Stony Brook University

Partial Synthesis of Bis-boronic Acid-based Glucose Sensor Incorporating FRET Signaling

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Abstract of the Thesis

Partial Synthesis of a Bis-boronic Acid-based Glucose Sensor Incorporating FRET Signaling

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2008

Diabetes is a disease affecting millions of people worldwide. Research to find a way to effectively and continuously monitor blood glucose levels in diabetes patients has become increasingly important. Current methods include the vastly used finger prick method and platinum based sensors that measure interstitial fluids in the body. For reasons including, calibration, inconsistency in crucial regions and the amount of time it can be worn, these devices have not become widely used. Current research is focusing on the synthesis use of an indicator molecule to be inserted into the body like a tattoo that can be used to continuously monitor blood glucose levels.

The computer program CAVEAT was used to design a scaffold structure on which to build a glucose receptor incorporating a pair of properly positioned boronic acid groups for selective glucose binding. The design also includes a pair of fluorophore groups such that a change in energy transfer or FRET between these two fluorophores is expected upon binding of the glucose. For synthesis, the potential glucose sensor was divided into a core scaffold structure and a pair of bis-aryl amine moieties incorporating the boronic acid and the fluorophore groups. The synthesis of the core scaffold structure was completed, bearing a couple of reactive methanesulfonate esters as eventual points of attachement of the bis-aryl amine groups. Several approaches to the synthesis of the bis-aryl amine were studied. A bis-aryl amine was eventually synthesized using palladium-catalyzed coupling that incorporates a protected boronic acid and a nitrile group. The nitrile will serve as the eventual point of attachment of the fluorophore groups. Thus synthesis of the designed glucose sensor is near completion.

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I) Background and Significance

i. Continuous Glucose Monitoring Systems

Research into blood glucose monitoring is focusing on the development of a continuous glucose monitoring system that is practical, convenient and affordable.[1,2,3] Continuous Glucose Monitors (GCM) are able to show trends in glucose level fluctuations, and can help prevent diabetes related complications. Worldwide diabetes is affecting approximately 246 million people, and annually 3.8 million deaths are attributed to the disease.[4] Normal blood glucose levels while fasting are just less than 6mM; levels of 7mM or more while fasting is a signal for diabetes.[5] Although glucose levels ranging from 1mM to 30mM can be observed in type I diabetic patients, to be able to avoid diabetes related complications the blood glucose level has to be kept between 3mM and 10mM.[6] By constantly monitoring the blood glucose levels, Continuous Glucose Monitoring helps avoids hyperglycemic and hypoglycemic ranges throughout the day, since these tend to occur at night while the patient is unaware.[7]

The most common way to keep track of blood glucose concentration is the finger pricking method. This method is painful, inconvenient and even the most vigilant patients only take between four and eight finger prick measurements per day.[8] For almost three decades now, researchers have been trying to find more effective ways to monitor the variations in blood glucose levels for diabetes patients. A "Continuous Glucose Monitor is capable of detecting unrecognized hypoglycemia and other patterns that may be undetectable by conventional glucose monitoring."[9] Noninvasive optical methods which measure interstitial fluids through the skin have been eagerly pursued. The problem is that since glucose has no significant spectral signal, it is very difficult to detect it using infrared spectra (NIRS). Using constant calibration and other multivariate techniques, it is possible to predict glucose concentrations, but this form of glucose monitoring has only reached moderate success because of the interference of the spectroscopic absorption by physiological conditions.[10]

Researchers have pursued the study of non-invasive glucose monitoring systems that measure glucose levels in the interstitial fluids (ISF). These measurements can be taken by inserting a needle into the subcutaneous tissue or by transferring interstitial fluids outside the body; they are considered minimally invasive because they do not puncture any blood vessels.[11] Although there is a direct correlation between the blood glucose and interstitial fluid glucose levels, the interstitial glucose level lags about 6.7 minutes behind the blood glucose level.[12,13,14] There are physiological factors that can affect and make this lag time inconsistent. This is problematic because "too long or to inconsistent a lag time could render interstitial glucose ineffective in controlling insulin delivery."[15] A continuous glucose monitoring system has to be consistent in lag time or

have a real time measurement, and be able to properly function without constant calibration, to be fully effective. The systems that are currently on the market require approximately four conventional finger-prick samples a day for calibration, and even with careful and thorough calibration they are not reliable in the hyperglycemic and hypoglycemic range.[16]

D-glucose + O_2 → gluconolactone + H_2O_2

Figure 1

Minimally invasive methods use biosensors in vivo to measure glucose concentrations in the body. Biosensors use the same concept as traditional glucose measuring devices by basing the electrodes on immobilized glucose oxidase and its sensitivity to glucose concentrations.[17,18] Using this concept, anodic oxidation of hydrogen peroxide was measured using a platinum anode (Figure 1) covered in enzyme glucose oxidase. Although it seems promising, physiological conditions in the body affect the implanted electrode and lead to unreliable results.[19] Physiological factors are also the problem when measuring oxygen consumption using an oxygen electrode and when measuring the change in pH due to the hydrolysis of gluconolactone.[13,19] Researchers have tried to bypass this problem by encasing the electrode in a semi permeable membrane, but this has also led to reliability issues. Another major problem with electrodes implanted into the skin is that generally they can only be implanted in certain areas of the body and remain in place for a few days due to infection and or inflammation at the site of the implant.[20,21,22] A way to have long term continuous monitoring has to be developed.

As of 2008, a number of Continuous Monitoring Systems have received approval from the Food and Drug Administration for use in the United States. The latest continuous glucose monitor to receive approval in the United States is the FreeStyle Navigator Continuous Glucose System (Abbott Diabetes Care). Approved in March 2008 it consists of a sensor, a transmitter and a receiver. The sensor is implanted into the back or the upper arm or abdomen, the transmitter is attached to a patch-like plastic mount on the sensor, and then wirelessly transmits the readings to the pager like receiver. This CGM system gives a minute to minute reading of glucose concentration and can be worn for up to five days, but is not meant to replace conventional glucose measuring techniques.[23] In 2007, the MiniMed Paradigm REAL-Time system became the first FDA approved sensor to integrate an insulin pump. The insulin pump is small and can behave like a healthy pancreas throughout the day. A small sensor is inserted into the body and can be worn up to three days; it wirelessly transmits to the insulin pump through radio frequencies and an alarm on the pump will sound to signal a problem. Again this system is not meant to replace conventional methods for diet decisions.[24] One of the major problems of these sensors are that they are for short term use. For this reason, currently,

DexCom Inc. is working on the first long term use continuous glucose monitoring system. The full history of continuous glucose monitoring systems is too vast to cover, but can be found online at Mendosa.com.[25]

ii. Research into Fluorescence Based Sensors

Alternatively researchers have been pursuing the study of fluorescence-based glucose sensors. Instead of using an immobilized glucose oxidase based electrode, this approach will use a glucose-sensitive fluorophore. Ideally the reporter molecule will be inserted into the skin, much like a tattoo, and glucose concentrations will be determined by measuring the fluorescence through the skin. Alternatively the tip of a tiny fiber can be filled with the indictor molecule and introduced into the skin. Fluorescent continuous glucose monitoring systems have a great number of advantages. Fluorescence techniques have great sensitivity; even single molecule detection has been reported using fluorescence.[26] Another advantage is that fluorescent measurements do not require the introduction of an electrode into the body, so with the appropriate fluorophore fluorescence change can be detected externally, limiting or eliminating damage to the body.[27] Having a fluorescence based system will eliminate the problem of long term use, the problems of calibration and stability, and the corrosion of the sensor by physiological factors, but it will still face problems of its own. These issues, although less problematic than those associated with electrode based systems, must be addressed to produce a fully functional continuous glucose monitoring system.

Any glucose receptor molecule that is useful in continuous diabetes monitoring must contain the appropriate dissociation constant and as well as sensitivity to and specificity towards glucose. The dissociation constant for a receptor must be approximately 6 mM, which is the normal blood glucose concentration, to be able to detect even the slightest deviation from the norm. In addition, these slight changes in glucose concentration must produce a change in fluorescence caused by one of a number of phenomena that would occur upon glucose binding. Photoinduced electron transfer (PET) is one phenomenon that produces a change in fluorescence upon saccharide binding. Upon bond formation, lone pairs that are normally able to be excited into an open shell of another atom, are tied up causing increased fluorescence. Another fluorescence phenomena that glucose monitoring research takes advantage of is fluorescence (or Förster) resonance energy transfer (FRET). FRET is caused by a dipole-dipole interaction that transfers energy from a donor fluorophore to an acceptor fluorophore that are within a few angstroms of each other; this is done without electron transfer.

Some of the first studies into fluorescence based glucose sensors were performed on the naturally occurring protein Concanavalin A (Con A). It is a lectin containing four separate glucose binding sites, that can be extracted from a jack bean.[28] "Con A sensors

are based on the competitive binding to the lectin of either glucose or a labeled carbohydrate derivative such a dextran, mannoside, or glycated protein."[29] In studies, rhodamine-Con A was used as an acceptor with dextran used as a donor; as glucose was introduced into the system, the donor and acceptor moved further apart decreasing FRET and in turn increasing the intensity of the donor fluorescence.[30] Sensing decay times using Con A was also advantageous in fluorescent based glucose monitoring studies. Glucose addition to the Con A donor-acceptor system increases the mean lifetime with decreased FRET. This study of the lifetime is important because it is independent of probe concentrations, light scattering and the absorption of the sample, which makes it attractive for in vivo use.[31] Although Con A was a good starting point, the problem is that it required competing molecules that could easily diffuse relative to each other. A membrane that would allow this diffusion is easy to manufacture, but a device would have to be implanted into the skin for in vivo use. Scientist soon went from studying naturally occurring molecules to synthesized molecules that would be more suitable for glucose monitoring in the body.

