Synthesizing Polycyclic Ether Precursors and Elucidating the Biosynthetic Mechanism of *P. reticulatum*

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Abstract

The biological synthesis of complex polyether molecules remains one of the few synthetic mysteries of organic chemistry - both the required enzymes and the mechanism used within the dinoflagellate cells. The current hypothesis of an endo-tet cascade mechanism using a polyepoxide precursor was tested through the synthesis of a cyclic-ether epoxide and its subsequent incubation with dinoflagellate extract. While the synthesis was performed with unfortunately low yields, the incubation with *P. reticulatum* extract was nonetheless possible and resulted in the formation of a cyclized product, which was tentatively concluded to be the 6-membered product. If true, it indicates the presence of an epoxide-opening enzyme, and that the epoxide methyl group (see Figure 3 on page 2) is not required for cyclization to occur.

1 Introduction

Polycyclic ethers have long been compounds of biological and chemical interest due to the prominent roles they play in mass fish killings during red tides, their complex structures, and the synthetic challenges they pose [3, 1] (see Figure 1 on page 2). *Protoceratium reticulatum* was the first unicellular organism recognized as a source of the polycyclic ethers known as yessotoxins in 1997[7]. Yessotoxins are of especial interest because of their propensity to accumulate in filter-feeders such as commercial mussels and scallops [6].

While the structural elucidation of the causative agents in red tides was the first hurdle[1] - and is still an ongoing difficulty for many related toxins including several compounds produced by other red tide dinoflagellates- the emphasis now lies in the synthetic reproduction of these compounds within the lab and discovering how the dinoflagellates themselves synthesize these molecules [5].

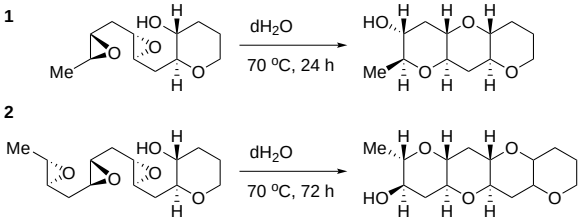

Figure 2: In two different experiments, *endo-tet* cyclization was induced by heating a poly-epoxidated cyclic ether in neutral, deionized water [9, 10].

The current hypothesis for the biological mechanism of polycyclic ether synthesis is the multi-step enzymatic reaction involving the polyepoxidation of a *trans*-polyene and a subsequent *endo-tet* cyclization as proposed by Nakanishi[8] (see Figure 1 on page 2). This synthetic method has been shown to work in water by Vilotijevic and Jamison, but required significant heating (see Figure 2 on page 1). In the absence of additional heat, the reaction required almost a month to go to completion, hence the existence of an enzyme would be required for this process to occur biologically [9]. Subsequent studies revealed that the reaction may proceed via a stepwise mechanism where each subsequent ring formation is faster and more stereoselective than the previous one[10]. However, confirmation of this reaction occurring naturally within cells has yet to be found. Ideally, isolation of the enzyme(s) responsible would corroborate the mechanism's validity, however this has yet to be accomplished. The current line of inquiry is to identify the possible substrates to such an enzyme, and then ideally work backwards to identify the enzyme(s).

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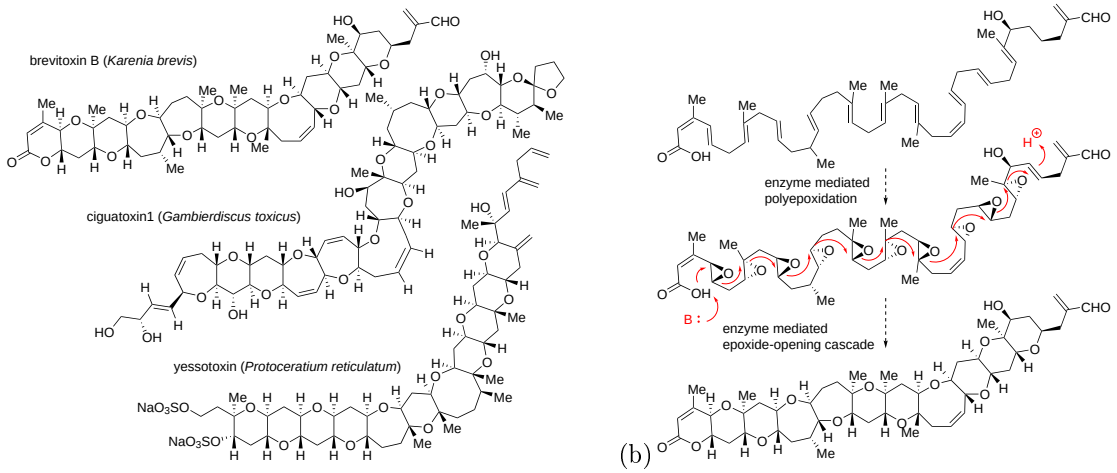

Figure 1: (a) Representative polycyclic ethers and the organisms from which they were isolated[2, 9, 6]. (b)The polyepoxidation and epoxide-opening cascade proposed by Nakanishi [8].

