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Application of click chemistry in modification of water soluble calix[4]arenes

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ABSTRACT

Click chemistry is a constantly developing field mostly because of the wide application of the reaction products from organic chemistry, through polymer chemistry and bioconjugation to drug development Calixarenes are group of macrocyclic oligomers with properties that find applications in biochemistry and drug delivery among many others. Click chemistry has already been applied in certain modifications of calixarenes and it make sense to pursue this path for several reasons. Calixarenes are generally synthesized by condensation of phenols and formaldehyde in the presence of base and they result in low to moderate yields. Click chemistry on the other hand results in very high yields, therefore the its application in modification of calixarene allows for minimal loss of the reagents. The following report presents preliminary data for the modification of the 5,11,17,23-tetracarboxylic acid-calix[4]arene. The scope of this work represents the first part of the multi-step project in modification of calix[4]arenes and the investigation of their potential biological applications.

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INTRODUCTION

Click chemistry is a term that was introduced by K. B. Sharpless back in 2001. Along with his coworkers, he presented the click chemistry as a simple and efficient method of connecting together small molecules by the use of heteroatom link (mostly N, O, and S). There are several criteria they described that need to be met in order to classify a reaction as "click reaction"[1]. To name a few of these criteria, the reaction cannot be specific to only a small group of compounds, but it must be wide in the scope, also should also require simple reaction conditions and readily or commercially available reagents. It must be modular and it must result in high or very high yields. Byproducts of the reaction (if any) should be removable from the mixture by nonchromatographic methods and also the purification of the product should be relatively easy and nonchromatographic as well. For that purpose such techniques as distillation or recrystallization can be utilized. Click reaction should also be stereospecific and obtained product should be stable under physiological conditions. Generally, click reactions are irreversible.

There are several known reactions where carbon – heteroatom bonds are formed that can be included in click reactions category:

- Epoxidation reaction – formation of three membered cyclic ether,
- Dihydroxylation – a reaction, where an alkene is converted to a vicinal diol,
- Aziridination – a conversion of an alkene into a three membered ring containing an amine group. They visually resemble epoxides, because of the structure of the three membered ring,
- Sulfenyl halide addition – used for formation of RS-O and RS-N bonds,
- Staudinger ligation – conversion of azides to primary amines
- Michael addition – 1,4- addition, a nucleophilic addition reaction of enolates to enones,
- 1,3- Dipolar cycloaddition – formation of a five-membered ring, by reaction of dipolarophile with a 1,3- dipolar compound,

- Diels- Alder – a reaction of diene with an alkene, also called a dienophile, resulting in formation of six membered ring that contains an alkene,
- Nucleophilic substitution reactions that include ring opening reactions of such compounds as epoxides, aziridines or episulfonium ions,
- Carbonyl chemistry – formation of such compounds as amides, hydrazones, aromatic heterocycles, ureas, thioureas and oxime ethers.

APPLICATIONS OF CLICK CHEMISTRY

Click Chemistry in Drug Development

The application of click reactions in drug discovery is of special interest, because it provides simple and efficient methods for synthesis of important compounds, often simplifying complicated multi-step and low yielding series of reactions. As examples of drugs synthesized by this method we can include peroxisome proliferator-activated receptor γ (PPAR-γ) agonists for the treatment of type II diabetes synthesized by Zhang and coworkers[2] (Figure 1 top left). Another example is an enzyme-bound inhibitor[3], where click reaction was carried in the presence of an enzyme acetylcholinesterase (AChE) and the triazole product of that reaction produced an inhibitor (Figure 1 right). Also, a product from reaction of 1,2,3-triazole with anti-HIV activity[4] (Figure 1 lower left).

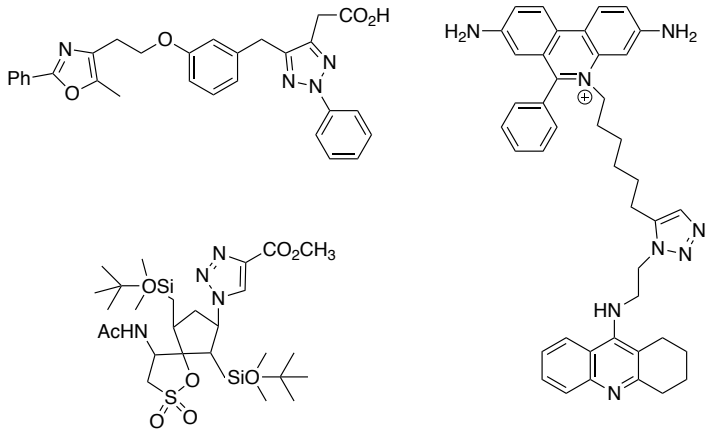


Figure 1. Examples of click reaction products with application in drug discovery.

Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis*, that every year kills between 1.2 and 1.8 million people worldwide. There are several available antibiotics and about thanks to them 85% patients are cured, however the mortality is still very high. In situ click chemistry was performed by Deprez and coworkers by probing the ligand binding domain of EthR, a mycobacterial transcriptional regulator known to control the sensitivity of *Mycobacterium tuberculosis* to several antibiotics[5]. Certain synthesized 1,4- sunstituted triazole compounds showed potent inhibition against EthR, therefore there is potential for them to be used for tuberculosis treatment.

Protein directed in situ chemistry enables medicinal chemists to explore the conformational space of a ligand-binding pocket and therefore it is a valuable design in drug delivery.

Click Chemistry in Bioconjugation

Bioconjugation or bioconjugate chemistry is a field of study, where biomolecules are linked together by either chemical or biological means. Any reaction that results in a stable covalent linkage between two biomolecules is considered as bioconjugation. The most common bioconjugations are coupling of small molecules to protein or protein- protein conjugations, amine coupling of lysine amino acid residues or photochemically initiated free radical reaction. Click reactions are of special interest in this field for several reasons. They are favorable because of the simple reaction conditions, high or very high yields and the lack of formation of toxic byproducts that can be potentially harmful to the living organism. Labeling of the proteins is also in the scope of interest when talking about click chemistry. Wang et al. were able to successfully label Cowpea mosaic virus (CPMV) with fluorescein[6]. It was done by first modifying the surface of the protein with either azide or alkyne and then by reacting it with fluorescein-bearing complementary groups.