iii. Boronic Acids as Glucose Sensors

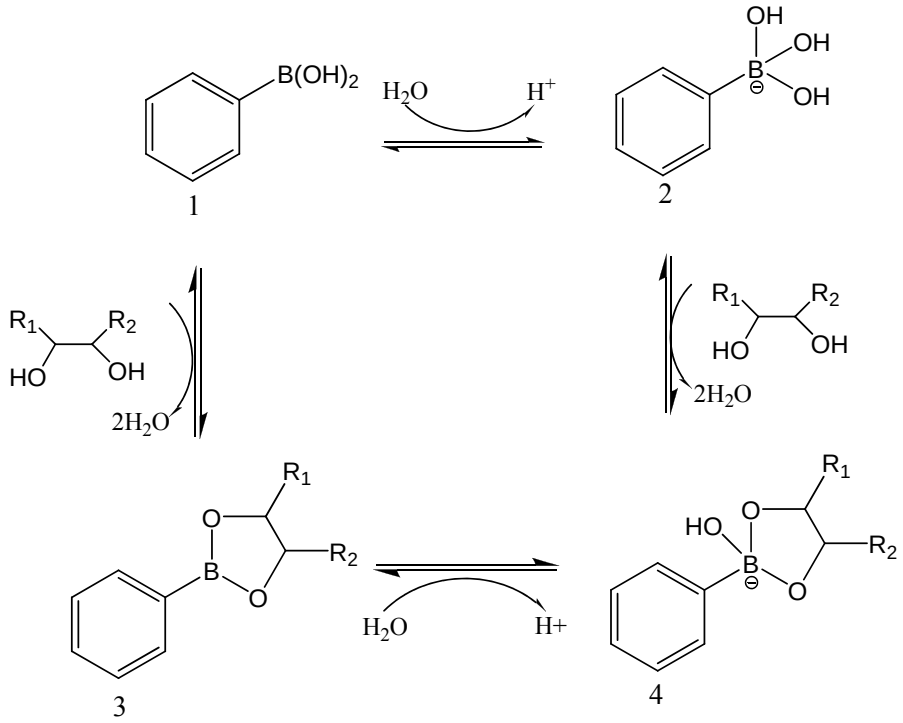


Figure 2. Saccharide-glucose bond[12]

Aryl boronic acids have been extensively studied over the last several years due to their ability to readily bind glucose. As Lewis acids, they can exist in the neutral trigonal form or in the anionic tetrahedral form as illustrated in Figure 2.[32] Boronic Acids are able to covalently bond 1,2 or 1,3 diols to form five (Figure 2) or six membered rings.

The affinity of boronic acids towards diols depends on the pK_a of both the boronic acid and the diol; the higher the pK_a of boronic acid the lower the affinity to a diol. Affinity is also dependent of the pH of the system. Being that this bond formation is reversible, boronic acids are ideal for glucose sensors.

The first two arylboronic acids synthesized for the purpose of glucose sensing were reported by Yoon and Czarnik.[33] Since they are monoboronic acids, these systems did not show the necessary affinity to or specificity towards glucose, but this work has been fundamental to research into the topic. Compounds **5** and **6** show high fluorescence when in their unbound state, but upon ester formation with the diol (**7** and **8**), the fluorescence decreases significantly. It was believed that the decrease in the fluorescence of these molecules is attributed to PET. In its unbound state, the boronic acid, due to its pK_a, should exist in the trigonal form (**1**, Figure 2). In this state the boronic acid has no negative charge and PET is not possible. When the arylboronic acid becomes bound to glucose, the pK_a changes causing it to change hybridization and gain negative charge. It has since been argued that this is not the system at play, but using the ideas explained above, researchers were able to develop compounds that are more useful in glucose monitoring.[34]

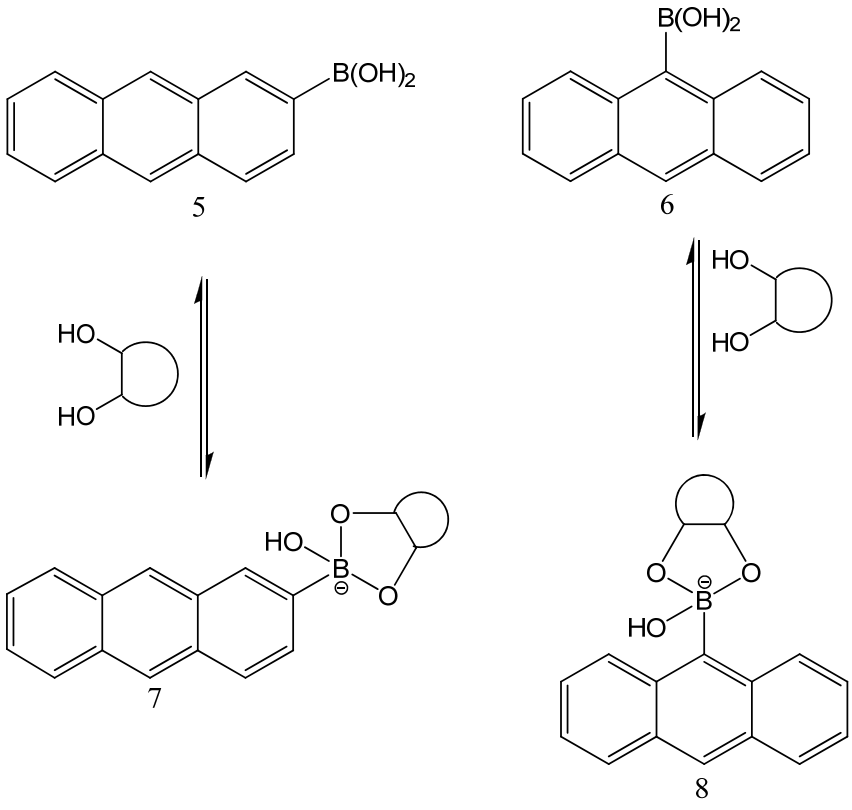


Figure 3

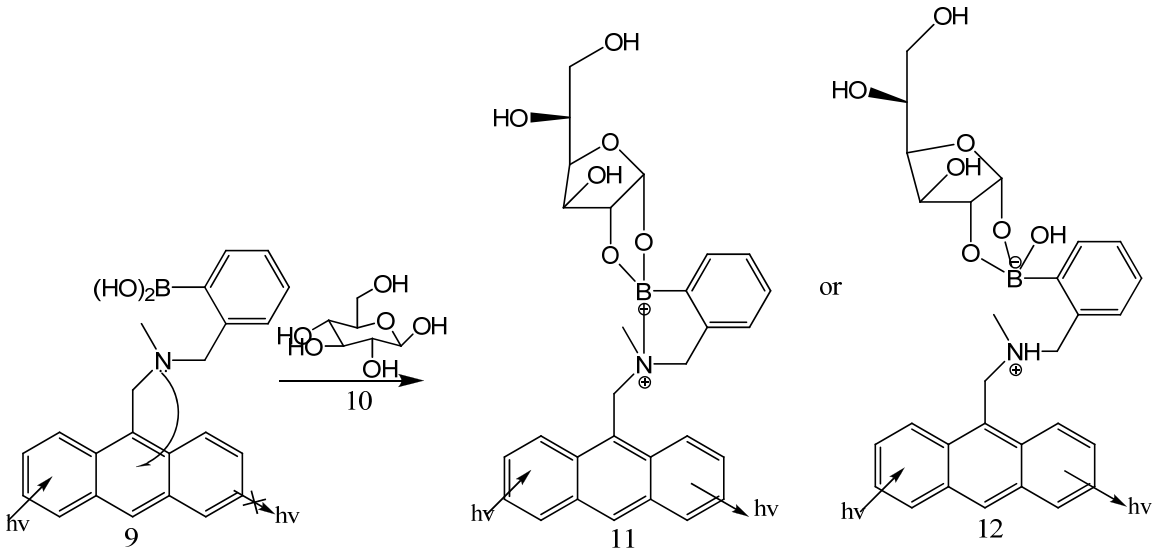
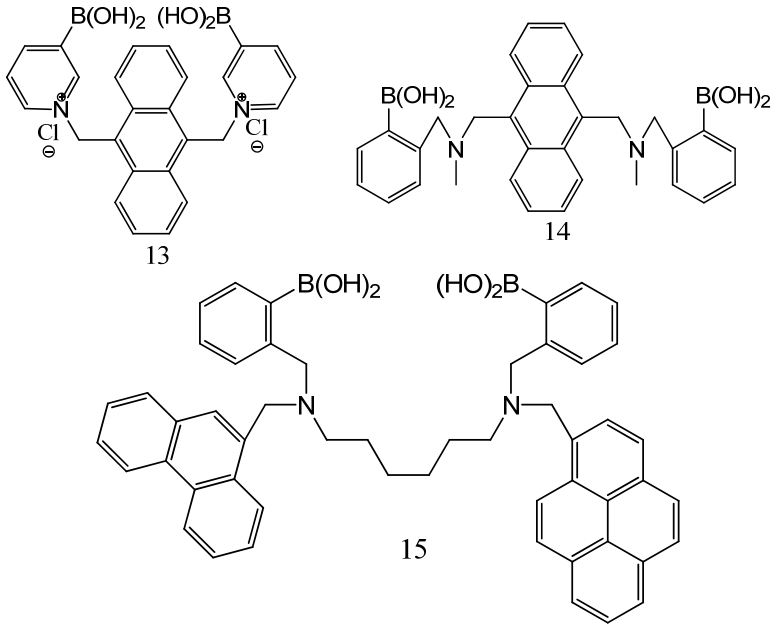


Figure 4

With PET fluorescence in mind, researchers developed compound **9** (Figure 4) to test in glucose sensing. In compound **9** the nitrogen and boron are positioned so that bonding can occur after the molecule becomes bound to glucose.[35] In its unbound state, **9**, has low fluorescence due to quenching by PET from the nitrogen lone pair to the fluorophore. But when bound to glucose, the nitrogen either engages in covalent bonding with the boron using its lone pair to form compound **11** or gets protonated to form compound **12**. In both scenarios the lone pair is lost and the system is no longer quenched causing a significant increase in fluorescence. This system did show a stronger affinity to glucose and fructose than previously reported[36] phenylboronic acids. Although it was a step forward, like all other previously tested monoboronic acids, it is still not sufficiently selective for continuous glucose monitoring. Simple boronic acids actually bind fructose about 50-fold more strongly than glucose.[37]

Diboronic acids were developed and are being aggressively pursued as a more logical choice to specifically bind glucose. Two appropriately spaced boronic acids are able to bind with glucose at two sites to form 1:1 complexes; in essence the glucose is a bridge between the two boronic acids. Compound **13** (**Figure 5**) is in its ionic state which makes it both highly water soluble and gives it a relatively low pK_a. The low pK_a makes it possible for it to bind with D-glucose at physiological pH's. Studies indicate that compound **13** has a 10-fold selectivity for glucose over galactose, but the dissociation constant with glucose is about 0.4mM which too low making the affinity about 15-fold too high for glucose sensing at physiological levels. A higher affinity towards glucose is seen in compound **14** which has an internal tertiary amine. Fluorescence changes about 7-fold upon glucose binding, and this compound has a 12.5-

fold selectivity for glucose over fructose and a 25-fold selectivity to glucose over galactose. Although the change in fluorescence and selectivity are good, the dissociation constant of 0.25mM is too far off from the desired dissociation constant of 6mM to match the normal physiological concentration of glucose. Both **13** and **14** were promising but proved to be irrelevant in physiological conditions.