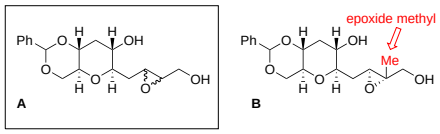

Figure 3: (A) The molecule currently under investigation. (B) A previously tested precursor for which cyclization was believed to have occurred.

A variety of precursors have been shown to produce cyclization when incubated with the dinoflagellate extract. The precursor (compound **A**) under study in this paper is of particular interest in order to determine whether the epoxide methyl is required for cyclization (see Figure 3 on page 2). **A** was synthesized through a multi-step procedure and the success of each step was determined using a variety of methods including TLC (thin-layer chromatography), FAB-MS (fast atom bombardment mass spectrometry), both ^{1}H and ^{13}C NMR (nuclear magnetic resonance), and 2D COSY (correlation spectroscopy) NMR. While TLC was mainly useful in deducing the presence or absence of product and by-product, FAB-MS and NMR analysis allowed for determining whether the appropriate compound was produced. SGC (silica gel chromatography) was also used after each step to purify the product.

Once **A** had been appropriately synthesized, it was incubated with *P. reticulatum* extract and then run through LC-MS (liquid chromatography mass spectrometry) to determine whether cyclization had occurred. LC-MS combines the concept of liquid column chromatography - the idea that compounds will be separated by the speed with which they elute through a polar environment - and mass spectrometry. LC-MS is particularly useful in characterizing complex mixtures such as the incubation solution which contains a veritable pantheon of proteins and organic molecules from within the *P. reticulatum* cells.

2 Materials and Methods

2.1 Synthesis of Compound A

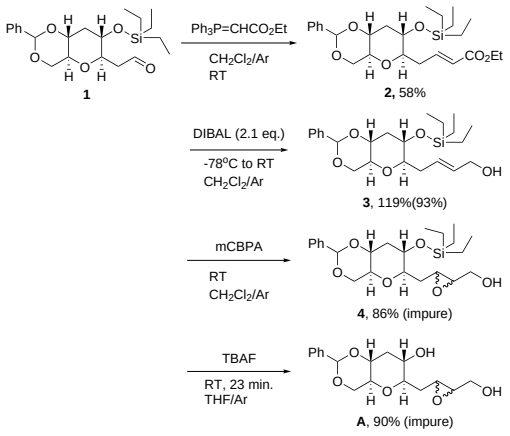

Figure 4: The synthetic method for **A**. The starting material **1** had previously been synthesized by Matoba in conjunction with the Tachibana Laboratory. Percentage yields are indicated on the right.

The conversion of compound **1**, which was obtained as the result of a prior synthesis, was completed in four steps as seen in Figure 4 on page 2. The Wittig

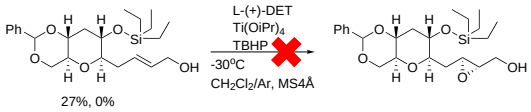

Figure 5: The attempted Sharpless Asymmetric Epoxidation reaction, with percentage of recovered starting material - based on ^{1}H NMR, no significant product was recovered - for the first and second trials, respectively.

reaction and reduction proved fairly uncomplicated - although the Wittig reaction had a low yield, presumably due to unfamiliarity with silica gel column chromatography. The purity of the product for both reactions was determined to be quite high using ^{1}H NMR (see Detailed Methods). The greater than 100% yield for the reduction step is probably due to the sample being too humid - the weight changed every time a measurement was taken. Hence, an approximation for the yield is given in parentheses based on the recovered amount of starting material and the fact that the ^{1}H NMR indicated that the sample was pure.

