. The flask was then capped with a rubber stopper, flushed with Argon, and then kept in a positive pressure inert atmosphere, similar to epoxidation. Next, 15mL diethyl ether (anhydrous) was injected, and rapid stirring was turned on. Finally, 650mg of dry epoxide was slowly added dropwise. The vial from which the epoxide was taken was rinsed with 2mL diethyl ether (anhydrous), which was then injected into the flask. The reaction ran for 4 hours; TLC, image 4 monitored reaction progression. After 4 hours, 650uL DI water was carefully added dropwise. This was followed by similarly careful addition of 650uL $NaOH_{(aq)}$ (20% wt.), followed by another addition of 650uL DI water. The resulting precipitates were filtered and washed with diethyl ether. The filtrate was collected in an Erlenmeyer and dried with $MgSO_4$. After another filtration, the solvent was removed by rotary

evaporation, with a maximum bath temperature of 25°C and a minimum pressure of 250mmbar so as not to evaporate the *cis*-cyclooct-4-enol (T_B = 90°C). The average crude mass of this reaction was 0.73g. An NMR spectrum of the crude product was collected before further purification.

Because the next step in this synthesis, the photoisomerization, demands a pure starting material, the crude products of two reduction trials were combined and purified through normal phase column chromatography with a mobile phase of 100% DCM. A 40g FlashPure EcoFlex silica gel column was used to purify 1.2g of crude product. After about 5 minutes of elution with 45mL of mobile phase per minute, the pure compound began to elute. Elution stopped after about 6 minutes, and the corresponding vials were combined into a 500mL round bottom. The DCM was removed, leaving 340mg of pure *cis*-cyclooct-4-enol. This somewhat unusual mobile phase is necessary because of the low boiling point of CCO-5-OH (T_B = 90°C). An ethyl acetate – hexanes gradient would provide superior separation, as evident through the TLC plate in **image 4**, but these solvents would not be removed without significant product loss. Nevertheless, DCM proved to be an effective gradient.

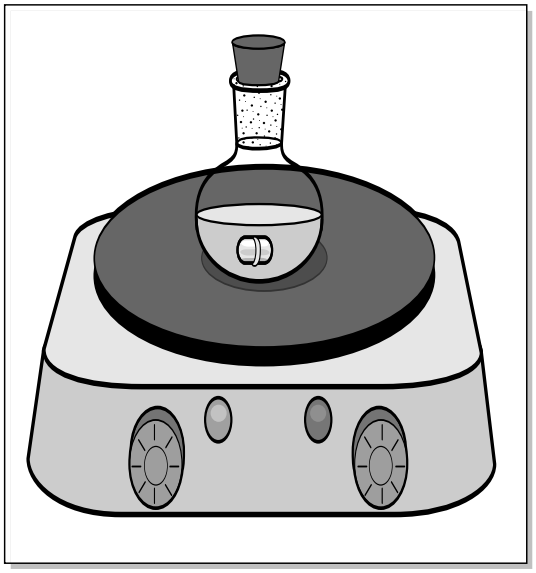


Figure 16: Depiction of reduction setup (not pictured: positive pressure Argon through an injected needle)

4.3 – No-Flow Photoisomerization Procedure

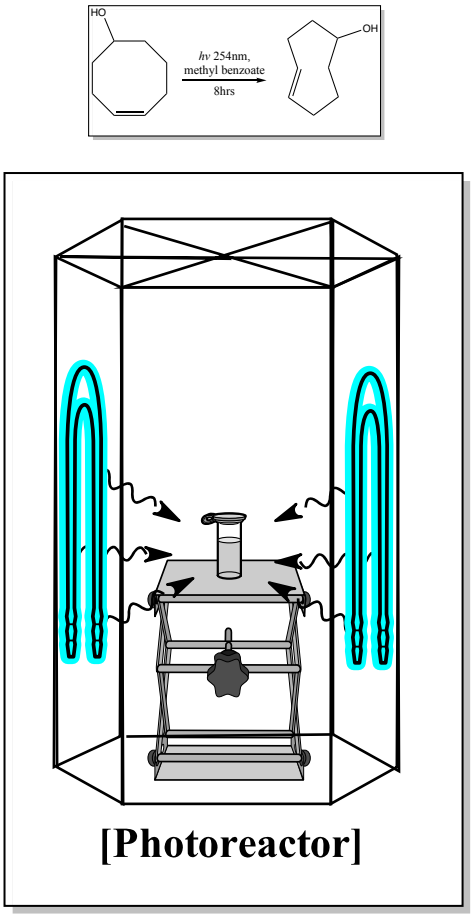


Figure 17: Description of no-flow photoisomerization reaction and setup

To isomerize the double bond on *cis*-cyclooct-4-enol (**3**), a solution of diethyl ether, hexanes**,** methyl benzoate, and (**3**) must be irradiated with ultraviolet light (wavelength = 254nm). Before investing in the materials needed for a flow-isomerization setup, evidence of a functional departmental photoreactor was required. This was tested through a no-flow design. Even though the equilibrium between *cis* and *trans* isomers heavily favors *cis*-, a functional photoreactor would bring the reaction mixture to this equilibrium. This equilibrium does not exist without recent exposure to 254nm irradiation. The no-flow setup was tested to see a difference in *cis*:*trans* isomers between the starting material and product.

To test the efficacy of the photoreactor, to a quartz cuvette, 100mg (0.8mmol) *cis*-cyclooct-4-enol was added along with 2mL diethyl ether and 2mL n-hexanes. The cuvette was capped with its plastic top. This connection was sealed with sealant and covered in aluminum foil, carefully avoiding covering below the solvent line of the cuvette. The sealed cuvette was then placed in a Rayonette photoreactor from the 1940s equipped with 254nm-emitting U-bulbs. Irradiation was turned on using two of the four bulbs. It was left like this for 3 hours. After 3 hours, the photoreactor was turned off, and the reaction solution was transferred to an 8mL vial. The solvent was removed by rotary evaporation. Lastly, a ^{1}H NMR spectrum was obtained.

To test the efficacy of the photoreactor more accurately, another no-flow reaction was performed, this time with one equivalent of methyl benzoate, as described in Fox et al.[7] To a quartz cuvette, 100mg (0.8mmol) *cis*-cyclooct-4-enol was added along with 101uL (0.8mmol) methyl benzoate, 2mL diethyl ether, and 2mL n-hexanes. The cuvette was capped with its plastic top. This connection was sealed with sealant and covered in aluminum foil, carefully avoiding covering below the solvent line of the cuvette. The sealed cuvette was then placed in the photoreactor, and irradiation was turned on, again using only two of the four bulbs. After 3 hours, the photoreactor was turned off, and half of the reaction solution was transferred to an 8mL vial wrapped in aluminum foil to prevent loss of TCO. The other half was returned for 30 more minutes of irradiation and then transferred similarly. The solvent was removed by rotary evaporation. The methyl benzoate was not removed. Lastly, a ^{1}H NMR spectrum was obtained for each sample.

4.4 – Flow Photoisomerization Procedure

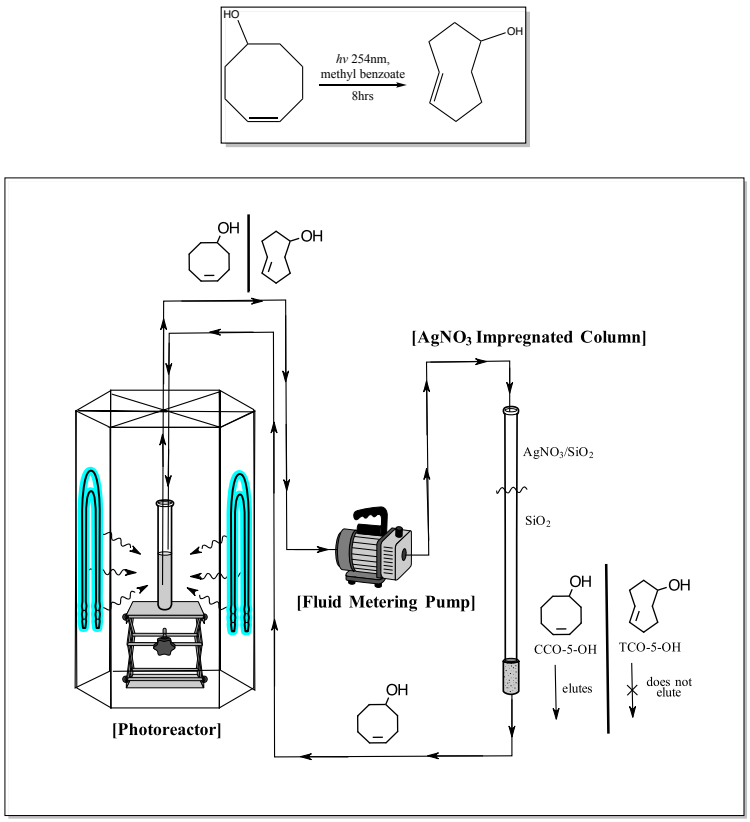


Figure 18: Flow setup for photoisomerization of *cis*-cyclooct-4-enol to *trans*-cyclooct-4-enol (CCO-5-OH to TCO-5-OH), derived from Fox et al.[5]

Three runs using the flow photoisomerization method, described in Fox et al.[5], were conducted using a low-quality fluid metering pump (**figure 19**) and a quartz tube (**figure 20**) sold as a protective sleeve for a hot tub sanitizing UV lamp; it can hold 45mL of liquid. The pump is capable of flow rates in the range of 8-70mL/min. These rates can be adjusted with the green dial. Each attempt differed slightly from the others but shared the same general procedure. Before the first attempt, 10% wt. silver nitrate impregnated silica needed to be prepared. This was done by adding 45g SiO_2 to 50mL DI water in a 1L round bottom flask. Then, 5g $AgNO_3$ was dissolved in 5mL DI water. This was then added to the round bottom and mixed vigorously. Water was removed by rotary evaporation. The round bottom was then placed in the back of a hood with no further

manipulations or aluminum foil covering. The first and second flow attempts used this gel.