13 14 15

Figure 5

The diboronic acid **15** was prepared to show Fluorescent Resonance Energy Transfer (FRET) upon glucose binding. This asymmetric compound has a donor fluorophore, phenanthrene, and an acceptor fluorophore, pyrene. In this system, long wavelength excimer emission are present due to the π-π stacking of the phenanthrene and the pyrene. This excimer emission changes to show FRET once the saccharide is bound due to the rigidity of the compound. The saccharide cause energy transfer from the donor fluorophore (phenanthrene) to the acceptor fluorophore (pyrene). Compound **15** has a 2-fold selectivity for glucose over fructose, but in conditions that mirror those in the human body the affinity for glucose is too high to affectively be used in continuous glucose monitoring.[38]

Diboronic acids containing amines (Figure 5) are more attractive to glucose sensing studies than their non-amine counterparts because of their pH independence in physiological conditions. While boronic acids such as **15** have dissociation constants that are highly dependent on the pH when the pH is below a pK_a of 8.8 (the pK_a of a phenylboronic acid), the pH of amine-coordinated boronic acids (Figure 5) is independent of pH at all pH's relevant to continuous glucose sensing. A problem common to all amine-coordinated boronic acids studied thus far is that the affinity for glucose is too

high. These compounds would never be able to accurately pick up slight glucose fluctuations in the body. The only way to eliminate this problem would be to alter the Lewis acidity of the compound, but that would decrease glucose selectivity. Adding appropriate substituent groups to the system that would efficiently change the Lewis acidity of the diboronic acid while still maintaining selectivity for glucose are possible and are being actively pursued.

iv. Previous Work from the Drueckhammer Group

For almost three decades now, there has been extensive research into continuous glucose sensing. Electrochemical sensors and fluorescent reporters have been two of the fields explored. To date, no truly effective way to monitor blood glucose has been developed. For this reason, research continues to be conducted in the field. The focus of the research that will be discussed in this paper is to find an appropriate FRET molecule for use in continuous glucose monitoring systems. Specificity for glucose and an appropriate dissociation constant are the crucial components needed in any molecule developed for this purpose, and should be the primary concern of researchers trying to synthesize such a molecule.

The CAVEAT computer program is a search engine that when used with a database can provide the user with molecules fitting specified parameters. CAVEAT treats a bond like a vector (**Figure 6**) and after the vector is defined, molecules from the database used that have bonds fitting the desired vector relationship are produced. A vector pair is defined uniquely by four parameters-the distance between the two base atoms, the dihedral angle between the vectors and the two exterior angles for each vector.[24] The Drueckhammer group at Stony Brook University were the first to design a glucose receptor molecule using CAVEAT. The database available at the onset of the design, TRIAD, is a database of tricyclic hydrocarbons. TRIAD gave the group approximately 300 tricyclic hydrocarbons that matched the specifications incorporated into the search. After extensive eliminations the group settled on compound **17** as the scaffold for synthesis. Replacement of the hydrogen atoms in bold gave structure **18**, the presumed glucose receptor.

Structure **18** went through several modifications before arriving at the structure that was synthesized and studied, **19**. One modification was the replacement of the fulvene ring with the furan ring; this was done because the furan ring is both more stable and synthetically accessible. Oxygen was introduced to bridge the phenyl ring containing the boronic acid to the second fulvene ring. Adding the bridging oxygen made the molecule more stable and avoided the out of plane twisting caused by the two hydrogen atoms that the oxygen replaced. A hydroxymethylene group was added to increase solubility in aqueous solution and the methyl group of the bridging portion was added merely because the commercially available starting material used contained it. The

proposed structure was synthesized and extensively studied for its fluorescent and glucose binding properties.

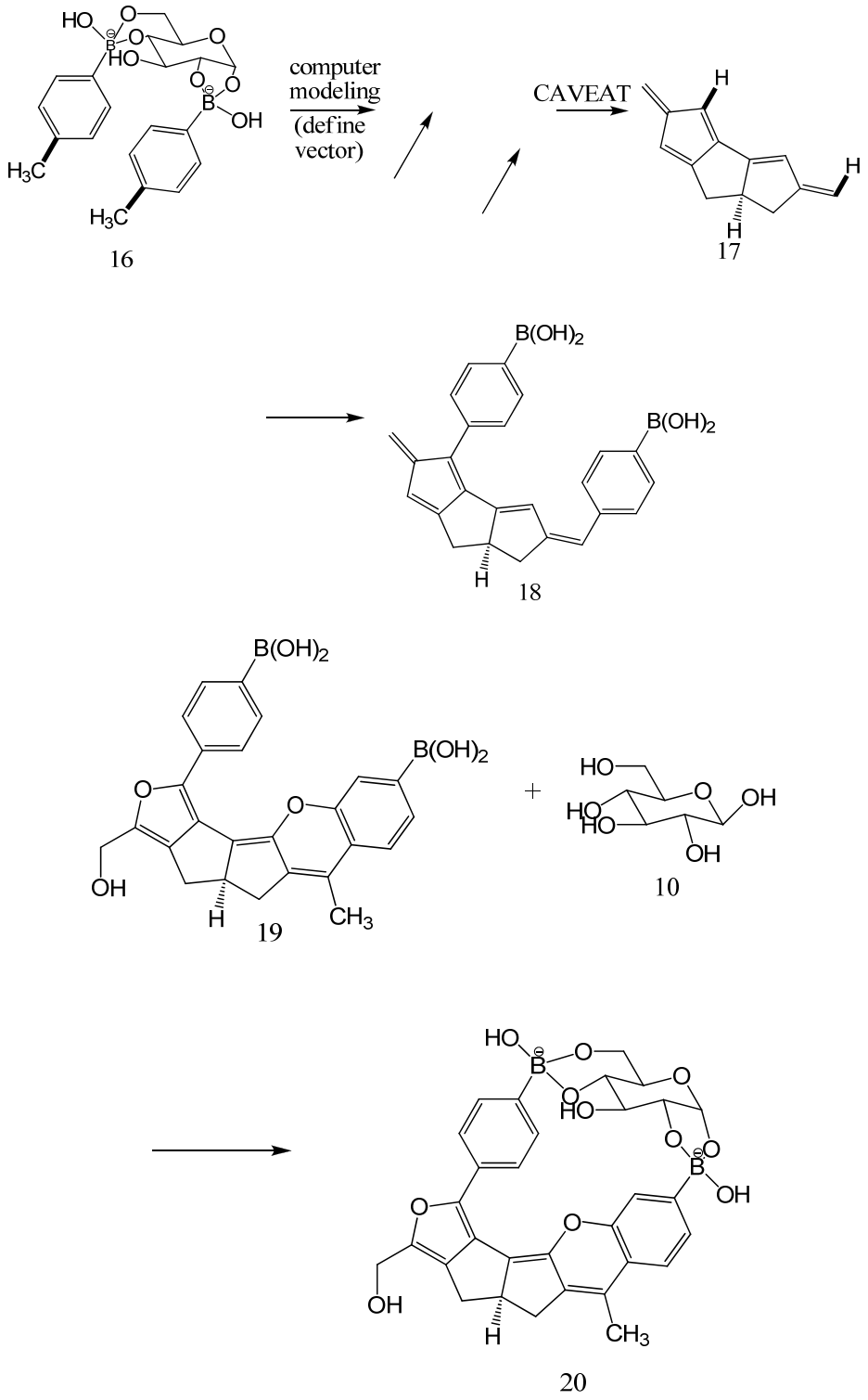


Figure 6

Structure **19** showed affinity to glucose that had only been seen in naturally occurring glucose binding proteins. Structure **19**'s affinity for glucose, fructose, galactose, and mannos studied by observing the decrease in fluorescence as a result of complex formation. The apparent dissociation constant for glucose is 2.5 x 10^{-5} M, and its fluorescence decreased 50% upon complex formation. Its affinity for glucose is 400-fold greater than its affinity for any of the other sugars, and its exhibited decrease in fluorescence is about 20% greater than that for any of the other naturally occurring sugars. The apparent affinity of **19** is 100-fold too high to be useful for physiological monitoring of glucose because it would exist completely in its complex form, **20**, at all relevant pH's. These results proved the usefulness of computer-aided design, but produced a molecule that could never be used in continuous glucose sensing. The group attempted to introduce electron donating groups to decrease the Lewis acidity of **19**, but this made an already complicated and lengthy synthesis nearly impossible. It was decided that the most logical thing would be to go back to CAVEAT and find another scaffold upon which to build.