Click Chemistry in Radiochemistry[7]

In radiochemistry, there is an inevitable physical decay of radioisotopes during the synthesis. For that reason, these reactions need to be designed in such way to maximize the reaction yield and to minimize time used for purification steps. As examples of these short-lived

isotopes suitable for radiolabeling reactions we can use ^{11}C, with half-life of about 20 minutes and ^{68}Ga, with half-life of about 68 minutes. Because reaction time is of essence in this field, click chemistry seems to be ideal. One of the first and still widely used applications of click chemistry in radiochemistry is radiolabeling with "clickable" prosthetic groups. These prosthetic groups are radiolabeled reactive small molecules that can be appended to biomolecules under benign conditions. Copper-Calatlyzed Azide-Alkyne Cycloaddition (CuAAC) is often the reaction of choice over the ruthenium catalyzed one, mostly because of the unfavorable conditions necessary to carry the latter one (organic solvent, elevated temperature and inert gas atmosphere). Also, CuAAC reaction does not result in formation of 1,5- disubstituted 1,2,3- triazoles, compounds that are metabolically active and can be degraded by enzymatic N^3 oxidation to produce highly reactive and potentially toxic metabolites. Although many different radiolabeled group have been a subject of research, click reactions have been most commonly applied for creation of ^{18}F-labeled prosthetic groups. For this purpose, different reactions were utilized, including CuAAC, strain-promoted azide-alkyne cycloaddition and inverse electron demand Diels-Alder among several others. One of less known click reactions in this field was utilized by Zlatopolskiy[8] et al. They synthesized a reactive nitrone using ^{18}F-fluorobenzaldehyde and phenylhydroxylamine and subjected it to [3 + 2] cycloaddition with a maleimide. The reaction took less than 15 minutes, however it was conducted at 80 ^{o}C, what obviously results in limitations for it when considering its application in labeling of biomolecules.

Click Chemistry and Polymers[9]

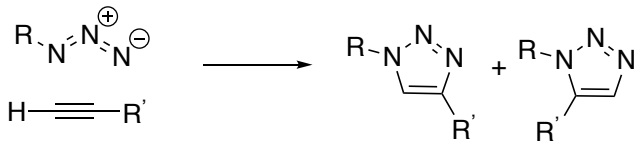
Out of all click reactions, copper- catalyzed azide- alkyne cycloaddition is the one that is most widely used. The application of click chemistry in polymer science is already known for the preparation of such materials as: terminal- and pendant- functional polymers, multi-block copolymers, micells, hyperbranched dendritic polymers, gels and polymers conjugated to nanomaterials among many others. One of the most common click reactions in polymerization is the use of functional initiators or transfer agents and the post polymerization modification of end groups, resulting in terminal functionality of the polymers. Click chemistry is also useful in preparation of block copolymers, especially in the cases when conventional polymerization

routes are not applicable. The first successful synthesis of block copolymers by combining ATRP (atom transfer radical polymerization) and copper catalyzed click reaction was achieved by conjugation of an azide with alkyne terminated polymers. It was done by incorporation of alkyne and azido groups using alkyne- modified ATRP initiator, and nucleophilic substitution of terminal bromine groups, respectively. The reaction proceeded successfully and no unreacted homopolymers were observed. Besides of the synthesis of the linear polymers, click chemistry is also known to be used for preparation of more complex structures, such as star, brush, graft and dendritic polymers. In the case of the first three types, click chemistry is usually utilized for linking pre-synthesized arms to a common core or adding the side chains to preexisting backbone. In the synthesis of dendrimers, click chemistry can be of special help because it allows to prepare hyperbranched macromolecules in one step, instead of a traditional time- consuming process in which the dendrimers are built one generation at the time. Gels, and especially hydrogels have also been prepared by click chemistry. They commonly have various applications in biomedical field and they can serve as scaffolds for tissue engineering, biosensors and responsive delivery systems. The click reactions are compatible with aqueous media and there are no side products to them and for those reasons it is expected that the interest in preparation of materials for biomedical applications will be only growing.

SYNTHESIS

Huisgen Reaction

There are several known chemical procedures that are classified as click reaction. Huisgen azide-alkyne 1,3-dipolar cycloaddition[10] (also called [3 + 2] azide-alkyne cycloaddition) that was introduced back in 1960's is one of them. This 1,3-Dipolar cycloaddition of aryl/alkyl azides with alkynes yields five-membered heterocycles. The reaction requires relatively high temperature, and it gives poor regioselectivity meaning that it results in the mixture of 1,4- and 1,5-disubstituted triazoles[11]. For this reason, the Huisgen reaction have many limitations and it is not applicable in drug discovery, biomedical or biomaterials field.

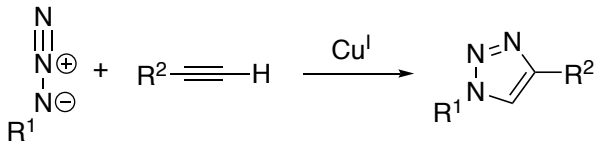


Scheme 1. Formation of 1,4- and 1,5-disubstituted triazoles (Huisgen reaction).

Because of the two reactions products resulting from Huisgen azide-alkyne 1,3-dipolar cycloaddition, there was a necessity of modification of the original reaction conditions.

Copper-Calatlyzed Azide-Alkyne Cycloaddition (CuAAC)

Sharpless and coworkers were able to modify those initial conditions designed by Huisgen and with addition of Cu(I) salt they were able to obtain the regioselectivity. Instead of the two 1,4- and 1,5-disubstituted reaction products, only one, 1,4-disubstituted 1,2,3-triazole was observed. This was a result of the use of Cu(I) catalyst. it is already known that copper catalysts have potential to lower the activation energy of the reaction and therefore they cause the reaction to be highly selective. Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAc) can be performed at lower temperatures than the original reaction and it is possible to perform it in a variety of solvents including the aqueous moieties. Copper (I) salts are also very favorable reagents compared to other metal catalysts because of their relative low cost and easiness of handling.

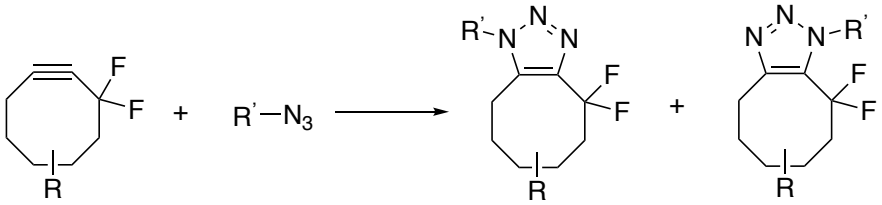


Scheme 2. CuAAC Reaction.

They have been widely used for terminal alkynes activation but besides of many advantages they also have some limitations, especially in such fields as bioconjugation and biological chemistry. Because of the toxicity of the coper metal, they are usually not compatible with living systems. To solve that issue, an alternative method for lowering of the activation energy for [3 + 2] cycloaddition was proposed by Codelli and coworkers[12].