In the first attempt, 100mg (0.08mmol) CCO-5-OH was added to the quartz tube along with 101uL (0.08mmol) methyl benzoate and about 40mL of ether:hexanes – 9:1. A 12g column was packed with about 11g of the silver nitrate impregnated silica gel from the first preparation. The pump was then connected to the ends of the column, and 50mL reaction solvent was pumped through the column before beginning the reaction. Once the column was uniformly wet, the quartz tube was placed into the photoreactor, and it and the pump were turned on after the tubing had been inserted into the reaction flask. This reaction failed within 10 minutes due to poor tubing-to-column connections.

In the second attempt, identical amounts of reagents and solvents were used. A longer, 12g, glass column with ports that formed a stronger connection with the tubing was used instead of the column from the first attempt. This reaction proceeded for 3 hours, but the solvent evaporated before completion. Additionally, the tubing and reaction flask were coated in an orange stain. This was attributed to leaching silver nitrate due to Ag(II) oxidation. The column was rinsed with 50mL of clean reaction solvent then emptied into an Erlenmeyer flask to which 10mL $NH_4OH_{(aq)}$ – 25% was added. This was mixed for 10 minutes then filtered into a separatory funnel. DCM was used to extract the organic layer which was collected into a round bottom for subsequent rotary evaporation. Once the solvent had evaporated, an NMR spectrum of the remaining oil was collected.

The second batch of silver nitrate impregnated silica was prepared using the identical amounts as the first with the same order of addition. The water was then evaporated, but the solids were washed with 100mL toluene. This toluene was then evaporated. This was followed by a second addition of 100mL of toluene. After evaporation, the flask was wrapped thoroughly in aluminum foil and placed under vacuum overnight. The flask was flushed with Argon the following day and capped tightly with a rubber stopper. Each time this cap was removed, the flask was flushed with Argon before returning to its storage place in the back of a fume hood, still encased in aluminum foil. This was used in the third attempt.

The third attempt used the same column, but the bottom 60% of it was filled with pure silica to act as a barrier, hopefully preventing the leaching observed in the second attempt. For the same reason, the column was wrapped in aluminum foil to avoid light-induced oxidation with air that managed to enter the column. A new batch of better-prepared silver nitrate impregnated silica was also used, as described above. The solvent was changed from 9:1 – ether:hexanes to 1:1 – ether:hexanes in hopes that a less polar solvent would decrease leaching. To slow the solvent evaporation rate, a section of the tubing was submerged in an ice bath for the entirety of the reaction. After 2.5 hours of irradiation and flow, the column was washed with 50mL of clean reaction solvent. The silica gel was worked up identically to the work up described above in the second attempt.

Figure 19: Exact model of quartz bulb cover used as reaction flask in all flow trials. Sundance Spas Replacement Quartz Tube – Model # 6472-859. $44.99. Images sourced from supplier, amazon.com.

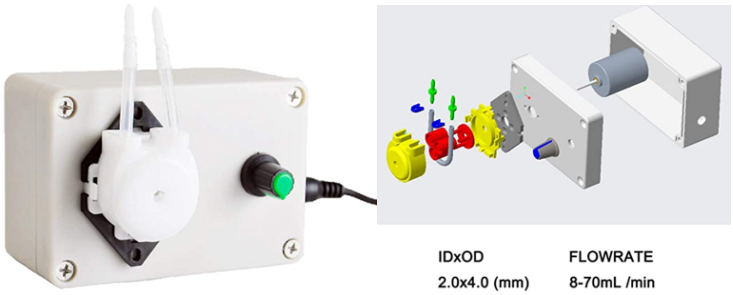


Figure 20: Exact model of fluid metering pump used in flow trials. Gikfun 12V Adjustable Peristaltic Dosing Pump Liquid Metering Pump with Adapter for Aquarium Lab Analytical – Model # EK1960. $32.98. Images sourced from supplier amazon.com.

5.0 – Results & Discussion

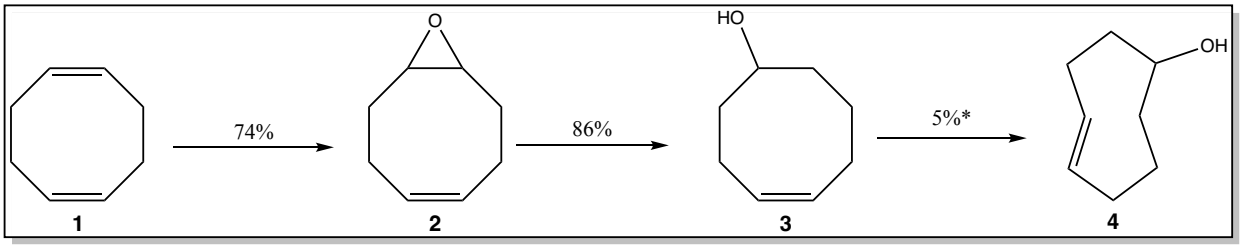


Figure 21: Average yield summary of synthesis; cumulative yield – 3%

5.1 – Epoxidation

Although only 0.98mmol mCPBA was used, di-epoxidation existed in every trial. Runs conducted in acetone-dry ice baths, cooling the reaction mixture to -78°C, gave the greatest percent yields (78-70%). Reaction progression was monitored by thin-layer chromatography. Disappearance of the limiting reagent, COD, indicated reaction completion; this can be seen in **images 1-2**.

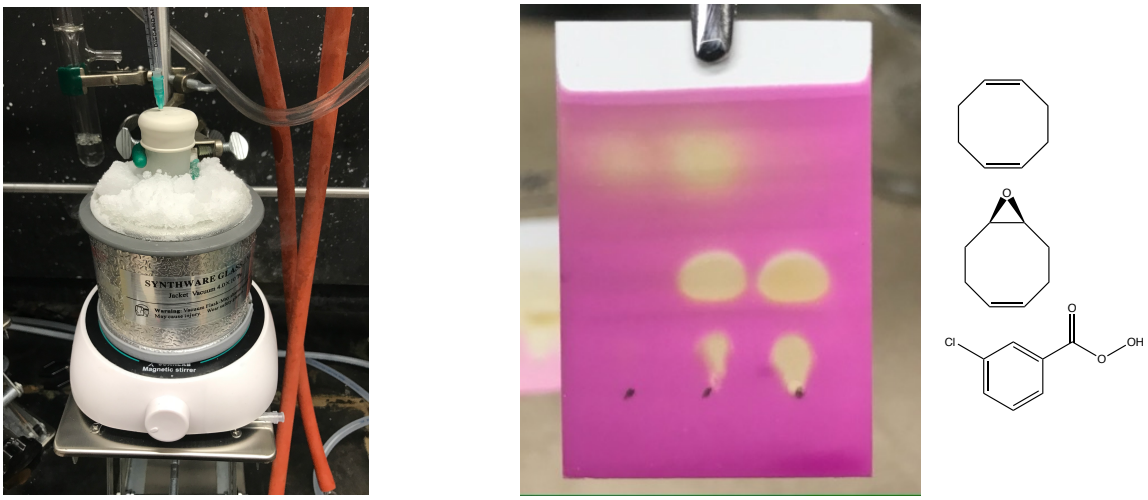
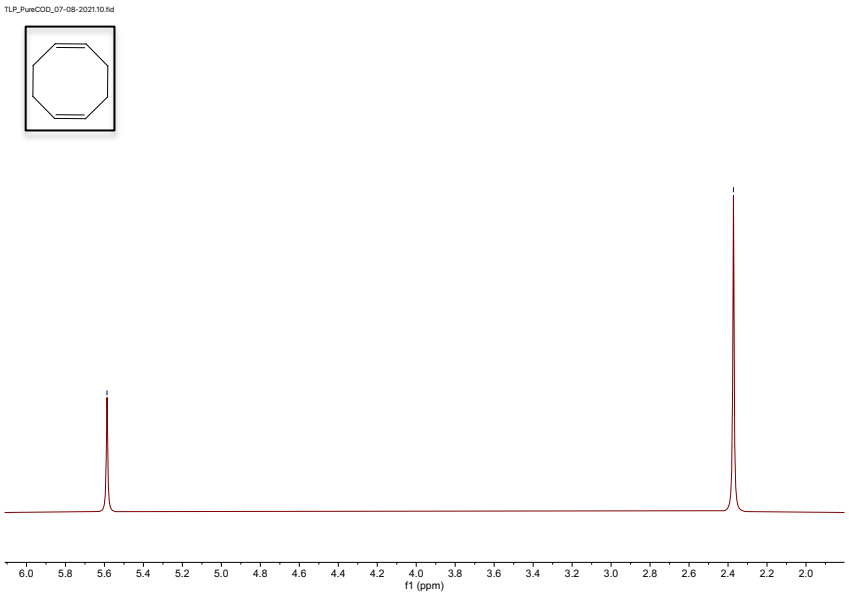