However, the epoxidation reaction proved more difficult to complete. The reaction was twice attempted using the Sharpless Asymmetric Epoxidation, but both times no product was recovered (see Figure 5 on page 3). It should be noted that the mass spectrum taken of the reaction mixture during the second trial did indicate the presence of product, however that product was lost during the purification process (see Detailed Methods). Hence, the reaction was changed to the peracid epoxidation which unfortunately produced a racemic mixture of both epoxide diastereomers, but did have the virtue of actually yielding product. However, it was deemed that in the interest of time and with the hope that the postulated enzymes could differentiate between the epoxides, they need not be separated before deprotection and incubation.

The deprotection step was concluded to have completed by TLC analysis, although SGC failed to separate the two products produced. Again however, in the interests of time, the unknown side product was believed to be harmless to the incubation and thus another purification was unnecessary.

2.2 Incubation of Compound A with *P. reticulatum* extract

Once the synthesis of **A** was complete, the postulated precursor was incubated with the prepared extract (see Figure 6 on page 3). The *P. reticulatum* cells were ultrasonicated to insure complete lysing of the cells and then suspended in buffer before the incubation. Controls were taken of both the substrate and the extract alone under the same reaction conditions. Samples were taken at 3, 6, and 21 hours to monitor for cyclization. After being quenched, centrifuged, and collected, the supernatant was analyzed by LC-MS. However, the first set of conditions yielded inconclusive results, so the column conditions were changed and the samples run again. This time, a peak separate from the epoxide peak was observed.

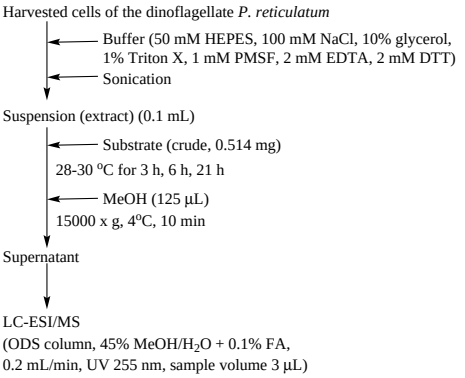

Figure 6: Incubation conditions and preparation of the *P. reticulatum* extract. The column condition was changed from 50% MeOH to 45% MeOH in order to better differentiate between the peaks.

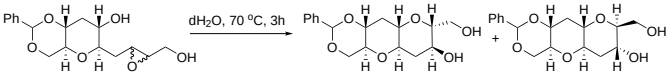

Figure 7: The desired *endo* product was synthesized using the method discussed by Jamison et al. [10] as a comparison for the results of the incubation.

A separate cyclization experiment was also undertaken using the method described by Jamison et al. [10]to provide a reference for the desired 6-membered product (see Figure 7 on page 3). This sample was also subjected to LC-MS using the second set of conditions. However, cyclization was unfortunately not observed. Whether the sample needed to be heated for longer or if the presence of the mCBPA prevented cyclization - since Jamison et al. were working with neutral water [9] - is not known.

3 Results and Discussion

While the synthetic yields could have been much higher, the incubation reaction required only a minute amount of substrate to proceed, so the low