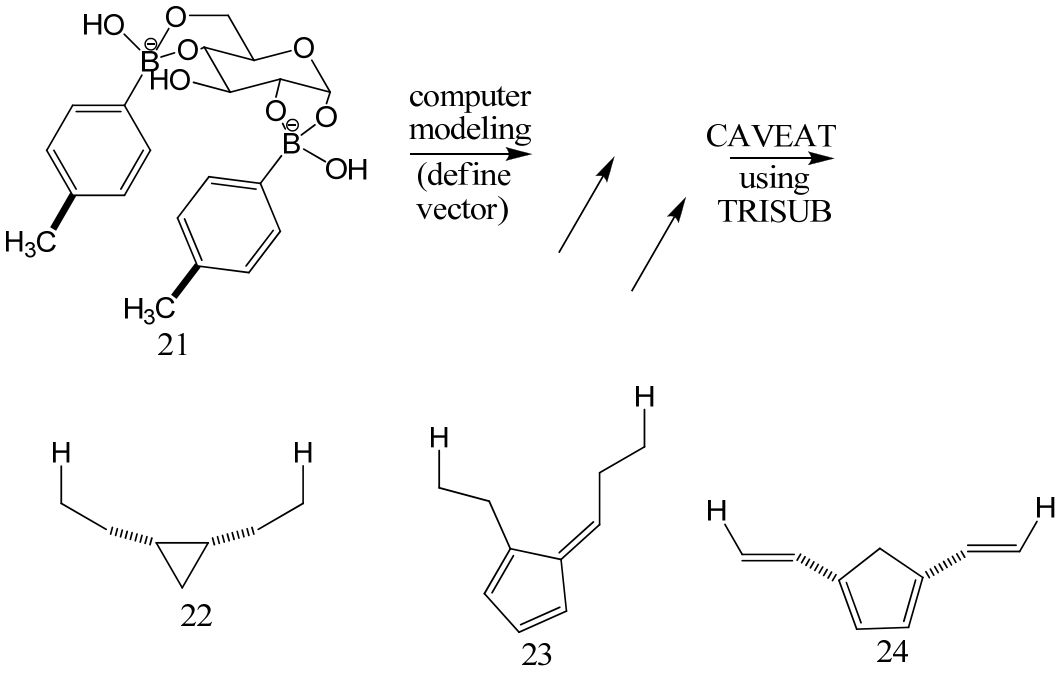


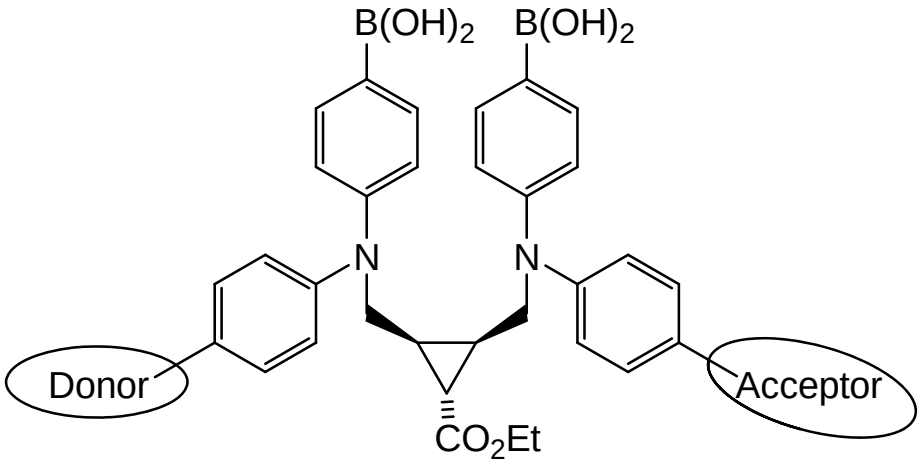
Figure 7

The group developed a new database TRISUB for use with the CAVEAT program. At the onset of research TRIAD was the only available database and provided structures that were highly complicated for synthesis. TRISUB is a database that contains less rigid and simpler molecules that would make synthesis easier and far more efficient. CAVEAT was now able to identify many more core structures. Scaffold

molecules **22**, **23**, and **24** were chosen for further study by the Group. The research which is the topic of this paper is based on the scaffold structure **22**. Other researchers in the group are currently working on synthesizing molecules based on the scaffolds **23** and **24**. These structures are a promising next step in continuous glucose monitoring devices.

II) Results and Discussion

i. Introduction into Current Research



25

Figure 8

Using scaffold **22,** a nitrogen containing diboronic acid, compound **25**, was designed to be a glucose receptor. The initial scaffold was slightly altered by adding a carboxylate group to the cyclopropane ring; this addition will both help make the synthetic route easier and make the compound more water soluble for subsequent analysis. Compound **25** contains a donor and acceptor fluorophore that will show FRET upon excitation of the donor. When the compound is free to rotate, the FRET will be at its maximum because the two fluorophores can achieve a minimum distance. When the compound is bound to glucose, the two fluorophores will be held at a distance and the extent of FRET will be less. Thus the ratio of donor to acceptor emission should increase when glucose is bound. With the two boronic acids strategically placed for optimal

glucose selectivity, it is believed that **25** will make a promising glucose receptor for continuous glucose monitoring.

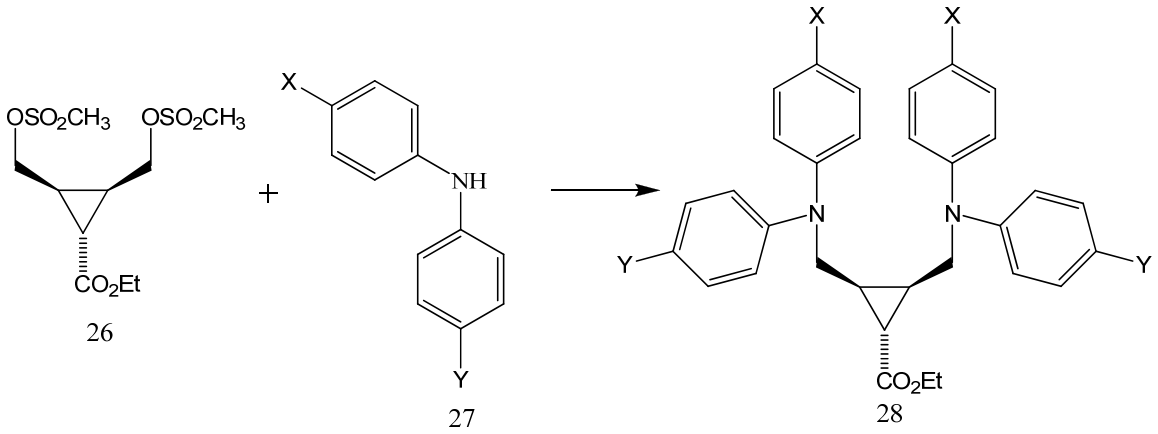


Figure 9

It was envisioned that **25** could be prepared by the general strategy shown in Figure 9. Compound **26** would be prepared from the known diol and coupled with diarylamine **27** to form **28**. In this figure X represents a protected boronic acid or a halide or a mesylate ester that can be converted to a boronic acid in a subsequent step. Y in turn represents fluorophores or functional groups to which the fluorophores can be attached in a later step.

Theoretically one could arrive at **28** using the in the route depicted in Figure 10 rather than the route depicted in Figure 9. Arriving at **28** via the synthetic route in Figure 10 would eliminate the need to protect and deprotect the diol necessary to synthesize the mesylate required by the process in Figure 9. This alternative was attempted prior to the known synthesis for the mesylate. Figure 11 shows the attempt at making the process simpler. Diphenylamine (**31**) was reacted with cis-1,4-dichlorobutene in the presence of LDA. The simple substitution reaction replaced the chlorine atoms with diphenylamine to form **32**, cis-1,4-di-(diphenylamine)butene. Compound **32** was then treated with ethyl diazoacetate in the presence of a catalyst, rhodium acetate, and heated to reflux in dichloromethane. After the slow and careful addition of the ethyl diazoacetate, ^{1}H-NMR showed that no cyclopropanation reaction had occurred. Thus, efforts turned to the approached shown in figure 9.