Strain Promoted Azide- Alkyne Cycloaddition Reaction

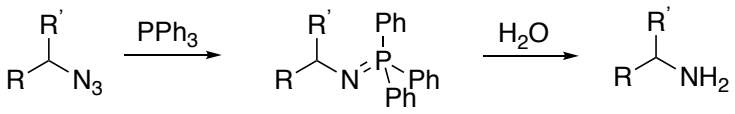
In their investigation of the click reaction, Codelli et al. used cyclooctynes- a molecules with intrinsically high strain. These highly strained compounds were then reacted with azides and the reaction resulted in a regioisomeric mixture of triazoles. They were able to carry the reaction under mild conditions that did not indicate toxicity. The purpose of this reaction was to avoid the toxicity observed in the reactions with use of copper catalysts, so it potentially can be utilized in living systems. The outcome however was only partially a success. They were able to design a click chemistry method that did not show any toxicity that is undesired when dealing with living organisms, but similarly like in Huisgen reaction, their design resulted in formation of two regioisomers, therefore its applications are limited, especially the compatibility with living systems.



Scheme 3. Strain promoted azide- alkyne cycloaddition reaction designed by Cedelli and coworkers.

Staudinger Ligation

Staudinger ligation[13] is another widely used type of click reaction. It was originally introduced in 1919 by Staudinger and Meyer. It utilizes PR_3 compounds, such as triphenylphosphine and water to convert organic azides to primary amines via an iminophosphorane intermediate. Staudinger ligation has been a reaction of interest because of its application in molecule labeling or even labeling of larger entities of biological importance.



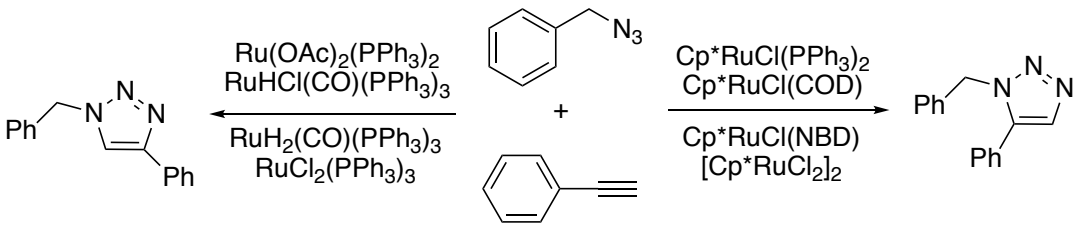
Scheme 4. Staudinger ligation.

Traceless Strudinger Ligation

Because of the high potential of the reaction in biological chemistry, it has been of interest of researchers to design modifications to the original method. Saxon and coworkers[14] reported the formation of the amide bond that uses the principle of the Staudinger reaction and they named it "traceless Staudinger ligation". And the biological potential of this modification is great, because the reaction does not result in formation of phosphine oxide. One of the suitable phosphine reagents that can be utilized in this reaction is diphenylphosphinemethanethiol.

Ruthenium-Calatlyzed Azide-Alkyne Cycloaddition (RuAAC)

Since 1,4- disubstituted 1,2,3-triazole is the only product formed during copper-catalyzed azide alkyne cycloaddition, there was a need for the development of another method to obtain the 1,5- disubstituted 1,2,3-triazole. Fokin[15] and coworkers demonstrated that by replacing the copper catalyst with ruthenium, formation of 1,5- disubstituted products is possible. However, 1,5- disubstitution is not the only possible product when using ruthenium as the catalyst in this reaction. Johansson and coworkers showed that by using different ruthenium catalysts, it is possible to get either 1,4- or 1,5-disubstituted triazole[16] exclusively, and not the mixture of regioisomers.



Scheme 5. Products of RuAAC reaction when various ruthenium catalysts are used.

CALIXARENES

Calixarenes are group of macrocyclic oligomers. They were first discovered in the second half of nineteenth century by Adolf von Baeyer. He heated *p*-substituted phenols with formaldehyde under either acidic or basic conditions and as the reaction product he received viscous compounds[17]. However, at that time, it was not possible for him to successfully characterize those compounds because of the quality of the analytical tools. About 50 years later, another scientist proposed the structure of the Baeyer's molecule[18] (Figure 2). The analytical tools that he was able to utilize again made the molecule characterization impossible. As we fast forward an additional 20 years, in 1970 Gutsche finally successfully characterized these molecules. He discovered that what was originally thought to be a single reaction product, was for a fact a mixture of cyclic tetramers, cyclic hexamers and cyclic octamers. He was also the one to give them the name "calixarenes" that we use today. In naming those compounds he saw similarities between their cup-like shape to the Greek vase, known as the calix crater. When naming calixarenes we need to distinguish the number of aromatic rings. Since they have been synthesized as tetramers, hexamers and octamers, the proper name includes a number inside brackets. Therefore a calixarene containing 4 monomers will be written as calix[4]arene etc. Gutsche was also able to establish experimental conditions and improve yields for these three products[19].

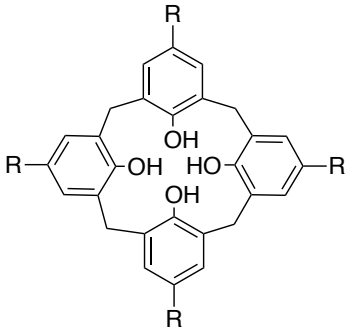


Figure 2. Structure of the calixarene proposed by Baeyer.

There are three distinct parts of the calixarene structure: the central part of the molecule that consists of the benzene rings connected with CH_2 links is called the annulus, Above the annulus, there is an upper rim and below it a lower rim. In the beginnings of the calixarene chemistry, before modifications to the original reactions were developed, only upper rim of the molecule was a subject of differentiation. It was possible by using different *p*-substituted phenols when reacting them with formaldehyde.

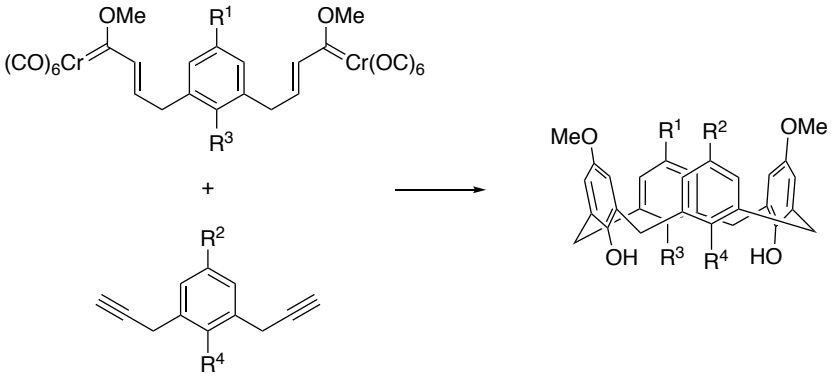
SYNTHESIS

The Original Synthesis

There are several different pathways for synthesis of calixarenes. Synthesis of monosubstituted calixarenes is relatively easy and it can be done by condensation of phenols with formaldehyde under basic conditions, and under elevated temperature[20]. The method that was originally designed by Baeyer and then modified by various groups depending on the molecules of interest. The exact reaction conditions and amounts of the base used vary for the synthesis of *p-tert*-butylcalix[4]arene, *p-tert*-butylcalix[6]arene and *p-tert*-butylcalix[8]arene. Also, this method has limitations because it requires *para*-substituted phenols. *Ortho*- and *meta*-substituted ones are not applicable. By using this method, it is not possible to obtain calixarenes with two or more different substituents on the upper and/or lower rim therefore there was a need for the development of a new reaction.