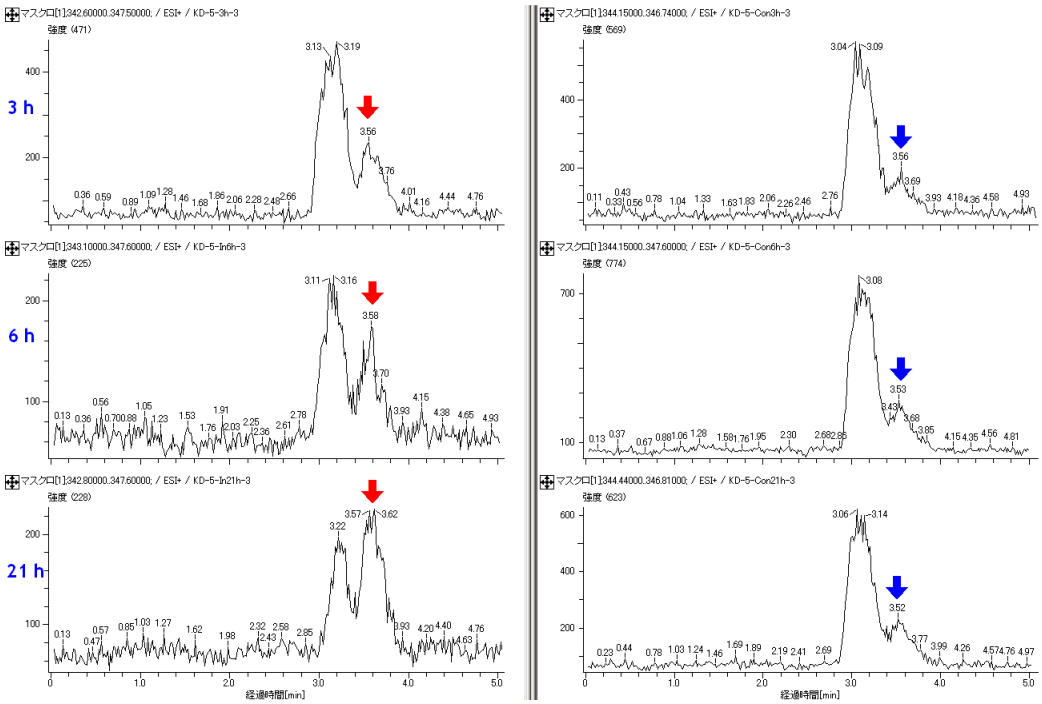

Figure 8: The LC-MS data indicates the formation of a cyclization product, but without further analysis, it is impossible to know whether it is the *exo* (5-membered) or desired *endo* (6-membered) product. The panels on the left are the experiment while those on the right are the control - substrate but no *P. reticulatum* extract. The red arrows mark the cyclization product, while the blue arrows mark the same value in the control. While the blue arrows do indicate the possible formation of a cycle in the control, the increase in height of the red peak over the time course does potentially indicate enzymatic action.

yield is inconsequential. However, it could potentially make the synthesis of subsequent precursors more difficult as 13 mg of starting material is a very difficult amount to work with. It is possible that the failure of the Sharpless Asymmetric Epoxidation the first time was due to the use of old reagents, or the addition of the molecular sieve too late in the reaction. Of course, this does not fully explain the disappearance of the starting material. This was probably due to general unfamiliarity with the proper technique for silica gel chromatography. The failure the second time was undoubtedly due to a mishap involving the vacuum apparatus (see Detailed Methods).

The formation of the 6-membered product during incubation would provide evidence in favor of the Nakanishi hypothesis. However, there is a reasonable concern for the spontaneous formation of the *exo* 5-membered ring. And the formation of this product would be highly likely given the delays in SGC which led to the deprotected product being at room temperature for much longer than is desirable. But, it is optimistically concluded that the second peak present in the LC-MS data is the 6-membered product (see 8).

However, the reaction mixture should be purified using HPLC (high performance liquid chromatography) and the second peak run through ^{1}H and ^{13}C NMR to determine whether the *endo* or *exo* product was formed. Regardless of the answer, many more precursors remain to be tested in the hopes of further identifying both the exact mechanism of cyclization and the enzyme(s) responsible.

4 Conclusion

Thus, we can tentatively conclude that the epoxide methyl group is not necessary for cyclization of the polycyclic ether. It is still unknown why the Sharpless Epoxidation reaction did not proceed, but the resulting low yield was still sufficient enough to obtain results via incubation. Of course, it still remains to be conclusively determined whether the 5-membered or 6-membered product was actually formed. While many precursors remain to be tested, these results lend credence to the Nakanishi hypothesis of an epoxide-opening cascade and hopefully someday, the responsible enzyme(s) will be isolated.

Acknowledgments

Firstly, I would like to thank UTRIP and FOTI for making it possible for me to be here. I would also like to thank Professor Tachibana for giving me the opportunity to work in his lab. I especially want to thank Professor Satake for his help and attention, as well as Raku Irie, Atsushi Mizukami, Yuki Takimoto, and Daisuke Naganuma for their assistance and attention in my laboratory endeavors. They were incredibly patient, helpful, and forgiving of my many mistakes. I would also like to thank everyone else in the Tachibana lab for being so welcoming to me and helping me experience Japanese culture, which I have to say I have fallen hopelessly in love with. Thank you all.

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Pledge

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A Detailed Methods

A.1 Wittig Reaction

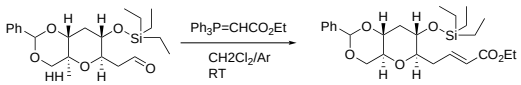

Figure 9: A Wittig reaction was the first step, performed with 58% yield.

Before conducting the Wittig reaction, the starting material was analyzed by ^{1}H and ^{13}C NMR to confirm that it had not undergone oxidation while in storage at $-30°C$ under benzene. The NMR was run using deuterated chloroform which was later confirmed to be contaminated. However, the sample was clear enough that the presence of the requisite aldehyde peak was still obvious.