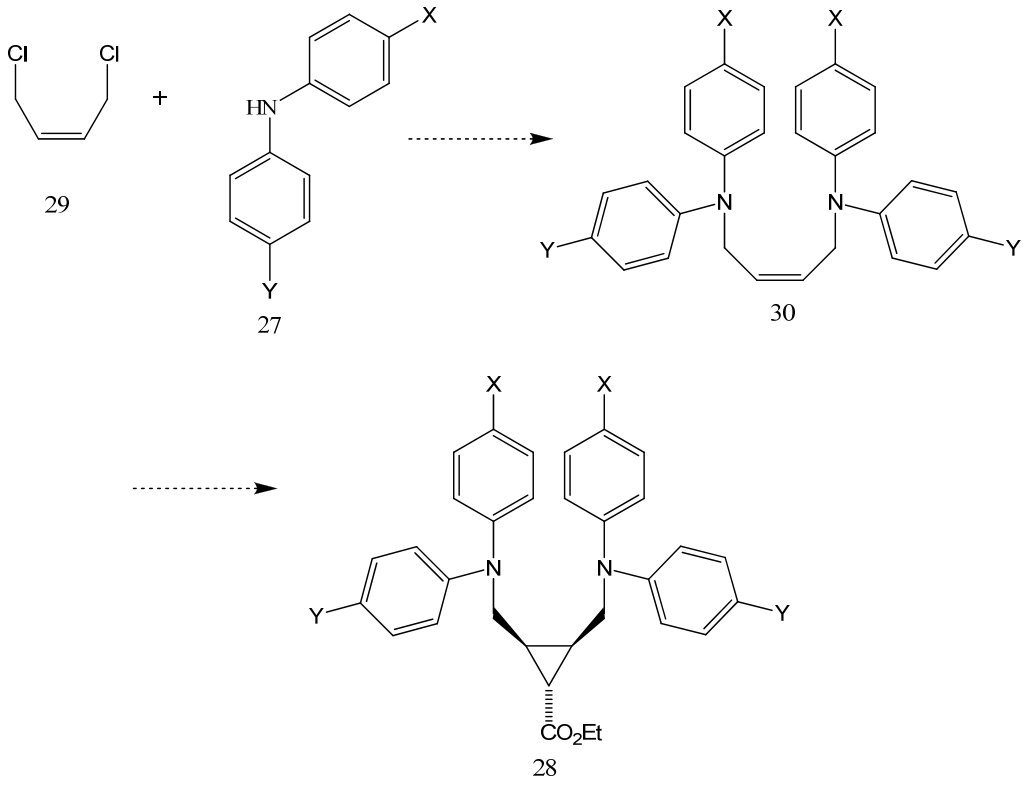


Figure 10

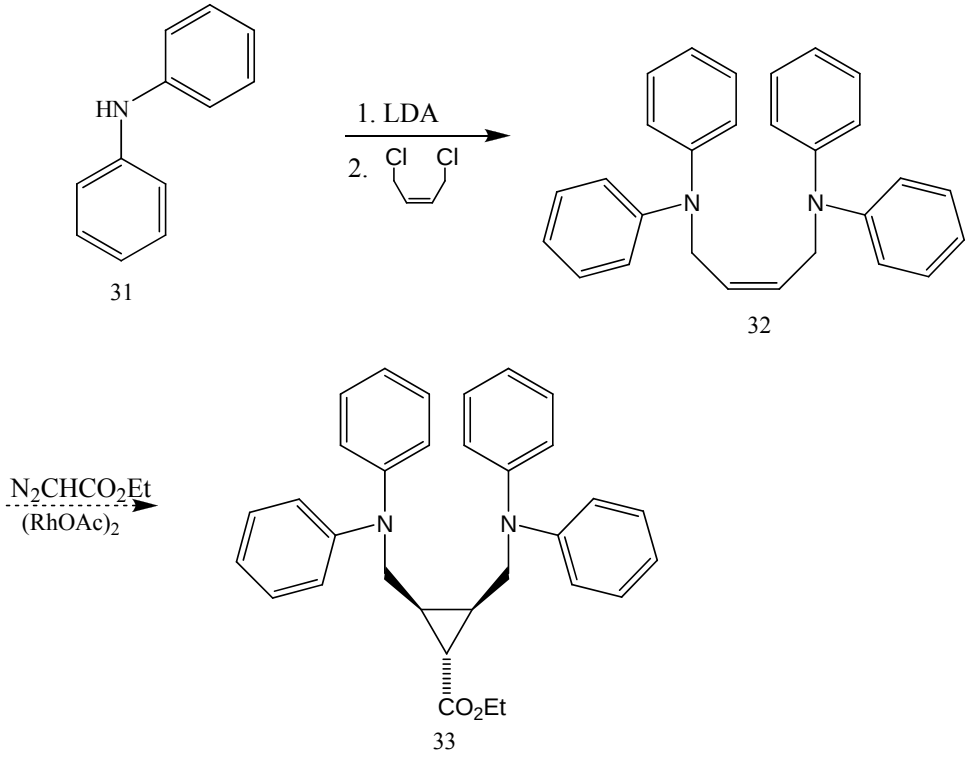


Figure 11

ii. Synthesis of the Mesylate

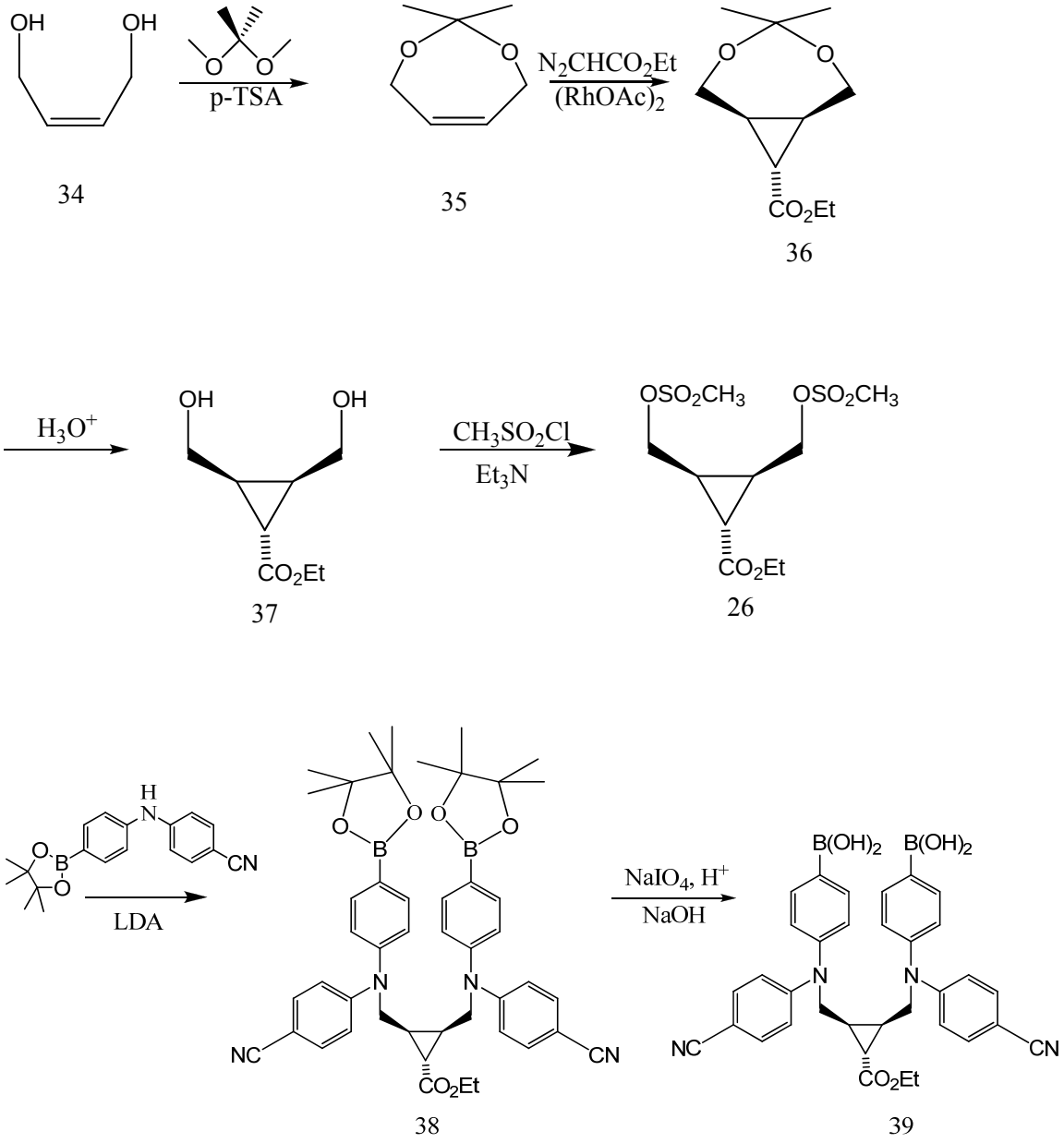


Figure 12

Figure 12 shows the overall synthetic route taken to arrive at the mesylate and then the subsequent attachment of the disubstituted diphenylamine. To be able to effectively cyclopropanate, it is necessary to protect the alcohols. For this purpose, cis-1,4-dihydroxybutene was reacted with 2,2-dimethoxypropane to yield the protected diol, cis-4,7-dihydro-2,3-dimethoxyepin (**35**). Compound **35** was obtained in 89% yield with methanol being the major byproduct. No purification was needed to continue because methanol was easily removed in vacuo after the product was neutralized and extracted.

The cyclopropanation step that followed is what determined the overall yield of the synthesis.

The literature contains a great number of examples of cyclopropanation reactions that are catalyzed by rhodium (II) acetate. These are usually quite tedious and yields are usually low, because the second reagent, ethyl diazoacetate, readily dimerizes to form dimethyl maleate and dimethyl fumerate. In an attempt to subdue this dimerization, the cyclopropanation was attempted using a copper catalyst. After several attempts, it was determined that no cyclopropanation was occurring.

Excess quantities of ethyl diazoacetate need to be used when cyclopropanating in the traditional manner because the reagent readily dimerizes. The problem is that it is an expensive reagent when purchased. In an attempt to reduce the cost of the cyclopropanation of the protected diol, ethyl diazoacetate was synthesized in the lab using sodium tetraborate decahydrate, sodium nitrite and ethyl glycine hydrochloride.[39]

The traditional cyclopropanation route, that uses ethyl diazoacetate as the reagent and rhodium (II) acetate as the catalyst, was used to obtain that desired cyclopropane product (**36**). As previously mentioned, the major problem of the reaction is the formation of the isomers dimethyl maleate and dimethyl fumarate from the dimerization of ethyl diazoacetate. This dimerization makes the reaction tedious; ethyl diazoacetate has to be added slowly over a long period of time in order to minimize the amount of the reagent in the flask at any given time. This slow addition lowers the yield of the byproducts and therefore increases the yield of the desired product **36**. Ethyl diazoacetate was added in 10 to 15 drop portions every 5 minutes over a period of six and a half hours. Even after the slow addition, the amount of the dimerized isomers was significant. It is difficult to gauge just how much of the final product is dimethyl maleate, dimethyl fumarate, the cyclopropane product or the unreacted protected diol due to the second problem that arises from dimerization.

In addition to lowering the yield of the desired product, dimerization of ethyl diazoacetate causes a problem in the purification. Because of the similarity in polarities it is difficult to separate dimethyl fumarate from the unreacted starting material. These two byproducts come off the column as a yellowish liquid mixture. Dimethyl maleate and the desired product **36** are also inseparable by column chromatography because of their similar polarities. They come off the column in same kind of yellowish liquid mixture. The fact that there is no separation makes it difficult to determine just how much yield is achieved through the cyclopropanation reaction although ^{1}H-NMR data confirms that **36** is the major component of the mixture in a ratio of about 3:1. Luckily, dimethyl maleate does not interfere with the deprotection of alcohol to form **37**, and therefore, the mixture obtained can be used in the next step without further purification.

The next step in the synthesis is the deprotection of the alcohol to produce a diol. This was achieved by refluxing compound **36** with hydrochloric acid and ethyl acetate. After the subsequent workup, the crude product was purified by column chromatography using 1:1 and then 3:1 Ethyl Acetate to hexanes. ^{1}H-NMR data obtained after the column was run showed that the product obtained was pure diol. The impurities form the earlier steps were successfully removed in the purification step. The mesylate (**26**) was then synthesized by treating the diol with triethylamine and methanesulfonylchloride in dichloromethane. The reaction only proceeded to completion when four equivalents of methanesulfonyl chloride were used as opposed to two, and excess triethylamine was also used. This suggests that maybe the diol was not completely pure and an impurity was consuming some of the methanesulfonyl chloride. After a subsequent work up, the crude product was purified by column chromatography, and it yielded yellow oil. This yellow oil was stored under refrigeration because the mesylate may not be entirely stable.

iii. Aryl Halide Amination

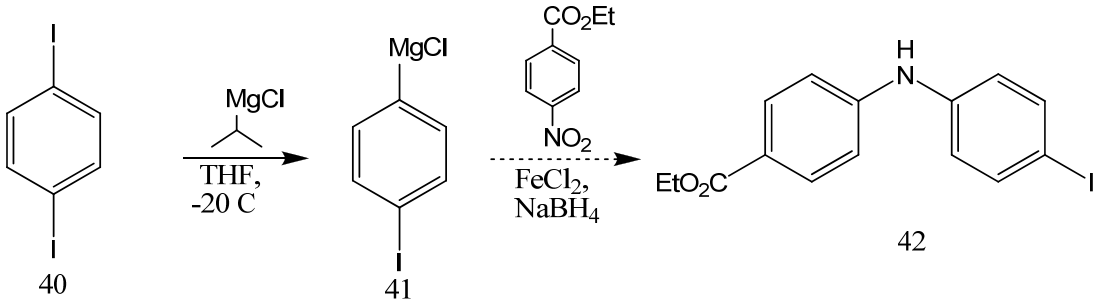


Figure 13[40]

Initially, a coupling reaction involving a Grignard reagent was attempted. Grignard reagent **41** was formed from **40** under dry conditions and nitrogen. The reagent **41** was then reacted with ethyl-4-nitrobenzoate ester in an effort to form **42**, the desired product. NMR data showed that the major product of the reaction was iodobenzene. This suggests that the first step of the reaction formed **41** as planned, but that the reaction with ethyl-4-nitrobenzoate ester was not successful. Many attempts were taken to ensure complete dryness of the reaction up to the workup step, involving the reaction with iron chloride and sodium borohydride, but the desired product **42** was never synthesized. The expected byproducts, iodobenzene and 4-iodophenol, were isolated indicating that some reaction did take place, but desired product **42** was never found.