Synthesis of Unsymmetrical Calixarenes

Gopalsamuthiram and Wulff presented a novel method for synthesis of unsymmetrical calixarenes[21]. They synthesized a series of bis-propagryl benzene derivatives and then used those to prepare bis-carbene complexes. In their method, two of the calixarene's benzene rings are formed in a convergent method. Their results showed that this is a valid method for synthesis of unsymmetrical calixarenes as they were able to obtain desired products in relatively low yields that ranged from 22% to 40%.



Scheme 6. Convergent method for synthesis of unsymmetrical calixarenes presented by Gopalsamuthiram and Wulff. Conditions: reactions carried in 1,2-dichloroethane at 100 ^{o}C. 2.5 mM in bis-carbene complexes with 1.0 equiv. of bis-propagryl benzene derivatives.

Green Synthesis of Calixarenes

Green chemistry application in the synthesis of calixarene have been investigated. The conventional methods for the synthesis of these compounds usually require high temperature. Because of the conventional methods of heating used for that purpose, the reaction is relatively slow and the energy transfer into the reaction system is inefficient. One of the alternative methods is the use of irradiation with a microwave. The heating is more efficient in this case because the microwaves penetrate the container and deliver the energy directly to the reaction mixture. That would lead to reduction of the overall reaction time. Such reaction was conducted by Baozhi et al. They used formaldehyde, *p*-methylphenol and KOH to synthesize *p*-methylcalix[6]arene. Instead of using conventional heating which would cause the reaction time to be up to two hours, they subjected the mixture to microwaves for 2-8 minutes. Then the mixture was refluxed for 40 minutes using dimethylbenzene. The overall yield of the product was recorded as 82%[22]. The same technique was used for synthesis of *p-tert*-butylcalix[8]arene with appropriate alternations to the conditions and to irradiation time. By using this method, it is possible to significantly reduce the reaction time without a significant reduction of the formed reaction products.

Generally, the synthesis of calixarenes require large amounts of solvents what leads to the large amounts of waste. There were attempts to minimize the amounts of solvents as well as designs for solvent-free reactions. One of the examples for solvent-free conditions was used to synthesize calixresorcinarene, a derivative of calixarene. It was done by mixing aldehyde and resorcinol with acid catalyst. Even though all used reagents were in their solid forms, once they are mixed, they are able to form either a thick liquid or reach paste like consistency. The acid catalyst can be then removed by washing the product with water. Unlike in the reactions involving solvents, no heating is required in this case, however it requires consistent scouring. The results showed that by using this method, it is possible to efficiently synthesize calixresorcinarene when different aldehydes are used[23]. Also, solvent- free conditions require shorter reaction time and yield higher percentage of the product than conventional methods.

APPLICATIONS OF CALIXARENES

Calixarenes are relatively large molecules. They also have a quite unique structure and basket-like shape, and thanks to those properties, they have many different applications. Those calixarenes that have two or more electropositive atoms such as germanium or tin have various potential applications that include catalysis, molecular recognition and anion sensing[24]. Another possible use of calixarenes include the complexation of radionuclides such as uranium and cesium[25-26], ion-selective electrodes[27], phase-transfer agents[28-29], chiral recognition devices, stationary phases or drug delivery/ discovery. Their structure also enables them to host guest molecules thanks to their internal cavity. For simple calix[4]arene ethers that have rather open structure and their cavity is only able to bind to a very limited number of guest molecules. The modifications of upper and lower rim of the molecule enable it to accommodate cations, ions and even small molecules[30]. It is also possible to control the overall shape of the calixarenes by modification of the lower rim and/or intramolecular bridges[31] and therefore to control their potential use. There are four possible conformations for calix[4]arenes: the “cone”, with four phenyl rings looking into the same direction, “partial cone”, where one phenyl ring is inverted, “1,2-alternate” and “1,3-alternate”, where phenyl rings look up and down, alternating. Compared to four conformations of calix[4]arenes, eight different conformations are possible for calix[6]arenes and even more for calix[8]arenes. The most stable conformation for all calixarenes is the cone.

Calixarenes In Drug Delivery

Calixarenes are considered examples of third- generation of host- guest supramolecular chemistry. Their structure, flexible nature and potential for modifications at their basic core and rims makes them great candidates as drugs or drug delivery systems. Along with their derivatives calixarenes are already known to possess antibacterial, antifungal, antiviral and anticancer activities. Calix[4]arene substituted with methylenebisphosphonic and hydroxymethylenebisphosphonic acid groups was proven to have an inhibitory effect on protein tyrosine phosphatase 1B, a protein involved in insulin and leptin transduction, and therefore it has antidiabetic and antiobesity activities[32].

Calixarenes as Anticancer Drugs

Calixarenes and their derivatives have been studied for their potential anticancer activities. These compounds have been investigated by different research groups around the world and tested against various tumor cells. Then their activity was compared to the standard drugs used for cancer treatment. Some of the calixarene based compounds showed great potential as anticancer drugs especially against lumphoblastic leukemia and melanoma cells. Nasuhi et al. worked with calix[4]arene and they synthesized its derivative functionalized with four platinum (II) centers. When their compound was compared with carboplatin, a chemotherapeutic agent, the new compound showed better activity against non-small cell lung cancer, hepatocellular cancer and breast cancer. Recently Fahmy et al. proposed that 4-sulfocalix[4]arene can be used in anticancer drug delivery[33]. In their investigations, they concentrated on complexation of nedaplatin and *para*-4-sulfocalix[4]arene. Nedaplatin can be used for decreasing toxicities induced by cisplatin, used for cancer treatment. It can be especially beneficial for patients suffering from urothelial cancer, esophageal cancer, uterine cervical cancer as well as head and neck cancer[34]. Their results show that such complex is possible and it had relatively high stability constant. Also, it does not involve the penetration of nedaplatin within the host and the stability is mostly established due to hydrogen bonding between the oxygen atoms of calixarene sulfonate moiety and hydrogen atoms of nedaplatin ammonia ligands.