Approximately 177.4 mg (0.452 mmol) - the number is approximate as the sample vial of starting material was originally recorded as containing only 160 mg of product, hence the 177.4 mg is regarded with a high degree of suspicion - was combine with 0.2647 (0.764 mmol) in 3 mL CH_2Cl_2 in a 20 mL recovery flask under argon atmosphere. The reaction was then left to stir overnight (approximately 18 h) at room temperature.

TLC indicated the presence of the desired product so the reaction mixture was rinsed with chloroform, rotovapped, and dried under a vacuum to produce 533.6 mg of crude product in the form of a yellow oil. The solution was stored under benzene at $-30°C$ until SGC was performed using a 10% mixture of EtOAc in hexane. The second batch of spots was collected, rotovapped, and dried to yield 119.9 mg of a clear oil product.

Both ^{1}H and ^{13}C NMR were taken under deuterated benzene to confirm the identity and purity of the product.

A.2 DIBAL Reduction

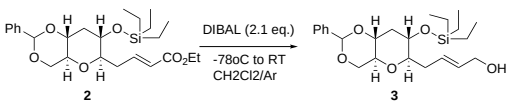

Figure 10: The second step was a DIBAL reduction, which had a maximum possible yield of 83%.

Using a 20 mL 2-necked recovery flask, 99.7 mg (216 μmol) of starting material was combined with ~5 mL of CH_2Cl_2at $-78°$C and stirred under argon. 755 μL(755 μmol) of DIBAL was added dropwise over the course of a minute. After stirring for two hours warming to room temperature, the reaction was quenched with a few drops of MeOH and then an equal volume of Rochelle salts was added over ice. The reaction mixture was then left to stir overnight.

Based on TLC analysis, the reaction had completed by the next morning (approximately 18 h). The mixture was then extracted with AcOEt, washed with Rochelle salts, washed with brine, and dried over $MgSO_4$ using a 50 mL, then 100 mL separatory funnel. The drying agent was removed via vacuum filtration. The solution was then rotovapped, dried under vacuum, and stored under benzene at $-30°$C until the next day.

SGC was performed using 15% EtOAc in hexane. 6.6 mg of starting material was obtained along with 108.4 mg of product, which is a 119% yield. While this would cause the purity of the product to be suspect, the ^{1}H and ^{13}C NMR - taken under deuterated benzene - were remarkably clean. Hence, the conclusion is that the humidity caused the weight of the sample to fluctuate. A COSY NMR was also taken so as to identify all of the peaks in the product.

A.3 Sharpless Asymmetric Epoxidation

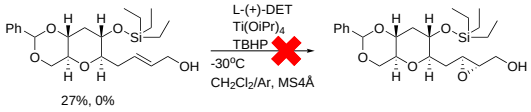

Figure 11: Though the Sharpless Asymmetric Epoxidation reaction was performed twice, neither time could a usable quantity of product be obtained.

A.3.1 First Attempt

The Sharpless Asymmetric Epoxidation reaction was attempted twice. The first time, 108.4 mg of starting material was combined with 2.5 mL of CH_2Cl_2 in a 30 mL 2-neck flask stirring at $-30°$C. 0.013 mL of L-(+)-DET (77 μmol) was added, then 0.014 mL of $Ti(OiPr)_4$ was added. 0.1166 g of 4Å molecular sieves was heated for 2-3 minutes with a heat gun 3 times under vacuum, and was added to the solution. It is possible that adding the molecular sieves at this point in the reaction was the cause of its failure. 0.23 mL (1.289 mmol) of TBHP was added one hour later and the reaction was left to stir at $-30°$C under argon overnight (approximately 16 h).

TLC analysis indicated the presence of two materials, an orange spot and a brown one, so the reaction was believed to have finished. The reaction mixture was then filtered through celite, quenched with Na_2SO_4on ice, extracted with EtOAc, washed with brine, and dried over Na_2SO_4. The drying agent was removed by vacuum filtration. The solution was then rotovapped, dried under vacuum, and stored in benzene at $-30°$C.

SGC was performed using first 20% then 40% EtOAc in hexane. Two different spots were collected, however the larger quantity of material recovered - 29.6 mg - was the starting material. Only trace amounts of product was recovered and that mixed with an unidentified contaminant. The starting material was recolumned using 40% EtOAc.