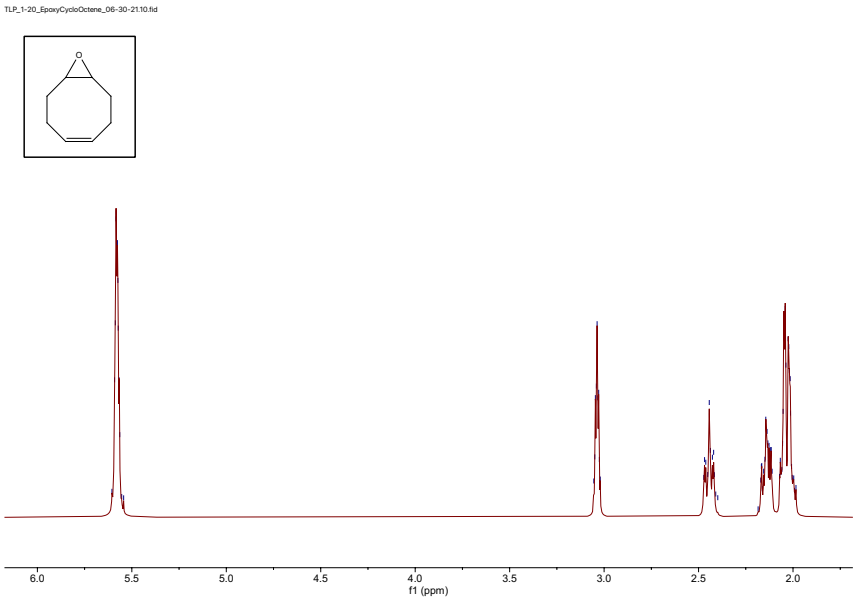
Image 1-2: Epoxidation setup and TLC plate indicating a completed reaction through the disappearance of COD spot in Lane 3.

(Left to right) Lane 1 – COD starter, Lane 2 – COD starter & reaction mixture, Lane 3 – reaction mixture



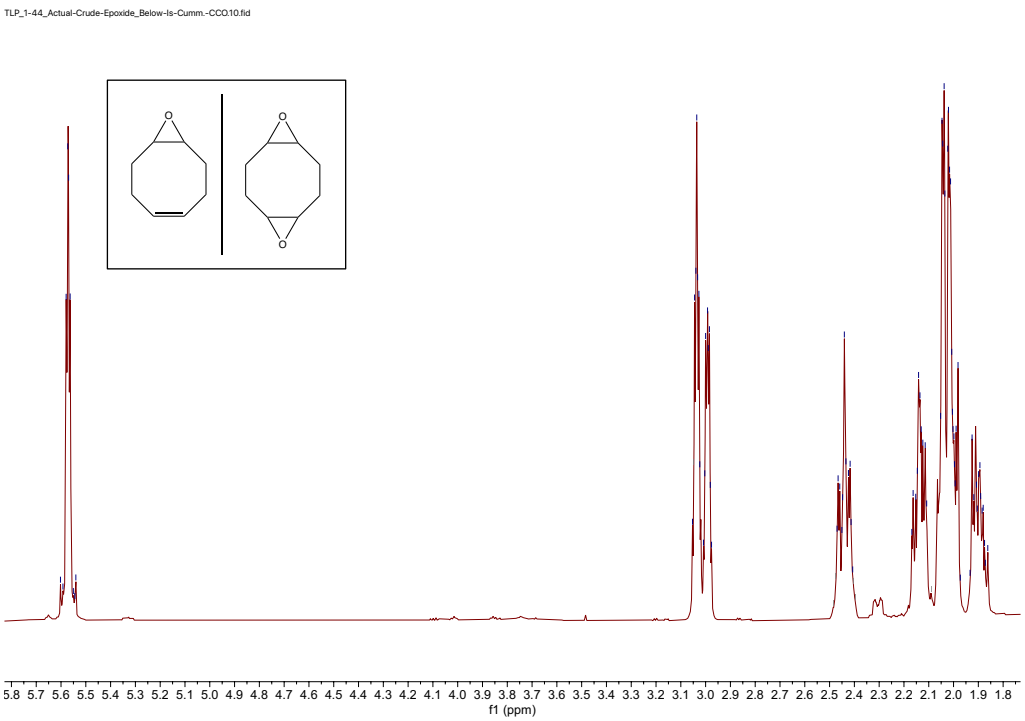
Spectrum 1: Starting material (**1**); 1,5-cyclooctadiene

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.59 (s, 4H), 2.37 (s, 8H).



Spectrum 2a: ^{1}H NMR spectrum given by epoxidation at -78°C, (compound **2**)

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.62 – 5.52 (m, 2H), 3.07 – 3.01 (m, 2H), 2.52 – 2.39 (m, 2H), 2.21 – 2.09 (m, 2H), 2.07 – 1.99 (m, 4H).



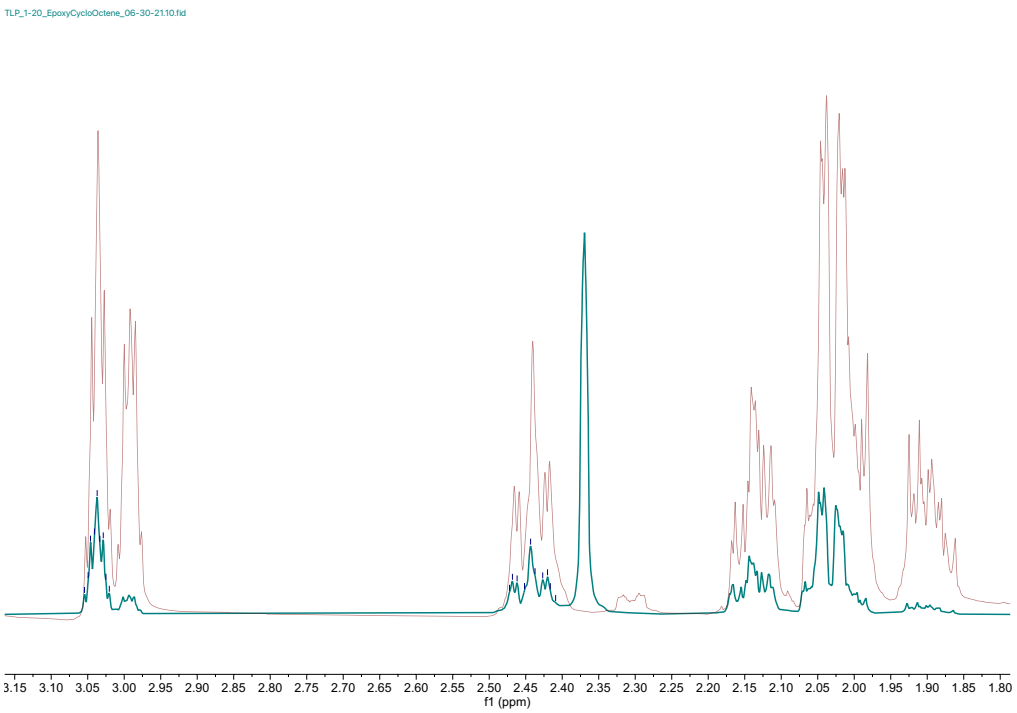
Spectrum 2b: ^{1}H NMR spectrum given by epoxidation at 0°C

Mono-epoxide (**2**) signals:

^{1}H NMR (500 MHz, CDCl3) δ 5.62 – 5.52 (m, 2H), 3.07 – 3.01 (m, 2H), 2.52 – 2.39 (m, 2H), 2.21 – 2.09 (m, 2H), 2.07 – 1.99 (m, 4H).

Di-epoxide signals:

^{1}H NMR (500 MHz, CDCl3) δ 3.01 – 2.95 (m, 4H), 1.96 – 1.84 (m, 4H).



Spectrum 2c: Superimposed view of resulting ^{1}H NMR data of epoxidation at -78°C (blue/spectrum 2a) vs. epoxidation at 0°C (red/spectrum 2b)

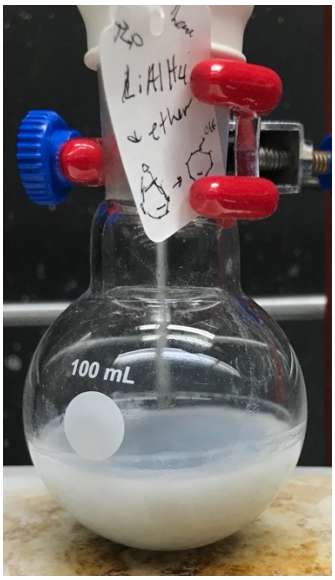
The differences in products given by the same reaction at different temperatures are more apparent by analyzing this superimposed image of the two spectra. The downfield di-epoxide signal (3.01-2.59ppm) is twice its actual intensity if all is normalized to the mono-epoxide. This is due to the two planes of symmetry experienced by the hydrogens of the di-epoxide, while those of the mono-epoxide experience only one. The result is a pair of visually misleading di-epoxide signals, the other being the multiplet upfield at 1.96-1.84ppm. The higher temperature gives complete epoxidation of COD, but much more di-epoxide (2.98,1.89ppm) is produced as a tradeoff at a 2.5:1 molar ratio of mono-:di-epoxide. The lower temperature yields a much greater molar ratio of mono-:di-epoxide (9:1), but about half of the starting material (2.37ppm) remains unreacted. Interestingly, the same relative amount of mono-epoxide (**2**) is produced in each since the molar ratio of unreacted starting material in the -78°C reaction is roughly equal to that of the di-epoxide in the 0°C reaction.