So far, there is not even one calixarene based compound that was approved by FDA in the United States. The US clinical trials database shows only one compound in Phase I study, a calixarene-based galectin-1 inhibitor with potential antiangiogenic and antineoplastic activity.

The host- guest properties of calixarenes can also be utilized in drug delivery. The cup- shaped molecules have potential to accommodate various chemical compounds by forming complexes through non- covalent bonding, van der Waal's interactions and hydrogen bonding. Anticancer drug, dinuclear platinum complexes with *para*-sulfonato-calix[4]arene were studied by Brown and coworkers[35]. The results of their investigation showed that besides of intramolecular and hydrophobic interaction, intermolecular hydrogen bonds between the calix[4]arenes and the dinuclear platinum compounds caused the stability of the host- guest complexes to increase

significantly. The activity of those complexes was tested against various cancer cells, and there were found to be active against one of the sublines of A2780 ovarian cancer cells which are cisplatin resistant cells.

Calixarenes as Electrochemical Sensors- Recognition Properties

The calix[4]arenes that have been esterified on their lower rim are known for their potential as electrochemical sensors. The calix[4]arene tetra ethyl ester, which was synthesized by McKervey[36] and coworkers is a commercially available compound that is used as sodium selective electrode. It is widely used in medical facilities for measuring sodium in blood. The same group also modified calixarenes that show potential as electrodes selective for potassium and cesium.

Low rim modified calixarenes containing such groups as carboxylic acids, amides, ketones or ethers show potential as complexing agents. Ungaro et. al were the first to investigate the properties of ethyleneoxy compounds[37], however their products showed only a modest degree of cation binding.

Calixarene-based molecules show cation recognition properties[38]. Of special interest here are crowned calixarenes. They are also called calixcrowns. Those calixarenes that contain oxygen atoms turned to be suitable candidates for selective binding of the alkali ions. Such ligands are more hydrophobic than crown ethers themselves and because of that the formed membranes are more stable. Nitrophenol or azophenol groups on calix[4]arenes that already contain ester groups can transform the Li/Na- selectivity in organic media into a batho-chromic shift from 350 to 425 nm with an assistance of an auxiliary base that supports deprotonation. The calixarene containing nitrophenylazophenol group is a flexible compound with multiple applications, it detects lithium ions in the presence of a week base, but it also has a property that enables it to detect weak bases such as volatile amines when Li+ is already present in the membrane.

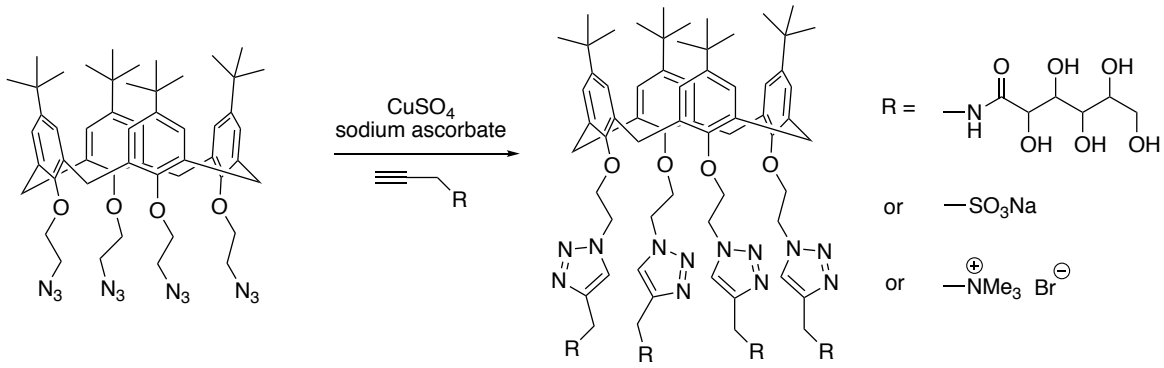
Ion selectivity of calixarenes

Crowned calixarenes are those that were modified using crown ethers. They have a high degree of molecular preorganization and thanks to that property, they achieve high selectivities especially when talking about alkali ions[39]. The crown moiety and calixarene molecule restrict

each other molecular flexibility, what is causing discrimination of ions by their size. Crowned calixarenes can be also utilized for the recognition of potassium ions. As an example we can use *t*-butylcalix[4]arene bearing crown-5 group on its lower rim that shows high K/Na selectivity. When the conformation of the calixarene is changed to partial cone, or alternating, the organization of the ligand becomes better for binding K+ ions. 1,3-alternate conformation of calix[6]arenes or calix[8]arenes also shows high potential for other ions such as cesium and rubidium selectivity.

Water-Soluble Calixarenes

In the last few years, water-soluble calixarenes have been the molecules of interest for scientists because of their potential biological applications. Water-soluble groups that have already been accommodated into alizarins and they include sulfonates[40], carboxylic acids, amines[41] and phosphonates. Ryu and Zhao proposed new efficient modifications to the lower rim of the calixarenes[42] to incorporate water-soluble groups.



Scheme 7. Synthesis of water-soluble calixarenes proposed by Ryu and Zhao.

In their investigation, they used commercially available *tert*-butylcalix[4]arene, converted it into azidocalixarene and then proceeded with further modifications by utilizing click reactions. In the synthesis of water-soluble calixarenes, the efficiency of the reactions was usually an issue, because the conversions were in general low yielding. Ruy and Zhao were able to isolate about 80% of the final products, what significantly improves the synthesis of those molecules.

Calixarenes as Stationary Phases

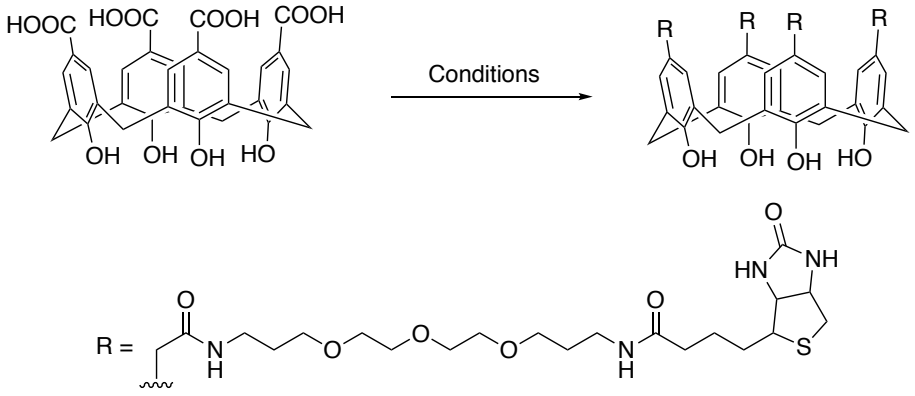
There are also some unusual applications of water soluble calixarenes. They can be used as stationary phases in certain situations. Silica bonded calixa[4]arene tetraester stationary phases can be used for amino acid esters and alkali metals[43]. That same group was investigating chromatographic studies using water soluble sulphonated calixarenes in mobile phases to determine nitrophenol compounds by the means of HPLC. They discovered that these sulphonated calixarenes are able to form host-guest complexes with a variety of cations, and therefore the selectivity of the separation increases.