A.3.2 Second Attempt

The Sharpless Asymmetric Epoxidation was then reattempted using 15.5 mg (35.5 μmol) of starting material under 1 mL of CH_2Cl_2 in a 20 mL 2-necked recovery flask. 27.3 mg of 4 Å molecular sieves was heated 3 times for 2-3 minutes using a heat gun and then added to the reaction mixture. The reaction mixture was then placed to stir in a $-30°$C water bath. Approximately 1.8 μL (10.7 μmol) of L-(+)-DET was added under argon atmosphere, followed ten minutes later by approximately 2.1 μL (7.1 μmol) of $Ti(OiPr)_4$. Note the operative word "approximately". A syringe small enough to differentiate such volumes was not available, so potentially much more of each reactant was added. After another six minutes, 0.052 mL (285 μmol) of TBHP was added, and the reaction was left to stir overnight.

FAB-MS was used to determine that the reaction had occurred - the presence of a 459 m/z peak indicated the formation of the epoxide - and so the reaction mixture was filtered through celite to remove the molecular sieves. Unfortunately, due to unfamiliarity with the vacuum apparatus, the reaction mixture was heavily contaminated with tap water. However, the resulting mixture was nonetheless extracted with

EtOAc, quenched with Na_2SO_3 on ice, washed with brine, and dried using Na_2SO_4. The drying agent was then removed using gravity filtration. The subsequent solution was rotovapped, dried under vacuum, and stored at $-30°C$ under benzene.

SGC was performed with the reaction mixture using 30%, then 40%, then 100% EtOAc in hexane. As only 2 of the 72 fractions obtained contained noticeable spots, all subsequent fractions were combined, rotovapped, and dried under vacuum. The ^1H NMR of the recovered material indicated that it contained neither the starting material nor the product - both the epoxide and alkene peaks were absent - so the reaction was scrapped.

A.4 Peracid Epoxidation

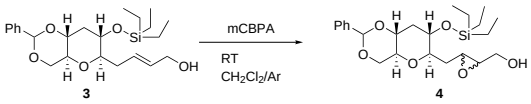

Figure 12: In place of the Sharpless reaction, a peracid epoxidation was performed instead with a yield of 86%, although that was unfortunately slightly contaminated with mCBPA.

After failure of the Sharpless reaction, it was decided that a peracid epoxidation would be attempted instead, despite the fact that it is not enantioselective. 18.2 mg of starting material (0.0433 mmol) was combined with 24.7 mg (0.130 mmol) of mCPBA in a 30 mL 2-necked recovery flask and dissolved in 3 mL of CH_2Cl_2 under argon. The reaction was then left to stir overnight (approximately 17 h).

After TLC indicated that the reaction had indeed finished, the reaction mixture was quenched with Na_2SO_3, extracted 3 times with EtOAc, washed with $NaHCO_3$, washed with brine, and dried over Na_2SO_4. The drying agent was then removed via vacuum filtration, and the subsequent solution was rotovapped, dried under a vacuum, and stored at $-30°C$ in benzene.

SGC was performed using 50% EtOAc in hexane yielding three subtly different sets of fractions. One set contained the racemic mixture of the desired products, the second (and larger fraction) contained a mixture of product and mCBPA, while the third contained unreacted mCBPA. Unfortunately, SGC was not performed with small enough aliquots to completely separate the mCPBA from the epoxide product. Hence the resulting 16.3 mg of product was not pure. However, a second column chromatography was foregone due to time constraints. Since the next reaction would involved column chromatography as well, it was hoped that the acid would be separated out at that stage.

A.5 Deprotection

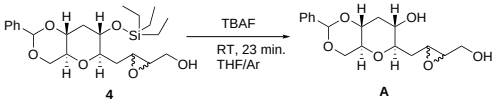

Figure 13: The final synthetic step was the deprotection of the alcohol group, which was performed with 90% yield.

For the last step, 19.7 mg of starting material (45.1 μmol) was combined with 0.2 mL of THF under argon. TBAF was then added dropwise while stirring at room temperature. After 23 minutes, TLC indicated the formation of product, so the reaction mixture was directly run through an SGC using 2% Et_3N in EtOAc. The polarity of the eluent was then increased by adding 2% MeOH, then 4%, then 10%. However, no noticeable spots ever appeared under TLC, so all the fractions were recombined and rotovapped and dried. The unfortunate result of this was that the mCBPA contaminant from the first step was not removed, so the sample remained impure.

The resulting solution was rotovapped and dried under vacuum to yield 13.03 mg of a yellow-gold solid. This solid was then dissolved under benzene and stored at $-30°C$. The synthesis of the desired precursor was thus completed.