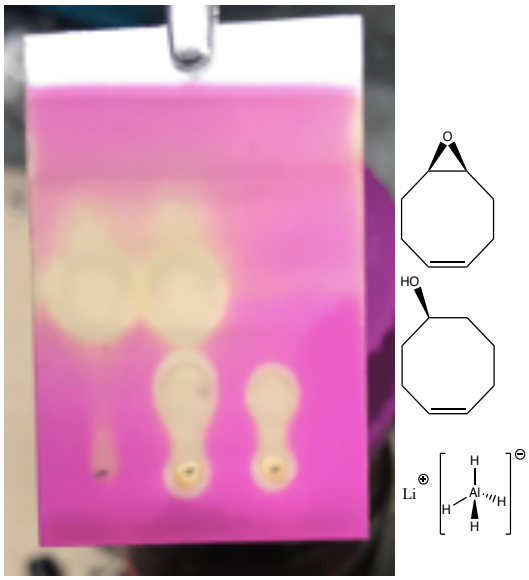
The superior reaction temperature proved to be -78°C. There are a few different reasons for this conclusion. The primary is that starting COD (**1**) is preferred to the di-

epoxide as a contaminant since it is unreactive in $LiAlH_4$ and has a more significant difference in polarity to the mono-epoxide (**2**). These contaminants are more difficult to remove than aqueous contaminants, such as meta-chlorobenzoate, which are quickly removed through a biphasic extraction. Removal of the di-epoxide would consist of normal phase column chromatography. This would cause a loss of at least some measurable amount of product due to the multiple transfers involved in this purification method. Though it is not a particularly wasteful or complicated technique, there is no reason to elect to purify after the first reaction of this synthesis solely to run a reaction that leaves no unreacted COD. Similar yields of mono-epoxide are exhibited at either temperature, so the more efficient course is to run the epoxidation at -78°C, do the biphasic extraction, remove the solvent, and proceed to the next step of the synthesis, reduction by $LiAlH_4$, after which contaminating COD will be removed by column chromatography, a purification that will be done regardless.

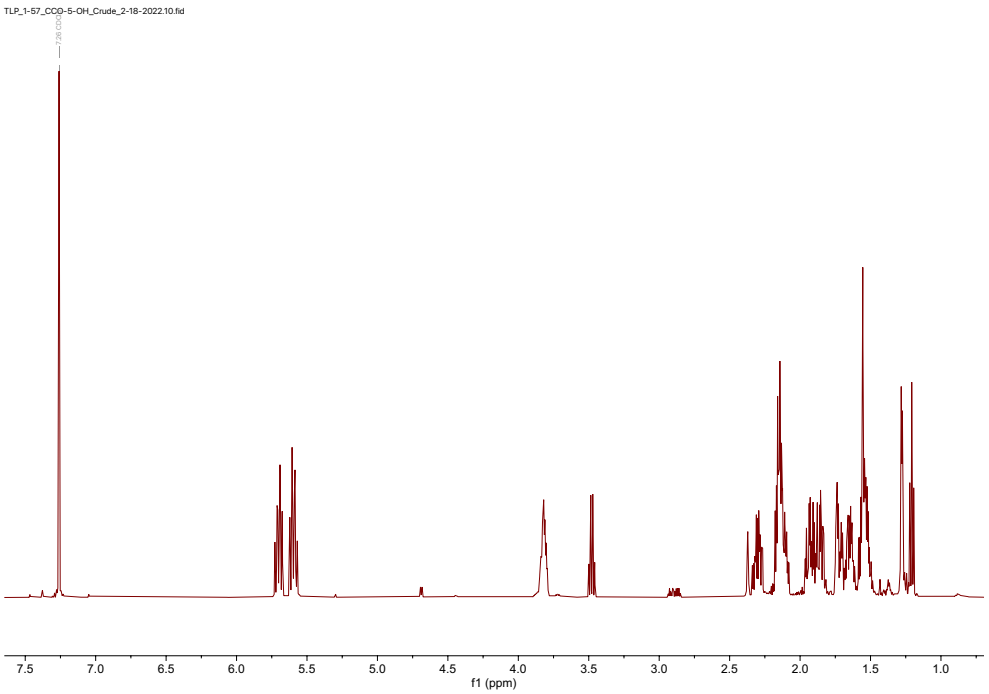
5.2 – Reduction

Although only 0.9 eq. $LiAlH_4$ were used, reduction of the epoxide proceeded quickly and thoroughly, as seen in the TLC plate in **image 4,** which was obtained just 2 hours after epoxide addition, half the allotted reaction time. This is because this reducing agent can be oxidized four times, so 0.9mmol $LiAlH_4$ can reduce 3.6mmol of mono-epoxide. Further, $LiAlH_4$ does not act on double bonds, so no significant manipulations to reaction conditions were needed. This reduction's products and side products are inert, so leaving the reaction running for longer than required did not impact the reaction negatively. The disappearance of the limiting reagent, the mono-epoxide, indicated reaction completion; this can be seen in **image 4**.





Images 3-4: Reduction setup (under Argon) and TLC plate indicating a completed reduction through the disappearance of epoxide spot in lane 3.
(Left to right) Lane 1 – epoxide starter, Lane 2 – epoxide starter + reaction mixture, Lane 3 – reaction mixture



Spectrum 3a: ^{1}H NMR spectrum of the crude product of the reduction (**3** crude), *cis*-cyclooct-4-enol

Because the next step in this synthesis, the photoisomerization, demands a pure starting material, the crude product of the reduction was purified through normal phase column chromatography with a mobile phase of 100% DCM; the resulting plot is included below in **figure 17**. This somewhat unusual mobile phase is necessary because of the low boiling point of CCO-5-OH (T_B = 90°C). An ethyl acetate – hexanes gradient would provide superior separation, as evident through the TLC plates in **images 4-5**, but hexanes and ethyl acetate cannot be removed without significant product loss. Nevertheless, DCM proved to be an effective gradient. **Spectrum 3b** supports the effectiveness of this method of purification, as the

Image 5: TLC plate of crude product (both lanes);
100% DCM in preparation for column chromatography

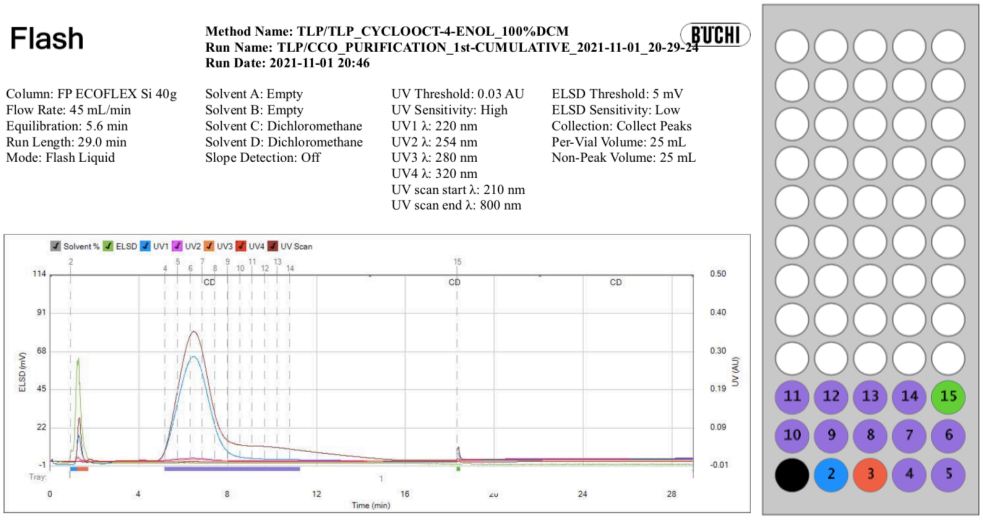
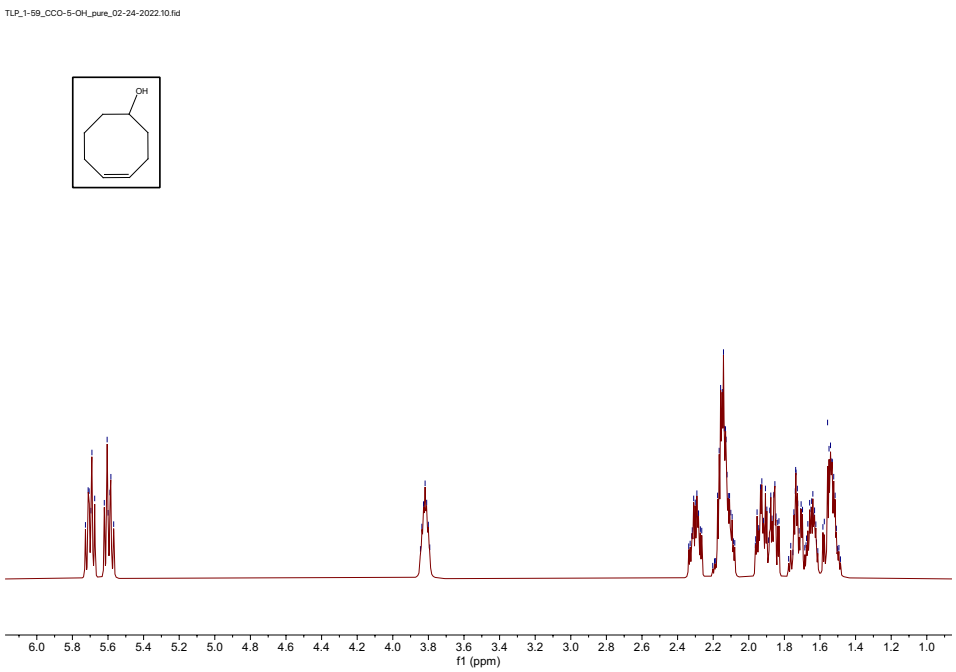