Water Soluble Calixarenes in Pharmacology

Water soluble calixarenes are of high interest in pharmacology and biological chemistry because of their high potential to be applicable in living systems. One of the problems that pharmaceutical industries face is the formation of two enantiomers where the probability that both of them are applicable is close to none. Usually only one of the formed enantiomers shows activity as potential therapeutic and in certain cases the second one may be harmful to the organism. Therefore, it is crucial to eliminate the unwanted enantiomer and to design reactions resulting in only one product. In certain cases, calixarenes can be of help in such situations. McKerver and coworkers investigated (S)-di-napthylprolinolcalix[4]arene and they found that it shows great discrimination between enantiomers of phenylglycinol, phenylethylamine and norephedrine[44].

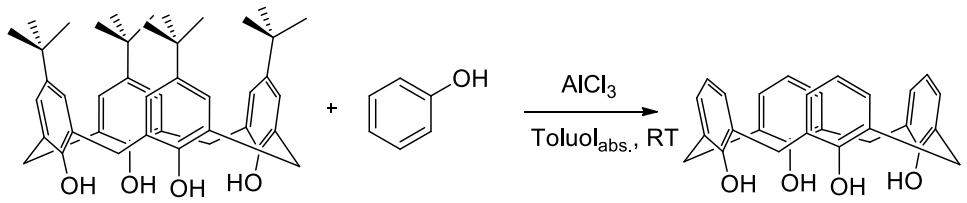
RESULTS AND DISCUSSION

The purpose of the first stage of this project was to modify 5,11,17,23-tetracarboxylic acid-calix[4]arene in such way that the carboxylic acid ends accommodate biotin connected to them using 4,7,10-trioxa-1,13-tridecane diamine as the link. First of all, we needed to synthesize 5,11,17,23-tetracarboxylic acid-calix[4]arene. Other mono functionalized calix[4]arenes such as tetrasulfonate calix[4]arene were also considered, however the molecule substituted with carboxylic acid seemed to have more potential for this project purpose, because of its higher water solubility compared to the tetrasulfonate calix[4]arene.



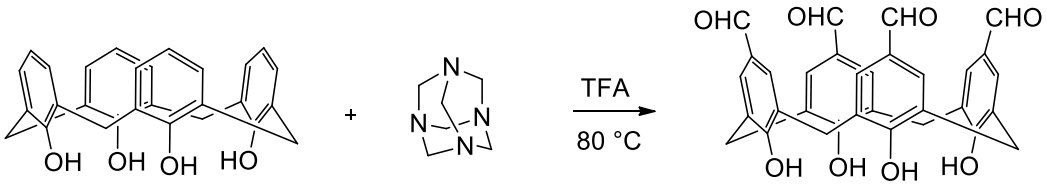
Scheme 8. Synthesis of 5,11,17,23-tetracarboxylic acid-calix[4]arene linked with biotin by 4,7,10-trioxa-1,13-tridecane diamine.

As the starting material in series of reactions aiming for the synthesis of 5,11,17,23-tetracarboxylic acid-calix[4]arene we used commercially available *para*-*tert*-butylcalix[4]arene. The first step in the series of reaction was to remove the *tert*-butyl groups from the molecule, and for that purpose retro Friedel-Crafts[45] reaction was performed, according to the procedure presented by Gutsche. Some alterations were applied in the reaction conditions to make it more efficient.



Scheme 9. Retro Friedel-Crafts reaction of *p-tert*-butylcalix[4]arene.

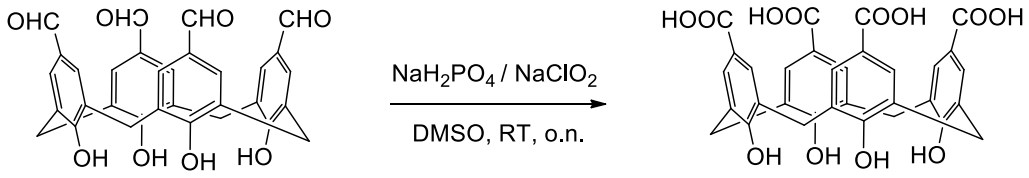
The reaction was proceeding as expected, however the product obtained from the first retro Friedel-Crafts reaction was not entirely pure and it was confirmed by NMR that the de-*tert*-butylation was not complete. The product of that reaction contained some of the desired fully de-t*ert*-butylated product, however it was also observed that partially de-*tert*-butylated molecules with various number of the *tert*-butyl groups we present in the mixture. In order to solve that problem, the same reaction was repeated using the crude material that was obtained from the first reaction and after work up, 46% yield of the desired calix[4]arene was recovered. Having the pure calix[4]arene ready, we proceeded to the next step, formylation.



Scheme 10. Formylation of calix[4]arene.

The formylation of calix[4]arene was done by performing Duff reaction[46] Duff formylation is a commonly used method for *ortho*-formylation of phenols and it involves an addition of HMTA and it requires an acidic medium, usually AcOH or TFA. In this case we did not pursue *ortho*-formylation because all *ortho* positions are already occupied by CH_2 that are connecting the benzene rings together. *Para* substitution was the goal of the reaction. The conditions used for our purpose were presented by Pur and Dilmaghani[47] and they were slightly altered. The reaction went to completion, resulting in formation of the desired 5,11,17,23-tetraformyl-calix[4]arene and there was no partially formylated products observed. With the formylation completed, there

was only one step left in the calix[4]arene modification: conversion of the formyl groups into carboxylic acids.



Scheme 11. Synthesis of 5,11,17,23-tetracarboxylic acid-calix[4]arene.

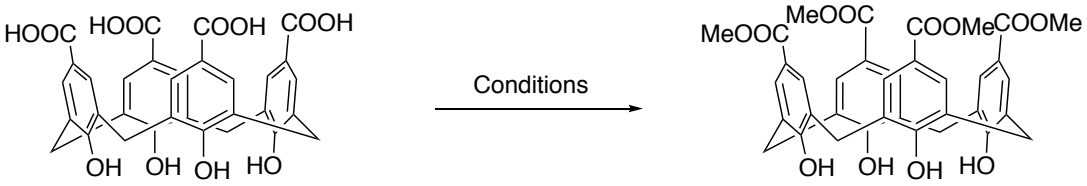
Pinnick oxidation reaction was utilized to obtain the final 5,11,17,23-tetracarboxylic acid-calix[4]arene. That reaction, also known as Lindgren oxidation is a relatively easy way of conversion of aldehydes to corresponding carboxylic acids[48]. The used reaction conditions were described by Pasquale[49]. The product resulting from that reaction was characterized as desired 5,11,17,23-tetracarboxylic acid-calix[4]arene, however the product was not entirely pure.