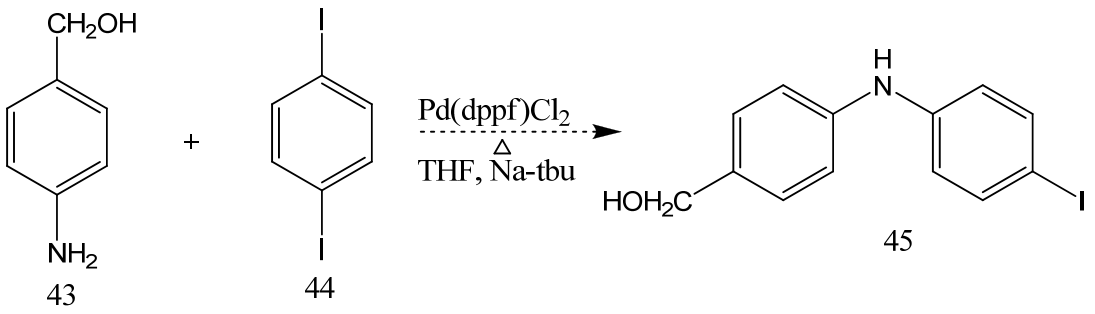


Figure 14

A more traditional approach to the type of desired coupling reaction is using a palladium catalyst. In this approach the aryl halide amination catalyst, palladium (1,1'-bis(diphenylphosphino)ferrocene) chloride ($Pd(dppf)Cl_2$), is used to obtain a secondary aryl amine (**45**) by coupling an aryl halide (**44**) with a primary amine (**43**). Although the literature on the subject suggested that this reaction should work, product **45** was never isolated using this synthetic route. Even after the reaction ran for several days while heating to about 150˚C, there was no conversion from starting material to products. The reaction was reattempted using different primary amines but the end result was always starting material. The literature also made reference to the same kind of reaction using different ligands to the palladium catalyst. Initially dppf was used as a ligand; the same kind of reaction was then run using triphenyl phosphine as a ligand. The reaction was run at 150˚C, but had the same end. It did not seem possible to form the disubstituted diphenylamine this way, so it was decided to just introduce groups to commercially available diphenylamine.

Compound **46** was synthesized from **31** by simple alkyl substitution at the para position of one of the benzene ring using benzyltrimethyl ammonium dichloroiodate as the iodization agent. The reaction was allowed to stir overnight at room temperature, and its end result, as seen in ^{1}H-NMR, was a 1:1:2 mixture of diphenylamine, 4,4'-diiododiphenylamine and N-phenyl-4-iodoaniline. Although the desired product was the main component of the mixture, purification proved to be complicated. Initially, column chromatography was attempted using 30:1 hexanes: ethyl acetate. The column was only able to partially purify the product. Because the polarity of a compound does not drastically change by replacing a hydrogen with an iodine, it was impossible to separate diphenylamine from N-phenyl-4-iodoaniline; the same difficulty was observed when trying to separate N-phenyl-4-iodoaniline from 4,4'-diiododiphenylamine.

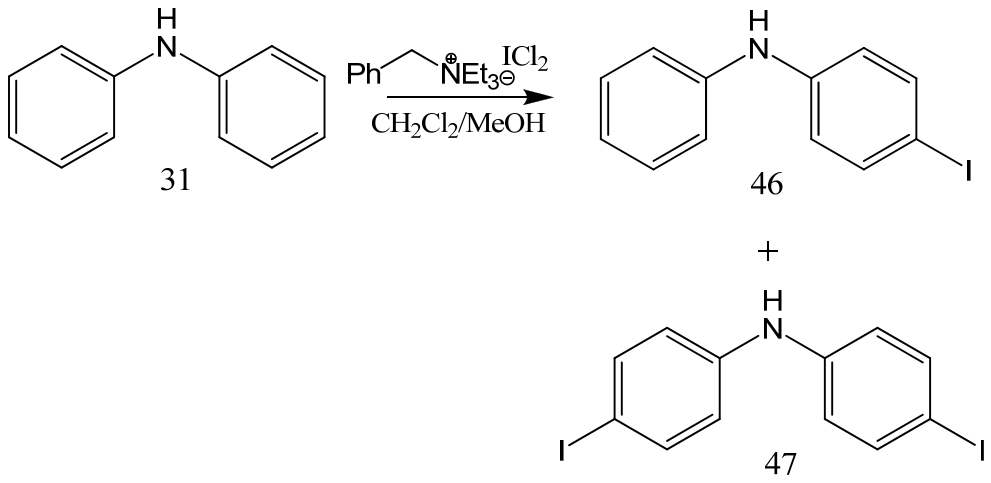


Figure 15

Although the introduction of iodine to the system does not drastically change the polarity, it has a significant effect on both the boiling and melting point of the compound. Diphenylamine (**31**) has a melting range of 48˚ C to 50˚ C. The melting point of the monosubstituted product, N-phenyl-4-iodoaniline (**46**), is twice as high; the range is 100˚ C to 102˚ C. Introduction of a second iodine leads to compound **47** having a melting point range of 125˚ C to 126˚ C. From that information, it is logical to assume that the boiling points of compounds **31, 46** and **47** will most likely be drastically different from each other due to the alkyl substitution. Taking advantage of this difference in boiling points is the only feasible way to get separation between diphenylamine and the desired compound **46**. The literature boiling point of diphenylamine is 308˚ C; it can be depressed with reduced pressure. A cold finger distillation was run at 0.05 torr, and diphenylamine boiled off at approximately 95˚ C. The white product formed a layer on the apparatus, and only the 2:1 mixture of **46**:**47** remained. Purification of this heavily colored product was achieved through simple recrystallization with ethyl ether: hexanes to afford compound **46** as white crystals.

^{1}H-NMR data for the iodination product cannot accurately distinguish the desired product from the byproduct and starting material so it was necessary to turn to ^{13}C-NMR data to interpret that the product was in fact pure. Since one aromatic ring on the monosubstituted diphenylamine (**46)** is indistinguishable from diphenylamine and the second is indistinguishable from 4,4'-diiododiphenylamine (**47**), the ^{1}H-NMR spectra for the crude 2:1:1 mixture of the products makes it seem like there is pure mono-substituted product. For this reason it was necessary to turn to carbon NMR which is different for each of the three compounds. Carbon NMR was used to determine that the white crystals isolated from the reaction above were not entirely free of diphenylamine. Carbon NMR data is not as quantitative as hydrogen NMR in integration, but at a rough estimate the sample still contained about an eighth of an equivalent of diphenylamine. It was decided

that a second functional group should be introduced to the para position of the second benzene ring in order to facilitate purification.

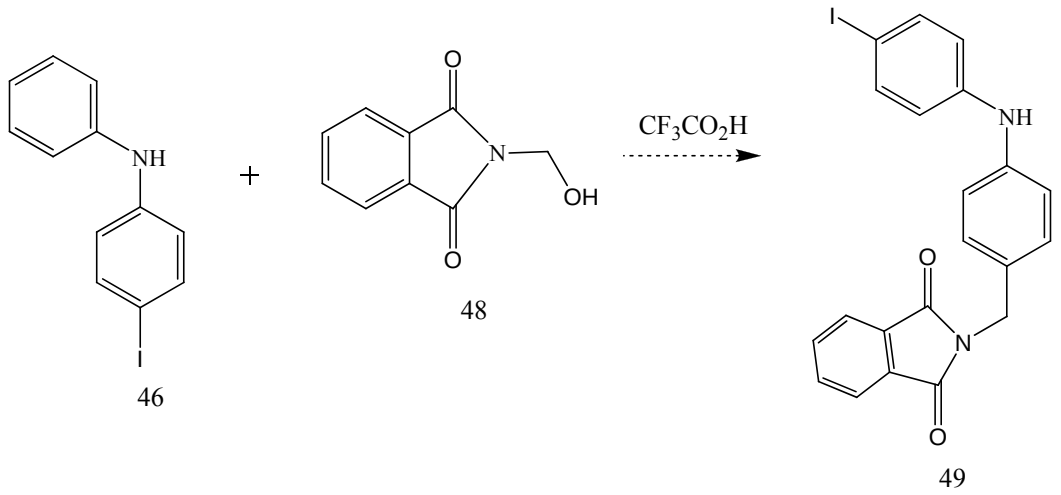


Figure 16

In an attempt to try to bypass this complicated purification, a series of Mannich reactions were attempted to introduce a second bulkier group at the para position of the second benzene ring. A Mannich reaction was prepared to introduce N-hydromethylphthalimide **48** to the crude product from the earlier iodoination step. The problem with this type of reaction is that it usually requires a strong acid catalyst. It would be impossible to use such a catalyst without protonating the amine group. Trifluoroacetic acid was used to run the reaction as it could be sufficiently acidic to catalyze the reaction yet not acidic enough to totally protonate the amine. However no reaction was observed suggesting that trifluoroacetic acid is not strong enough to run an effective Mannich reaction. It was later attempted using boron trifluoride diethyl ether complex, but this was also not an effective acid. A similar reaction was attempted to add a chloromethyl group at the desired position, but that reaction also failed. An attempt was made to add a diethylamine R group at the second para position of compound **46**. The diphenylamine was reacted with diethylamine, hydrochloric acid and formaldehyde to try to produce the disubstituted product but this reaction also failed. At this point an alternative coupling reaction was attempted.