Figure 22: Plotted elution detection throughout the flash liquid normal phase column chromatography used to purify CCO-5-OH (purple)



Spectrum 3b: ^{1}H NMR spectrum given of (**3**) after purification

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.78 – 5.65 (m, 1H), 5.64 – 5.51 (m, 1H), 3.89 – 3.75 (m, 1H), 2.37 – 2.24 (m, 1H), 2.19 – 2.07 (m, 3H), 1.98 – 1.82 (m, 2H), 1.80 – 1.42 (m, 5H).

5.3 – No-Flow Photoisomerization

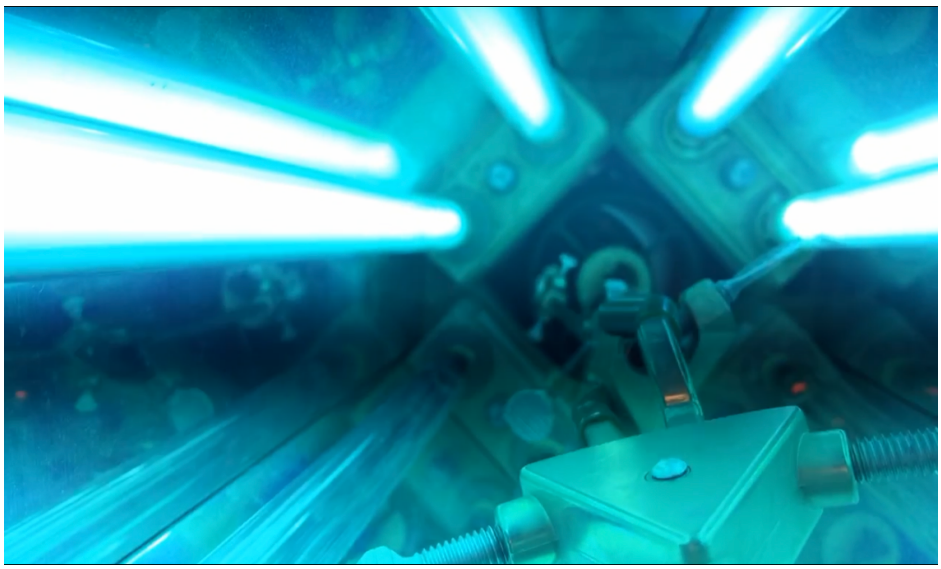
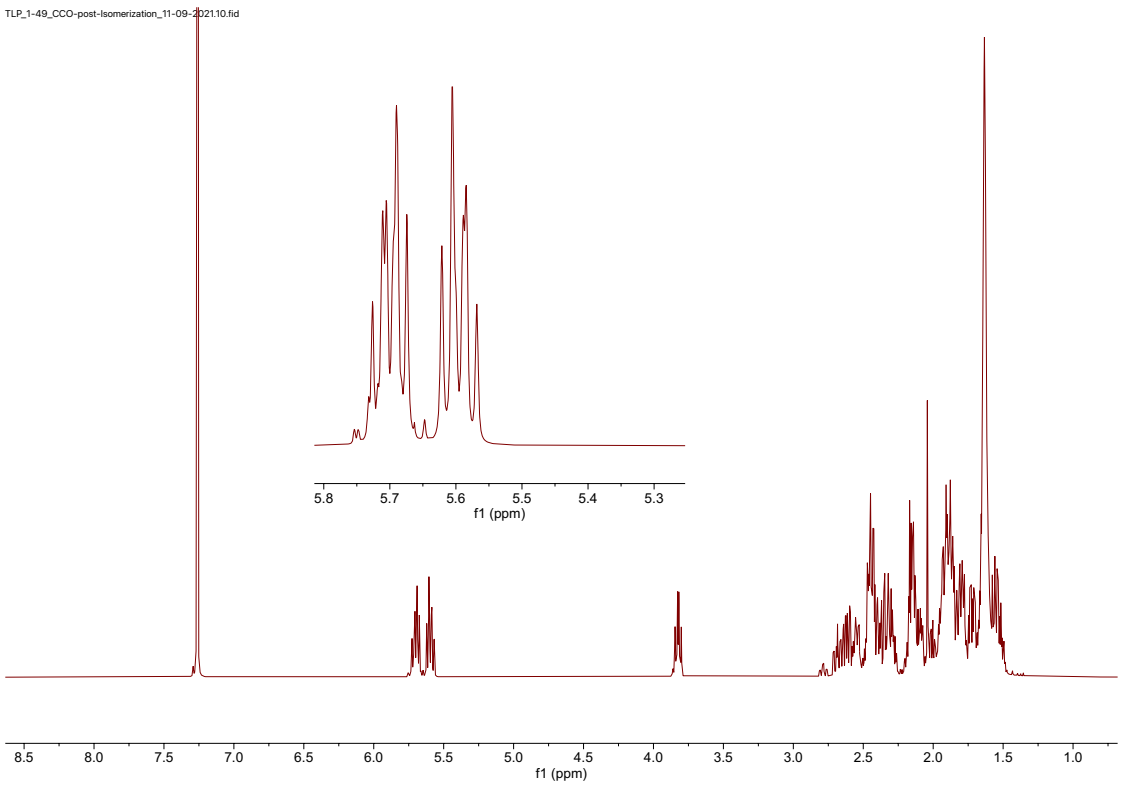
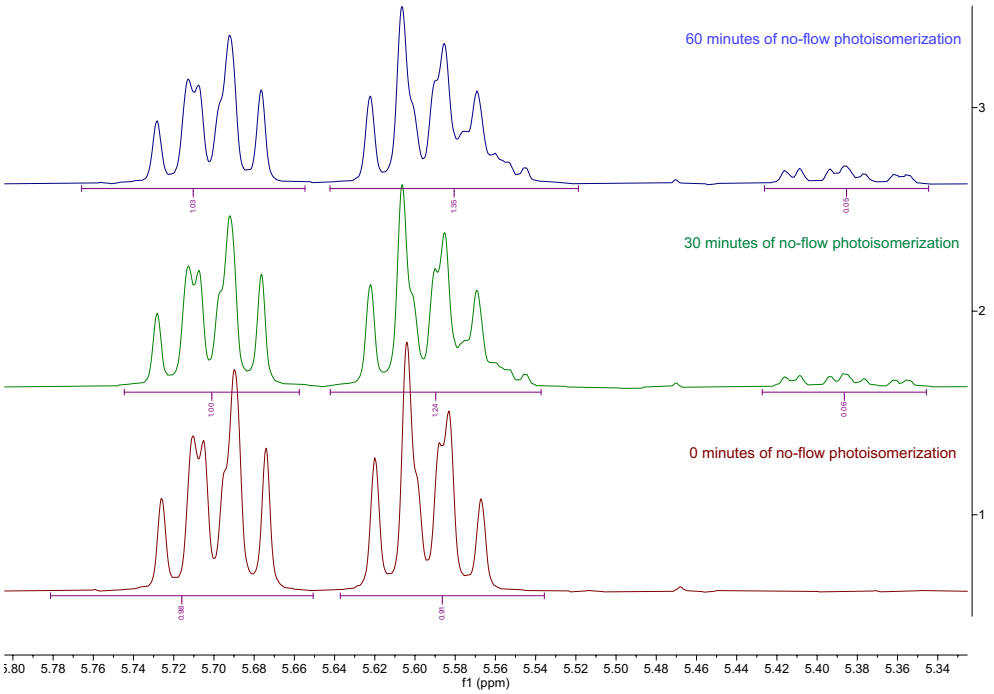