Having the 5,11,17,23-tetracarboxylic acid-calix[4]arene synthesized, we moved on to the next step in the project. The aim of this part was to connect the calix[4]arene with biotin by using 4,7,10-trioxa-1,13-tridecane diamine as the linking molecule.

The unknown was in what order the reactions should be performed as well as the conditions for those reactions. There were two possible pathways to pursue: 1. To react 5,11,17,23-tetracarboxylic acid-calix[4]arene with 4,7,10-trioxa-1,13-tridecane diamine and then attach the biotin at the amine end of the resulting molecule, or 2. To synthesize 4,7,10-trioxa-1,13-tridecane diamine and biotin adduct followed by its connection to 5,11,17,23-tetracarboxylic acid-calix[4]arene.

To determine the correct order of reactions, we decided to run several test reactions to establish the necessary conditions.

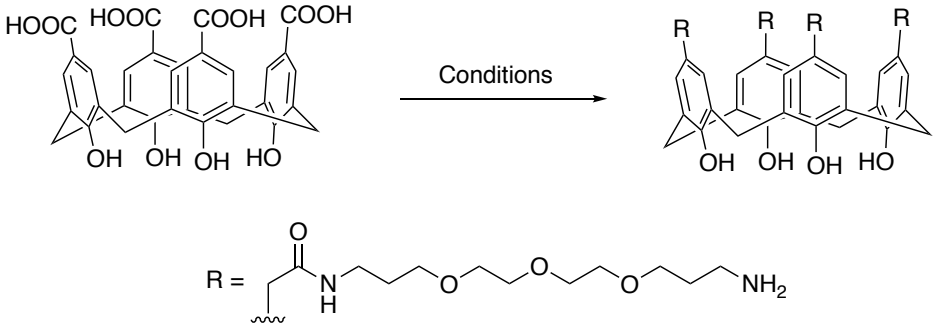
One of the reactions that we investigated was conversion of carboxylic acid groups on the calix[4]arene onto methyl ester that potentially would allow for an easy attachment of 4,7,10-trioxa-1,13-tridecane diamine in the next step.



Scheme 12. Methylation of 5,11,17,23-tetracarboxylic acid-calix[4]arene

We considered two different reaction conditions. In the first attempt we tried simple methylation of the 5,11,17,23-tetracarboxylic acid-calix[4]arene using methanol and sulfuric acid as catalyst. After the reaction did not proceed at all, we decided to try one more method. The second conditions included an addition of DMAP and DCC. This method also turned to be unsuccessful, and no formation of the desired product was observed.

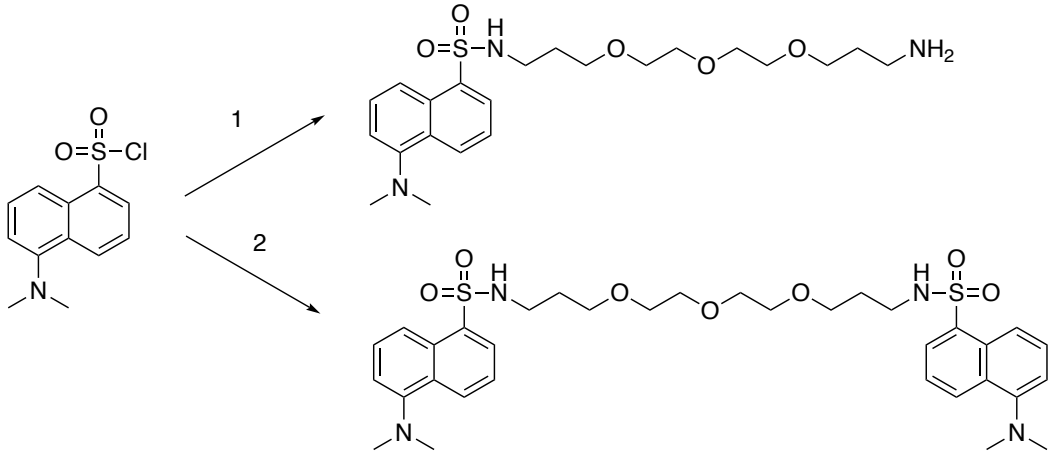
With those negative results, we abandoned the attempt of conversion of carboxylic acid into a methyl ester and instead we considered direct amidation of the carboxylic acid part of the 5,11,17,23-tetracarboxylic acid-calix[4]arene. One reaction was designed to check that pathway.



Scheme 13. Amination of the 5,11,17,23-tetracarboxylic acid-calix[4]arene.

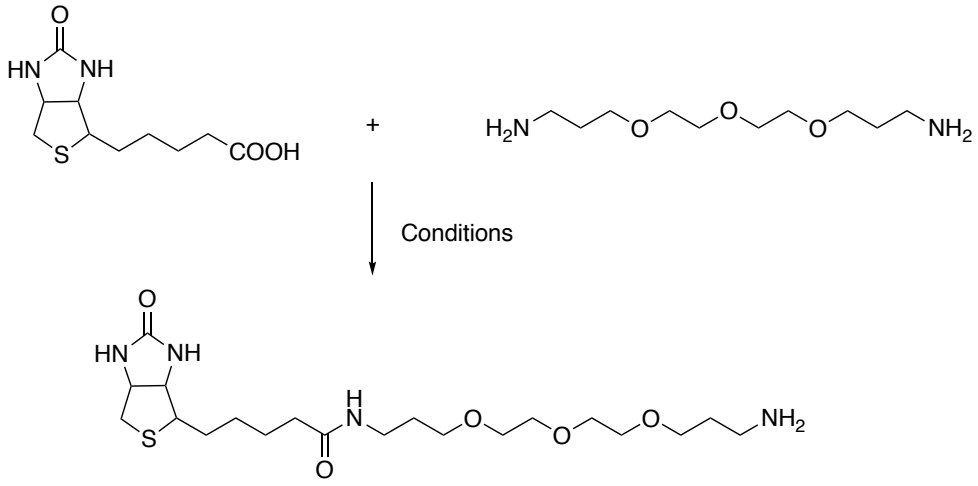
Simple amidation of the 5,11,17,23-tetracarboxylic acid-calix[4]arene dissolved in DMSO with an addition of 4,7,10-trioxa-1,13-tridecane diamine was unsuccessful and we decided to move away from this pathway of reactivity and proceed with biotin- 4,7,10-trioxa-1,13-tridecane diamine linkage.