Coupling an aryl bromide with an amine seems to be the easiest and most logical approach to synthesizing the kind of diphenylamine product that is desired. After exhausting other options, a coupling reaction that uses palladium acetate as a catalyst, 1,2-bis(diphenylphosphino) ethane [DPPE] as a ligand and toluene as a solvent was attempted. Initially the reaction was attempted using aniline and bromobenzene as the reactants and xylenes as the solvent, but there was no conversion from starting materials. Xylenes were substituted for toluene because of their higher boiling point, but this

coupling seems to require toluene as a solvent. The process was then attempted using toluene at a lower temperature (100˚ C) than suggested by the literature, but there was only about 50% conversion to the product, diphenylamine; when the temperature was increased to110˚ C there was almost full conversion. Using aniline and bromobenzene to test the reaction helped us determine that it was highly sensitive to the use of toluene and a temperature of 110˚C or greater. We also discovered that the DPPE ligand is highly sensitive to the air, so extra precaution needs to be taken to seal the reaction from the atmosphere. Nitrogen was actually bubbled into the solution being heated, and the condenser that attached was then sealed to the air and connected to a bubbler.

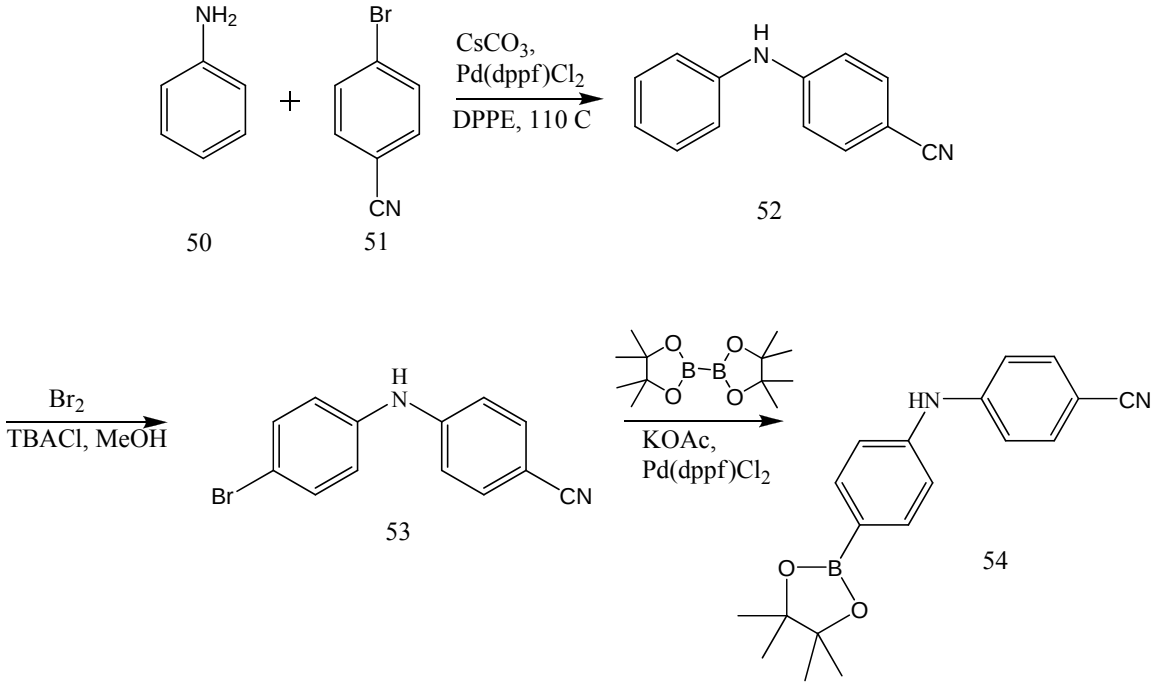


Figure 17

With the successful synthesis of diphenylamine from aniline and bromobenzene, it was decided that the coupling would be attempted using methyl-4-bromobenzoate and 4-iodoaniline as the reactants. There was only minimal conversion from starting materials to products even after taking the precautions described above, so we decided to change one of the starting reactants to see if we would achieve better conversion. Instead of 4-iodoaniline, aniline was used with the methyl-4-bromobenzoate. This time there was approximately 75% conversion from starting materials to product suggesting that the electron donating effect of the iodine made the amine less reactive. Since having a nitrile group at the para position of one of the benzene rings would be more convenient for future reactions, it was decided that we would use 4-bromobenzonitrile (**51**) instead of methyl-4-bromobenzoate as a reactant. This pair of reactants actually produced complete conversion from starting material to product (**52**) showing that the more electron

withdrawing the aryl bromide the better the conversion rate. An attempt was made to use 4-bromobenzonitrile with 4-iodoaniline but there was no conversion of starting material, so proceeding with the synthesis via 4-(phenylamino)benzonitrile (**52**) was our most appealing option.

Although it was partially purified, and useable in further synthesis, a pure sample of 4-(phenylamino)benzonitrile (**52**) could not be obtained in large yield. Some pure product did precipitate out of solution as needle like white crystals after sitting for approximately a day, but the majority of the sample remained in solution. Aniline (**50**) which was present in excess during the reaction was removed by extracting with concentrated sodium hydrogen sulfate solution and brine. Separation by column chromatography would have been very difficult since the two compounds have almost identical R_f values on TLC. The remaining material was run through a column using a 2:1 hexanes to ethyl acetate ratio. The result was an off-white to yellow solid. ^{1}H-NMR showed that the solid was still about 25% impurities, but it was decided that it was pure enough to use in the next step. Compound (**52**) was now ready for the introduction of a halide into the para position of the second benzene ring.

iv. Bromination

Introduction of a halide to the para position of the second benzene ring was the focus of the next set of experiments. It was initially attempted by using the same procedure as we did to iodinate diphenylamine (Figure 15), but the reaction yielded no product; only starting material remained in the flask. The withdrawing effect of the nitrile group must have lowered the electron donating effect of the amine enough to prevent the reaction from taking place. In order to try to get the reaction to go, heating at reflux was attempted. Even after several days no reaction had taken place, and it was decided that iodination would not be the best option, so attention was diverted to bromination.

Bromination seemed like the most logical approach to introduce the halide at the desired position. Bromine isn't as effective a leaving group as iodine, but it would be adequate for the subsequent reactions. A reaction was set up using a dichloromethane as a solvent and tributylammonium chloride and methanol as catalyst. Bromine was added dropwise to a flask containing 4-(phenylamino)benzonitrile (**52**) and the catalyst and solvent as described above. After approximately one hour there was complete conversion from **52** to 4-(4-bromophenylamino)benzonitrile (**53**). The crude compound was run through a column to produce a yellowish sticky solid. ^{1}H-NMR analysis showed that this product contained the same kind of aromatic impurities as the **52**. TLC analysis showed that the polarity of the brominated product was similar to that of both 4-(phenylamino)benzonitrile (**52**) and aniline (**50**) making further purification of the solid difficult. The product was pure enough to continue to the addition of the protected

boronic acid. The polar group should change the polarity enough to obtain a pure product.

v. The Boronic Acid

Since synthesizing a boronic acid is the goal of this research, an important step in the synthetic route is the replacement of the bromine atom with the protected boronic acid group. This substitution reaction is achieved by using a moderate base, potassium acetate, a palladium catalyst, [1, 1'-bis(diphenylphosphino)ferrocene] dichloropalladium(II), and a polar solvent, dimethyl sulfoxide (DMSO) with bis(pinacolato)diborane as the boron source. The more polar the solvent used the more smoothly the reaction will proceed. After running for approximately 9 hours at 80˚ C, the reaction yielded the protected boronic acid, 4-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenylamino)benzonitrile (**54)**. The crude reaction mixture was extracted with water to remove the high boiling solvent, DMSO, and purified by column chromatography. NMR data confirmed the conversion to boronic acid.

After the introduction of the boronic acid, modifications have to be made to have a molecule that can perform the task of a Continuous Glucose Monitor. First, the nitrile groups in the aromatic compound, would have to be reduced to amines. A fluorophore containing a carboxylic acid would then be attached to the amine via an amide bond. Once the fluorophores are attached, the two pieces can be combined. The boron could then be deprotected and converted to a boronic acid. Analysis on glucose binding and FRET for use with a Continuous Glucose Monitor can then be conducted on this bisboronic acid.

vi. Conclusion

The two individual pieces that compose the final desired molecule were synthesized. Using a widely known cyclopropanation technique, the mesylate was synthesized from a diol. The aromatic amine was synthesized by using aryl halide amination, bromination and a simple substitution. In the end, both ethyl (2RS, 3SR)-2,3-dimethylsulfonate-methylcyclopropane-carboxylate and 4-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenylamino)benzonitrile were successfully isolated and stored. Attachment of fluorophores followed by the combination of the two pieces and a subsequent conversion to a bisboronic acid should yield a product that can be useful in a Continuous Glucose Monitor.