Image 6: No-flow photoisomerization setup



Spectrum 4a: Product of no-flow photoisomerization using no methyl benzoate

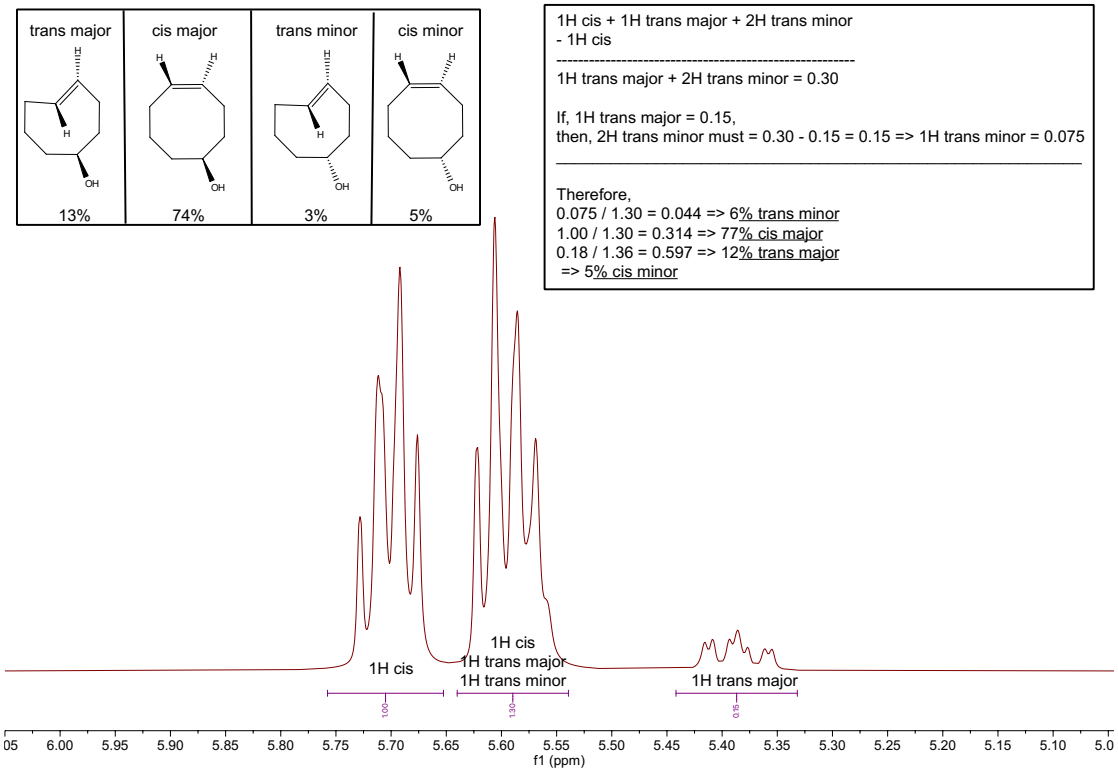
The photoisomerization conducted with neither methyl benzoate nor a flow system failed to produce any *trans*-isomers. This is evident in the absence of signals upfield to the *cis*-isomer signal pair at 5.70 and 5.60ppm. Adjustments to the procedure were made, and one equivalent of methyl benzoate was implemented into the photoisomerization procedure, as indicated by Fox et al.[5]



Spectrum 4b: Stacked spectra from intermittent monitoring of no-flow photoisomerization using methyl benzoate

With the addition of one equivalent of methyl benzoate, the *trans*-isomers exhibited measurable proton signals after irradiation with a no-flow setup. This is because methyl benzoate acts as a singlet sensitizer, bringing the pi-electrons of double to their excited state, effectively lowering the energy barrier and preventing the more favorable *cis* conformation from converting to the highly strained *trans* conformation. As indicated above, in **spectrum 4b**, ^{1}H NMR spectroscopy data were collected before irradiation, after 30 minutes of irradiation, and again after 60 minutes of irradiation. The resulting spectra provided two valuable pieces of information: the photoreactor can do this isomerization, and a flow setup is necessary if any significant quantity of TCO-5-OH is produced. The flow setup is needed for two reasons. The first, and most obvious reason when analyzing **spectrum 4b**, is because the equilibrium constant of this isomerization is nearly roughly 0.05. The second reason is because this equilibrium was achieved relatively quickly since there was no measurable change in *cis*:*trans* isomers after the first 30 minutes. Manually passing the reaction solution through an $AgNO_3$

impregnated column would be highly inefficient. Parts for the flow setup were then ordered and were used in all subsequent photoisomerizations.



Spectrum 4c: Repeat ^{1}H NMR with 1024 scans (instead of 64) of the no-flow product featured in **spectrum 4b**

^{1}H NMR (500 MHz, CDCl3) δ 5.70 (dt, J = 10.4, 7.8 Hz, 1H), 5.60 (dt, J = 10.7, 8.1 Hz, 1H), 5.39 (ddd, J = 15.6, 11.1, 3.6 Hz, 0H).

5.4 – Flow Isomerization

The flow isomerization method was much more complex than any previous reactions. Silver nitrate impregnated silica gel must be perfectly prepared, stored in an inert atmosphere, and protected from light to prevent Ag(II) oxidation. Many aspects of the setup also needed to be fashioned from materials designed for other functions. These were points of error, and their poor management negatively impacted the isomerization. For example, a rubber stopper was used to cap the quartz tube, but a hole needed to be punctured through it to allow the two ends of the tubing to reach the

reaction solution. A large micropipette tip was cut in half and inserted through the cut that had been made through the stopper. The micropipette tip provided structure to the hole, creating gaps through which solvent vapor escaped despite thorough aluminum foil covering. The second and third flow attempts consisted of modifications to the preceding attempt.

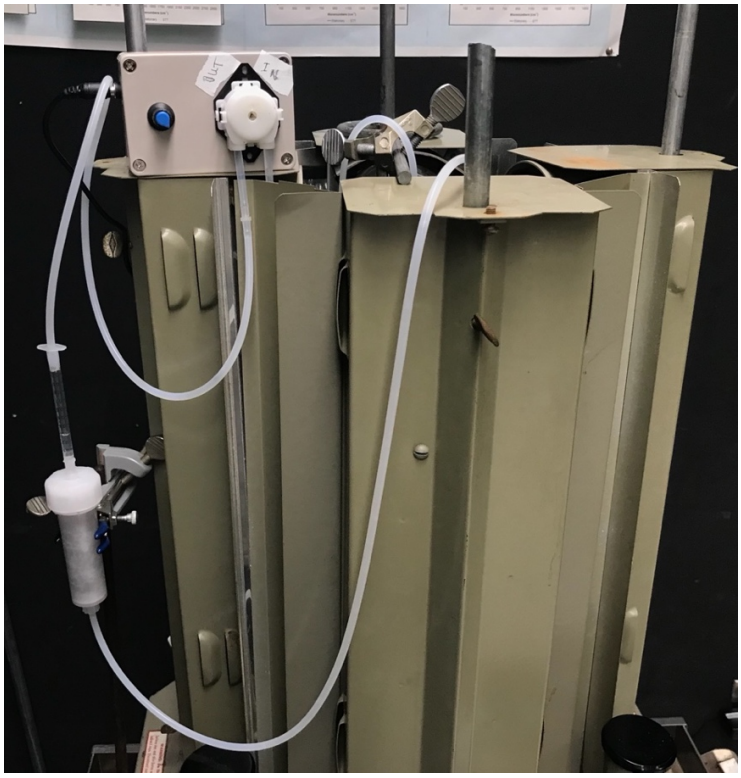


Image 7: First attempt at flow photoisomerization.

The first attempt at flow photoisomerization failed due to poor connections between the column and tubing (2mm ID x 4mm OD). Both tubing ends eventually came off their column ports, releasing the reaction solution onto the laboratory floor. No NMR data was acquired from this trial since it was stopped after less than 10 minutes. A second error in this attempt was present; the $AgNO_3$ impregnated silica gel was contaminated with water. Unfortunately, due to the premature stoppage, this error went unnoticed until the second attempt, a trial that used $AgNO_3$ impregnated silica gel from the same batch as the first.

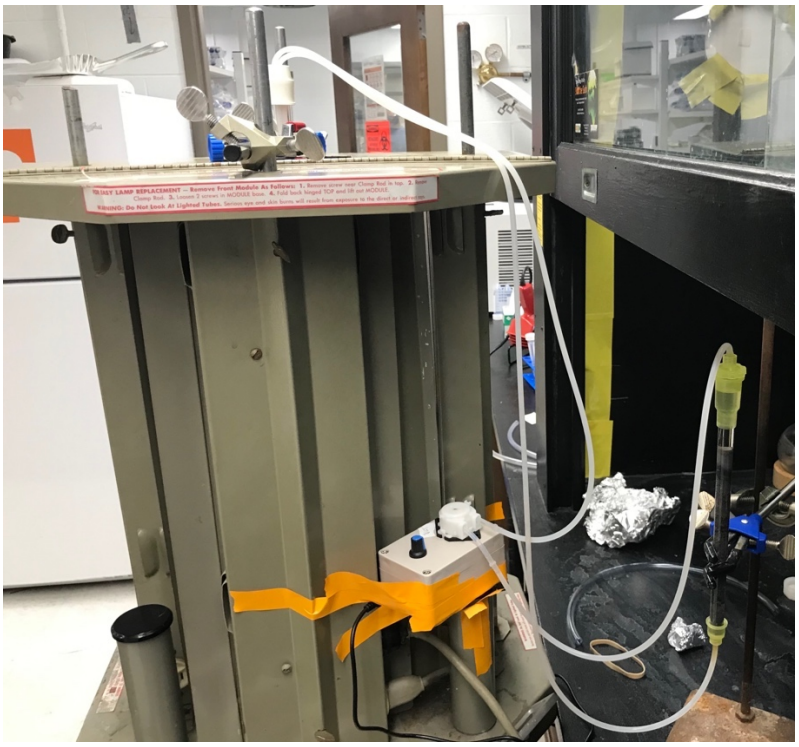
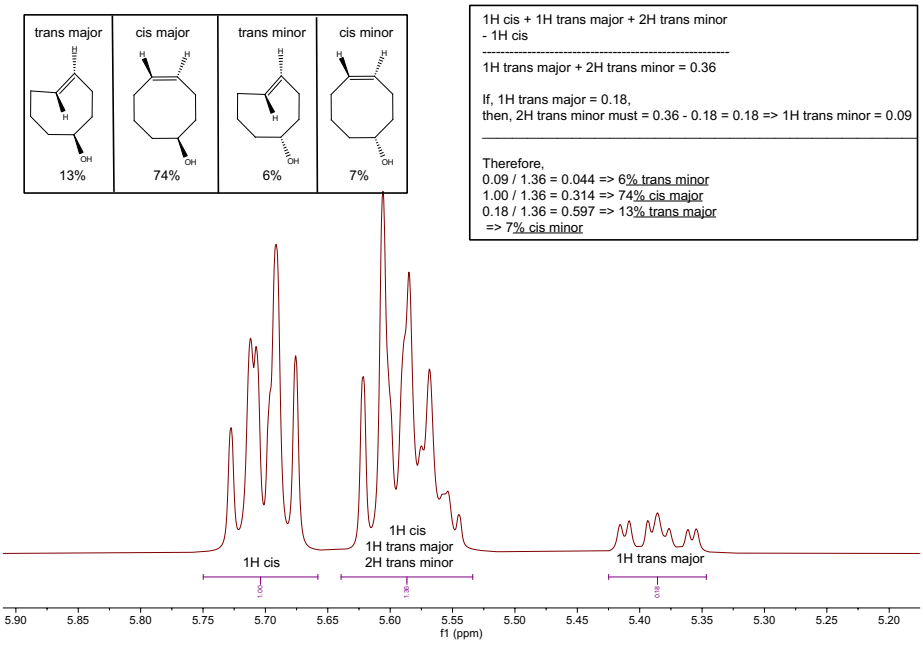


Image 8: Second attempt at flow photoisomerization

The second attempt at flow isomerization was also unsuccessful. As indicated above, the water contamination in the $AgNO_3$ impregnated silica gel led to the oxidation of $Ag^{+2}{}_{(aq)}$ to $Ag_{(s)}$. This not only removed $AgNO_3$ from the column, but it also caused the leaching of solid silver from the column. This resulted in stained orange tubing and an orange coating of the reaction flask. This effectively stopped any further isomerization. Leaching was likely compounded by the use of 9:1 – diethyl ether:hexanes as the reaction solvent. Though this level of polarity provides superior elution speeds for CCO-5-OH, it proved to be too polar for this context, exacerbating the elution of oxidized silver through the column and tubing and into the reaction flask. **Spectrum 5a** supports these inferences since it exhibits no significant differences from the no-flow data shown in **spectra 4b**. Because the equilibrium ratio of *cis*:*trans* is seen, active removal of the *trans*-isomer did not occur. Besides this leaching, another flaw was the rapid evaporation of solvent from the quartz tube due to the imperfect seal between the rubber stopper and the flask. The perimeter of the flask's opening was well-sealed, but the holes that were cut into the rubber stopper to make room for the

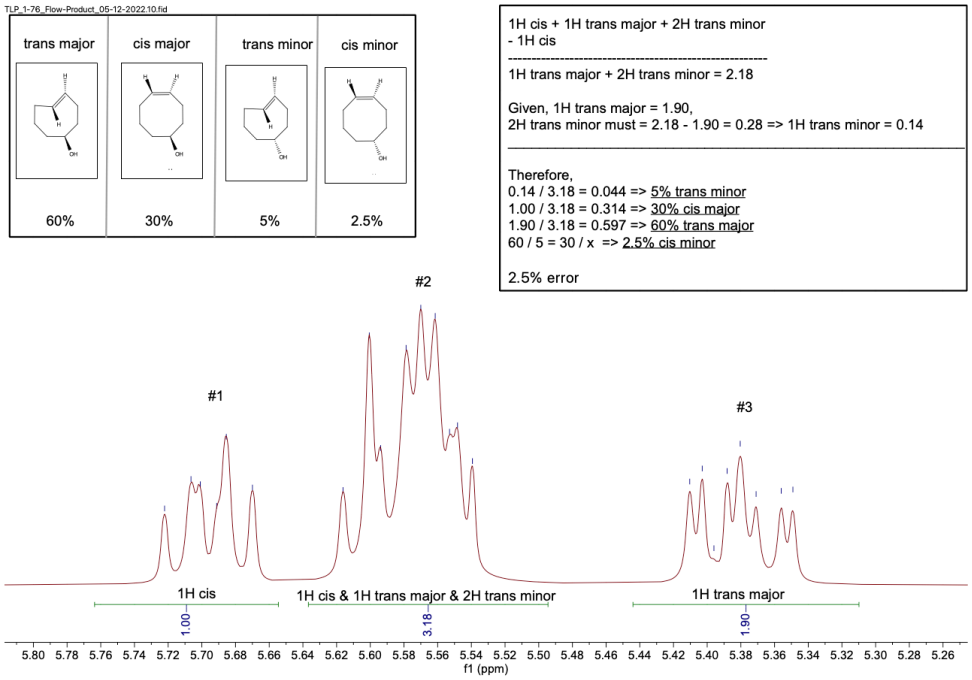
tubing were not. This allowed significant evaporation, and at some point, between 1.5 and 3 hours of reaction time, 100% of the solvent has been evaporated, leaving an empty flask coated in a thin layer of methyl benzoate, leached orange material, and CCO-5-OH.



Spectrum 5a: Racemic product (**3**,**4**) of second flow photoisomerization, (E)/(Z)-cyclooct-4-enol, no observable difference exists between **spectra 5a** and **4c**

^{1}H NMR (500 MHz, CDCl3) δ 5.70 (m – *cis* only, J = 10.8, 7.8 Hz, 1H), 5.59 (m – *cis* & *trans*, J = 11.1, 7.7 Hz, 1H), 5.39 (m – *trans* only, J = 15.6, 11.0, 3.6 Hz).

Image 9: Third attempt at flow photoisomerization



Spectrum 5b: Racemic product (**3**,**4**) of third flow photoisomerization, (E)/(Z)-cyclooct-4-enol; correct shifts, per Stanimirov et al.[8]