III) Experimental

1,4-didiphenylamine-cis-2-butene (32) To a stirred solution of diphenylamine (5.5mmol, 0.85g) in anhydrous THF (10 ml) was added dropwise lithium diisopropylamide (1.5M in hexanes, 3.67 ml). The addition was done in an ice bath, and the reaction was allowed to stir for 45 minutes. A solution of cis-1,4-dichloro-2-butene (0.25 g, 2 mmol) in anhydrous THF (10 ml) was added dropwise and allowed to stir overnight.[41] The crude reaction mixture was diluted in water (3 ml) and then extracted with diethyl ether (3 X 3ml). It was then dried over $MgSO_4$ and concentrated in vacuo. It was purified by column chromatography (30:1 hexanes: ethyl acetate) to give a white powder. ^{1}H-NMR ($CDCl_3$, 300MHz): 7.25 (t, J= 1 Hz, 8H), 6.97 (m, 12H), 5.74 (t, J= 1.3 Hz, 2H), 4.34 (d, J= 1.3 Hz, 4H)

Ethyl Diazoacetate To a solution of 18.9 g (135 mmol) of sodium tetraborohydrate in 380 ml of water was added 98.2 (1423 mmol) of sodium nitrite and 190 g (1355 mmol) of ethyl glycine hydrochloride. The solution was warmed to 20˚ C. To the aqueous solution was added 440 ml of toluene and the reaction mixture was cooled to 0˚ C in an ice bath. A phosphoric acid solution (18.9 g in 930 ml of water) was added slowly. It was allowed to react for several minutes. The reaction was than extracted with water and sodium bicarbonate solution. It was dried over $MgSO_4$ and concentrated in vacuo. No further purification was needed for use in cyclopropanation reaction. ^{1}H-NMR ($CDCl_3$, 300MHz): 4.08 (s, 1H), 4.25 (q, J= 4 Hz, 2H), 1.28 (t, J= 4 Hz, 3H)

Cis-4,7-dihydro-1,3-dimethoxyepin (35) To a solution of 2-butene-1,4 diol (1.5 g, 17 mmol) in 2,2-dimethoxypropane (20.8 ml, 170 mmol), p-toluenesulfonic acid (0.15 g, 5 mol%) was added with stirring. After 15 minutes the mixture was diluted with aqueous sodium bicarbonate (0.85 mmol) and was extracted with diethyl ether (1 x 50 ml and 2 x 25 ml). The organic phase was separated, dried and concentrated in vacuo to yield a colorless to yellow product which was used in the next step without further purification. ^{1}H-NMR ($CDCl_3$, 300MHz): 5.63 (t, 2H), 4.23 (d, 4H), 1.41 (s, 6H)

Ethyl (2SR, 3RS)-2,3-dimethoxyepin-methylcyclopropane-carboxylate (36) To a solution of cis-4,7-dihydro-1,3-dimethoxyepin (7.04 g, 55 mmol) in CH_2Cl_2 (20 ml) was added $(RhOAc)_2$ (250 mg, 5 mol%). The resulting mixture was allowed to stir vigorously at reflux while a solution of ethyl diazoacetate (9.47 g, 83 mmol) in CH_2Cl_2 (10 ml) was added drop wise over a period of 5-6 hours. After the addition was completed, the reaction was allowed to stir for 15 minutes followed by cooling to room temperature. The mixture was filtered and concentrated in vacuo, and the residue was purified by column chromatography (AcOEt/hexanes 1:10 and then 1:5) to afford compound **2** as a mixture with dimethyl fumerate. The product was used in the next step without further purification. ^{1}H-NMR ($CDCl_3$, 300MHz): 1.1-1.3 (m, J= 7.1 Hz, 9H), 1.65 (bs, 2H), 1.85

(t, J= 3.5 Hz, 1H), 3.70 (dd, J= 12 Hz, 2H), 4.0-4.1 (m, J= 7.1 Hz, 2H), 4.1-4.2 (m, J= 4 Hz 2H)

Ethyl (2RS, 3RS)-2,3-dihydroxy-methylcyclopropane-carboxylate (37) To a solution of ethyl (2RS, 3SR)-2,3-dimethoxyepin-methylcyclopropane-carboxylate (3.65 g, 17 mmol) was added 0.5 N HCl/EtOH (10 ml, 5 mmol) and the solution was allowed to reflux for 2-3 hours at which time the solvent was removed in vacuo. The residue was taken up again in EtOH (20 ml), and the resulting solution was neutralized with solid $NaHCO_3$. The mixture was filtered and then concentrated to afford crude product which was purified by column chromatography (AcOEt/hexanes 1:1 and then 3:1) to give the product as colorless oil. ^{1}H-NMR ($CDCl_3$, 300 MHz): 1.23 (t, J= 2.3 Hz, 3H), 1.49 (bs, 1H), 1.86-2.00 (bs, 1H), 3.30-3.45 (bs, 2H), 4.06-4.14 (m, J= 2.3 Hz, 4H)

Ethyl (2RS, 3SR)-2,3-dimethylsulfonate-methylcyclopropane-carboxylate (26) To a solution of ethyl (2RS, 3SR)-2,3-dihydroxy-methylcyclopropane-carboxylate (0.39 g, 2.24 mmol) in CH_2Cl_2 (20 ml) at 0◦C was added triethylamine (0.68 g, 6 mmol). Then methanesulfonylchloride (0.656 g, 2.5 mmol) was added drop wise over a period of 15 minutes. After the addition was complete, the reaction was allowed to stir for 2-3 hours during which time the reaction was monitored by TLC (AcOEt/hexanes 1:3). The reaction mixture was washed with 1N HCl (5ml, 5mmol) and saturated $NaHCO_3$ solution (5ml). The solution was dried over $MgSO_4$ and concentrated in vacuo to afford a yellow solid product. ^{1}H-NMR ($CDCl_3$, 300MHz) 1.25 (t, J= 1.9 Hz, 3H), 1.84 (t, J= 3 Hz, 1H), 2.02-2.06 (m, 2H), 3.06 (s, 6H), 4.08-4.16 (m, 4H), 4.41-4.46 (m, J= 1.9 Hz, 2H)

Benzyltrimethylammonium dichloroiodate Bleach (5.25% NaClO, 480 ml, 350 mmol) was added to sodium iodide (52.5 g, 350 mmol) and concentrated sulfuric acid (120 ml) contained in an Erlenmeyer flask. The solution was then gradually introduced into a solution of benzyltrimethylammonium chloride (700 mmol) in water (220 ml). The resulting light yellow precipitate was collected by filtration, washed with water (100 ml, 3X) and dried in vacuo for 12hrs to afford light yellow crystals.[42]

N-phenyl-4-iodoaniline (46) Diphenylamine (1.69 g, 10 mmol), benzyltrimethyl ammonium dichloroiodate (3.5 g, 10 mmol) and sodium bicarbonate (1.69 g, 20 mmol) were added to a round bottom flask equipped with a magnetic stir bar, and flushed with nitrogen. A 20 ml 1:1 solution of dichloromethane: methanol was also flushed with nitrogen and added to the round bottom flask. The solution was allowed to stir overnight. The solution was poured into 20 ml of water and extracted (3x10 ml) with water. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The product was then partially purified by column chromatography (AcOEt/hexanes 1:30) to afford a 1:1:2 mixture of diphenylaniline:diiododiphenylamine:N-phenyl-4-iodoaniline.[43] ^{1}H-NMR ($CDCl_3$, 300MHz) 7.47(d, J= 2.1 Hz, 2H), 7.27 (d, J= 4 Hz, 2H), 7.05 (d, J= 4.1 Hz, 2H),

6.94 (t, J= 4 Hz, 1H), 6.81 (d, J= 2.1 Hz, 2H), 5.6 (bs, 1H); ^{13}C-NMR ($CDCl_3$, 300MHz) 143.4, 138.3, 129.7, 122.1, 119.6, 118.8, 82.4

4-(phenylamino)benzonitrile (52) A solution of palladium acetate (5 mol%, 11 mg) and 1,2-bis(diphenylphosphino)ethane (10 mol%, 40 mg) in 5 ml of toluene is heated at 110˚ C for 10 minutes in a round bottom flask. Then benzonitrile (1 mmol, 0.103 g), aniline (2 mmol, 0.187 g) and cesium carbonate (1.1 mmol, 0.358 g) were add to the round bottom flask. Nitrogen was then bubbled through the solution and the condenser attached was sealed off to the air. The reaction was allowed to run for approximately 36 hours at 110˚C which point it was cooled to room temperature and concentrated in vacuo. The crude product was taken up in ether (50 ml) and extracted with 2M $NaHSO_4$ (1x25 ml, 1x15 ml) and brine (1x15 ml). The product was concentrated in vacuo and then purified by column chromatography (3:1 hexanes: ethyl acetate) to afford an off white to yellow solid. ^{1}H-NMR ($CDCl_3$, 300MHz) 7.51 (d, J= 3 Hz, 2H), 7.43 (t, J= 2.5 Hz, 2H), 7.24 (d, J= 2.5 Hz 2H), 7.18 (t, J= 2.5 Hz, 1H), 7.05 (d, J= 3 Hz, 2H), 6.63 (bs, 1H); ^{13}C-NMR ($CDCl_3$, 300MHz) 148.0, 139.9, 133.6, 129.4, 123.6, 120.9, 119.9, 114.7, 100.7

4-(4-bromophenylamino)benzonitrile (53) Under nitrogen, to a solution of 4-(phenylamino) benzonitrile (1 mmol, 0.194 g), tetrabutylammonium chloride (0.1 mmol, 0.028 g) and methanol (0.1 ml) dissolved in dichloromethane (1 ml) was added dropwise, a solution of bromine (1 mmol, 0.16 g) dissolved in 1ml of dichloromethane. At room temperature, it was allowed to stir for an hour at which point it was dissolved in ether (5ml) and extracted with aqueous sodium bicarbonate (3x5 ml). It was dried over $MgSO_4$ and concentrated in vacuo. It was purified by column chromatography 3:1 (hexanes: ethyl acetate) and the result was a light brown powder. ^{1}H-NMR ($CDCl_3$, 300MHz) 7.48 (d, J= 2.9 Hz, 2H), 7.44 (d, J= 2 Hz, 2H), 7.03 (d, J= 2.9 Hz, 2H), 6.96 (d, J= 2Hz, 2H), 6.17 (bs, 1H); ^{13}C-NMR ($CDCl_3$, 300MHz) 147.4, 139.2, 133.5, 132.2, 122.1, 119.7, 115.0, 101.4

4-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenylamino)benzonitrile (54) A solution of bis(pinacolato)diboron (1.1 mmol, 0.278 g), $Pd(dppf)Cl_2$ (5 mol%, 0.024 g), 4-(4-bromophenylamino)benzonitrile (1 mmol, 0.274 g) and potassium acetate (3 mmol, 0.294 g) in DMSO (4 ml) was heated to 80˚ C under nitrogen. After 9 hours the heat was removed and the reaction was allowed to cool to room temperature. It was then dissolved in ether (10 ml) and extracted with water (3x10 ml). It was dried over $MgSO_4$ and concentrated in vacuo. Purification was carried out using 1:1 ethyl acetate: hexanes and it afforded yellow oil. ^{1}H-NMR (DMSO, 300MHz): 7.58 (d, J= 2.8 Hz, 2H), 7.54 (d, J= 2.5 Hz, 2H), 7.10 (d, J= 2.8 Hz, 2H), 6.98 (d, J= 2.5 Hz, 2H)

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