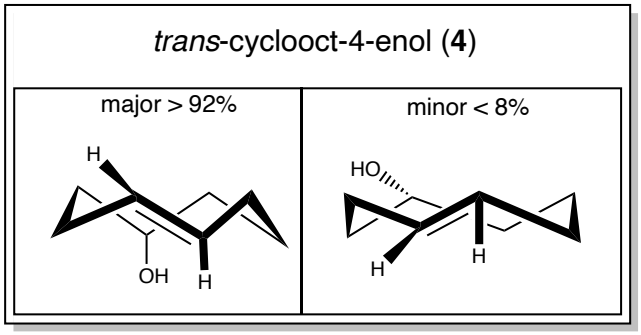
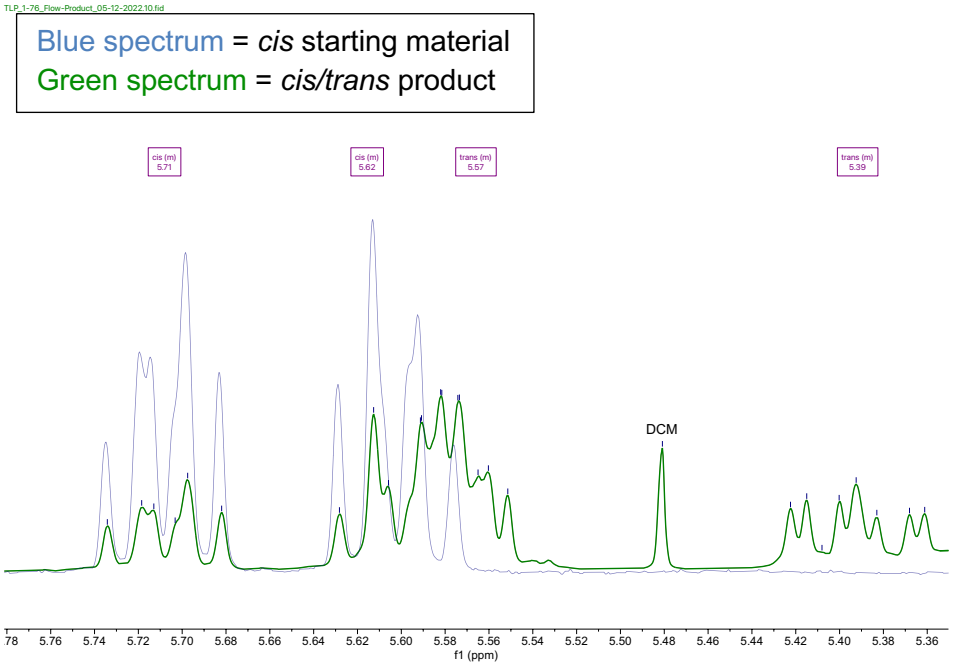
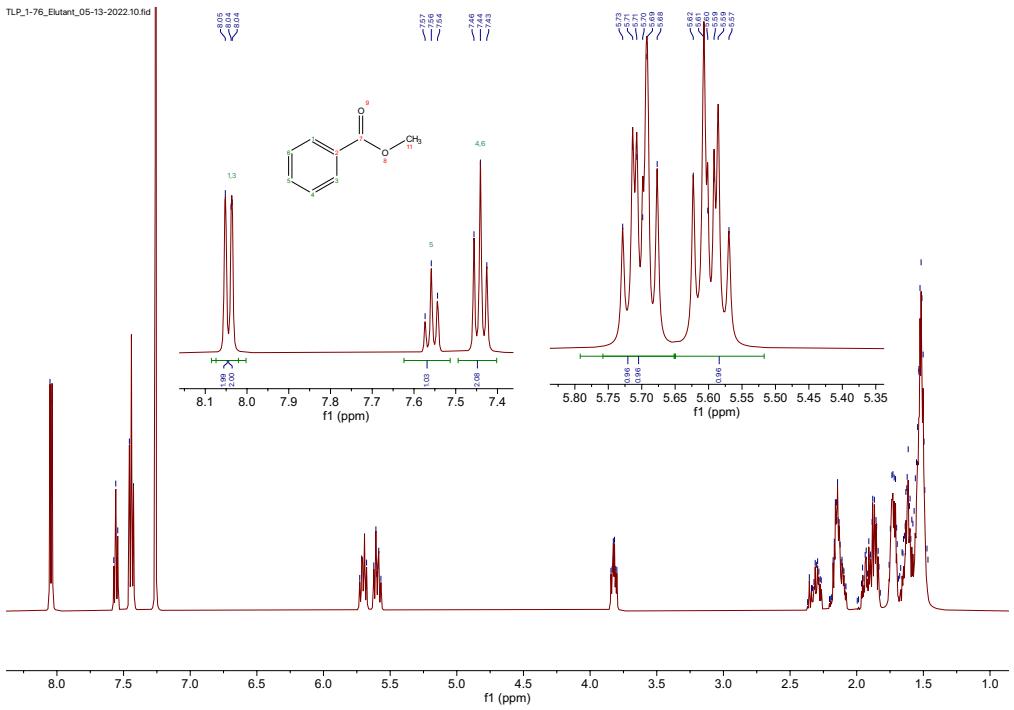


Figure 23: Conformational isomers of (**4**), *trans*-cyclooct-4-enol, with their experimentally determined abundances



Spectrum 5c: Superimposition of spectrum of the starting material, *cis*-cyclooct-4-enol, (blue) and *cis/trans*-cyclooct-4-enol (green); the product of the third flow isomerization

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.74 - 5.67 (m, 1H, *cis*), 5.64– 5.57 (m, 1H, *cis*), 5.61 – 5.52 (m, 1H, *trans*), 5.44 – 5.34 (m, 1H, *trans*).



Spectrum 5d: Elutant (1:1 – CCO-5-OH:methyl benzoate) from third flow photoisomerization

methyl benzoate signals:

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.09 – 8.00 (m, 2H), 7.56 (t, 1H), 7.44 (t, 2H).

cis-cyclooct-4-enol (**3**) signals:

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.78 – 5.65 (m, 1H), 5.64 – 5.51 (m, 1H), 3.89 – 3.75 (m, 1H), 2.37 – 2.24 (m, 1H), 2.19 – 2.07 (m, 3H), 1.98 – 1.82 (m, 2H), 1.80 – 1.42 (m, 5H).

The *trans*-isomer did not elute through the $AgNO_3$-impregnated SiO_2 column. This was determined through an analysis of ^{1}H NMR data collected from the elutant, **spectrum 5b**. There were no measurable *trans*-alkene hydrogen signals in this spectrum; however, there was not perfect separation of the two isomers inside the column. No *trans*-isomers escaped the column, but not all *cis*-isomers eluted. Nearly one-third of all cyclooct-4-enol molecules that were trapped in the column were *cis*-isomers. This was determined by the calculations included in **spectrum 5a**.

The unanticipated retention of the *cis* isomer in the $AgNO_3$-impregnated column was likely a result of incomplete column washing, not of a metal complexation between *cis* and $AgNO_3$. This method of separating *cis* and *trans*-isomers is commonly used because of its effectiveness and broad applicability, so such a significant portion being *cis* must be an effect of experimental error. Further, with a crude yield of 5%, i.e., 5mg, and ^1H NMR data, shown in **spectrum 5b**, indicating a ratio of roughly 2:1 – TCO:CCO, it can be reasoned that the silver nitrate impregnated silica gel only retained about 1.65mg of CCO. Such a small amount of compound can easily be attributed to not wholly drying the gel before the extraction with 25% NH_4OH.

There are various potential reasons for the poor yield of this third and final trial. The slow flow rate of the fluid metering pump (10-20mL/min) and an abbreviated reaction time from 12 hours to 3 hours most likely combine to give this minimal yield, a yield no different than that of the successful no-flow isomerization (4-6%). However, it is important to note that this third flow isomerization attempt provided the highest degree of confidence in the success of future trials with more refined methods. These refinements include attaching an outlet plug-in timer to the photoreactor's power cord which would alter continuously between 1 hour of its ON state and 30 minutes of its OFF state, allowing the reaction solution to cool intermittently. This would limit evaporation enough for a cumulative 12 hours of photoirradiation to be reached; this is the reaction time used in the literature procedures followed in this synthesis.[6,7] The fluid metering pump would continue to pump the reaction solution through the column so that any potential implications of dry silver nitrate impregnated silica gel could be avoided. If this refined trial yielded results like the third, then a larger mass of silver nitrate impregnated silica should be used in conjunction with a larger column, still filling the bottom 60% with pure silica to avoid any leaching of oxidized silver, which is known from the second flow attempt to stop isomerization.

6.0 – Conclusions

In summary, this synthesis proved to be only conditionally successful, but further refinement of the flow isomerization would likely result in a significantly improved yield. It demonstrated that a high-quality photoreactor is not needed to convert the *cis* isomer to *trans*. It also showed that quartz glass used in covers for sanitation lights works as a quartz reaction flask. The failures arose in the active removal of *trans*-isomers over a sustained period, i.e., 6-12 hours. There was no significant improvement in yields between the flow and no-flow setups, but each flow reaction had identifiable and fixable flaws. Due to time constraints, the third flow attempt could not be improved with a fourth attempt. The primary adjustment, a series of 60-minute irradiation periods followed by 30-minute cooling periods, automated through a timed outlet attachment, would allow the solvent to cool, allowing for a reaction time equivalent to those in literature.[5,7]

References

1. Kolb, H.C., Finn, M.G. and Sharpless, K.B. (2001), Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angew. Chem. Int. Ed.*, 40, 2004-21. DOI: 10.1002/1521-3773.
2. Fox, J.M., Royzen, M., Blackman, M.L. (2008). Tetrazine Ligation: Fast Bioconjugation Based on Inverse-Electron Demand Diels-Alder Reactivity. *Journal of the American Chemical Society.* 130(41), 13518-9. DOI: 10.1021/ja8053805.
3. Handula, M., Chen, K. T., & Seimbille, Y. (2021). IEDDA: An Attractive Bioorthogonal Reaction for Biomedical Applications. *Molecules* (Basel, Switzerland), 26(15), 4640. DOI: 10.3390/molecules26154640.
4. Schubert, U.S., Hoogenboom, R., Becer, R.C. (2009). Click Chemistry beyond Metal-Catalyzed Cycloaddition. *Angew. Chem. Int. Ed.*, 40(27), 4900-8. DOI: 10.1002/anie.200900755.
5. Fox, J.M., Royzen, M., and Yap, G.A. (2008), A Photochemical Synthesis of Functionalized *trans*-Cyclooctenes Driven by Metal Complexation. *Journal of the American Chemical Society*. 130 (12), 3760-376. DOI: 10.1021/ja8001919.
6. Mushtaq S, Yun SJ, Jeon J. (2019), Recent Advances in Bioorthogonal Click Chemistry for Efficient Synthesis of Radiotracers and Radiopharmaceuticals. *Molecules*. 24(19), 3567. DOI: 10.3390/molecules24193567.
7. Fox, J.M., Darko, A. (2014) Conformationally strained *trans*-cyclooctene with improved stability and excellent reactivity in tetrazine ligation. *Chem. Sci.*, 5(10), 3770-6. DOI: 10.1039/C4SC01348D.
8. Stanimirov, S.S., Todorov, B.R. (2021), Simplified synthetic procedure for (Z) to (E)-cyclooct-4-enol photoisomerization. *Bulgarian Chemical Communications*, 53(2), 228-33. DOI: 10.34049/bcc.53.2.5346.
9. Devaraj, N.K. and Finn, M.G. (2021) Introduction: Click Chemistry. *Chem. Rev.* 121(12), 6697-8. DOI: 10.1021/acs.chemj.