To start, we tried to link dansyl chloride (in place of biotin) with 4,7,10-trioxa-1,13-tridecane diamine. Dansyl chloride is a widely used reagent used in reactions with primaty and secondary amines. It is often used for modifications of amino acids as the products of the reactions are fluorescent and it serves as fluorescent die. First reaction conditions that were tested used 1.0 equiv. of dansyl chloride and 2.0 equiv. of 4,7,10-trioxa-1,13-tridecane diamine dissolved in DCM. That reaction went to completion, however it resulted in formation of two different products. Once separated, it was confirmed that one of the products was the desired



Scheme 14. Two products formed from synthesis of dansyl chloride and 4,7,10-trioxa-1,13-tridecane diamine reaction.

dansyl chloride-4,7,10-trioxa-1,13-tridecane diamine adduct, however the amount of that product was not satisfactory, while the second, major product was containing dansyl chloride attached at both amine ends of the diamine. In order to eliminate the undesired product, reaction conditions were slightly modified. In the second reaction 1.0 equiv. of dansyl chloride and 5.0 equiv. instead of 2.0 equiv. of 4,7,10-trioxa-1,13-tridecane diamine were used. Additionally, 0.5 equiv. of trimethylamine was added. This time the reaction resulted in formation of only one product, there was no disubstituted molecule present. Having that molecule synthesized, we moved away from dansyl chloride and decided to do an amidation of biotin.



Scheme 15. Synthesis of biotin 4,7,10-trioxa-1,13-tridecane diamine adduct.

The first conditions that were tested included an addition of carbonyldiimidazole onto the biotin that was dissolved in DMF followed by an addition of 4,7,10-trioxa-1,13-tridecane diamine. This reaction did not proceed at all and there was no desired product forming.

The second reaction conditions used for this experiment were previously reported by Senevirathne and Pflum[50]. Since the reaction was already known, it was expected that it will give the desired product in a moderate yield. Monitored by TLC, the progression of the reaction showed two different products forming. The crude product of this reaction was not purified and the products were not characterized, however it is expected that the two different reaction products are diamine substituted with biotin on one amine end and one substituted at both ends. What would be similar to the two products obtained from the reaction of dansyl chloride with 4,7,10-trioxa-1,13-tridecane diamine.

The next suggested steps in the project are the purification and characterization of the two products obtained from the last reaction of biotin and 4,7,10-trioxa-1,13-tridecane diamine. If the suspicion that the reaction products are in fact mono and disubstituted compounds, the alterations to the reaction conditions may be necessary in order to optimize it and receive the highest possible yield of the desired product and to minimize or to eliminate the formation of the unwanted one. Once biotin-4,7,10-trioxa-1,13-tridecane diamine adduct is successfully

synthesized in a satisfying yield, there will be one more unknown that will need to be addressed. Because the reaction conditions for linking that adduct to 5,11,17,23-tetracarboxylic acid-calix[4]arene are still unknown and the reaction where we attempted to link the diamine to the calixarene failed, it will be necessary to design a series of test reactions to establish and then optimize the conditions.

Once the satisfactory reaction conditions are known for all steps and the molecule is completed, it will be then possible to move on to the next stage of the project, testing of the biological activities of the obtained new compound.

EXPERIMENTAL

Synthesis of calix[4]arene from p-tert-butylcalix[4]arene

In a Shlenk tube equipped with a magnetic stir bar were placed *p-tert*-butylcalix[4]arene (5.03 g, 1.0 equiv.), phenol (3.51 g, 4.77 equiv.) and anhydrous aluminum chloride (5.392 g, 5.16 equiv.). Dry toluene (46 mL) was added and the mixture was stirred at room temperature under nitrogen conditions for 4 hours. During the duration of the reaction, the precipitate formed inside the flask. After 4 hours, the reaction was quenched adding 100 mL of 0.2 M hydrochloric acid. The organic layer was then separated and the aqueous work up was performed by washing it twice with 140 mL of $NaHCO_3$- and then twice with 240 mL of brine. Organic layer was then dried over Na_2SO_4, most of the solvent was evaporated (about 25% of the solvent was retained) and the solution was diluted with methanol and placed overnight in the fridge in order to facilitate the crystallization. The following day formed precipitate was filtered out, rinsed with methanol and ethyl acetate and then dried. The purity of the product was not sufficient and it included molecules that contained various number of *tert*-butyl groups, therefore the de-*tert*-butylation reaction was repeated one more time using the obtained crude compound by applying the above conditions. The second reaction monitored by TLC was complete in 5 hours and yielded pure desired product in a form of white solid in a moderate 46% yield. No partially de-*tert*-butylated products were observed.

Synthesis of 5,11,17,23-tetraformyl-calix[4]arene from calix[4]arene

In a Shlenk tube equipped with a magnetic stir bar were placed calix[4]arene (0.95 g, 1.0 equiv.), hexamethylenetetramine (4.70 g, 15.0 equiv.) and trifluoroacetic acid (70 mL). The mixture was stirred at 80 ^{o}C and monitored by TLC. The color of the solution became red upon heating. After 26 hours, the reaction was complete. The flask was then allowed to cool down and once it was brought to room temperature, the mixture was poured into 250 mL of deionized water in order to quench it. Once on water, the solution became cloudy yellow, with fine precipitate forming. The precipitate was then filtered out on a Buchner funnel, dried and recrystallized using methanol. It was confirmed by NMR that the precipitation is in fact the desired product. The remaining crude compound was dried and the recrystallization using methanol was repeated.

The product obtained from recrystallization of the crude material was also the desired fully formylated calix[4]arene.

Synthesis of 5,11,17,23-tetracarboxylic acid-calix[4]arene

The three neck round bottomed flask was equipped with a magnetic stri bar, addition funnel and a reflux condenser bearing a drying tube. 5,11,17,23-tetraformyl-calix[4]arene (599.3 mg, 1.0 equiv.) and dimethylsulfoxide (14.0 mL) were placed in the flask and stirred until solid dissolved completely. A solution of sodium hydrogen phosphate monohydrate (212.6 mg, 1.38 equiv.) dissolven in 11 mL of deionized water was added to the flask. Then a solution of sodium chlorite (1.41 g, 1.38 equiv.) dissolved in 8 mL of deionized water was placed in the addition funnel and added dropwise to the reaction mixture over the duration of 4 hours. The reaction was left stirring overnight at room temperature. Upon completion, hydrochloric acid was slowly added to the reaction mixture, until the pH of the solution dropped to about 1. During that addition, the precipitation formed. The precipitate was then separated from the liquid by filtration, washed with water and dried under reduced pressure. Obtained product was beige solid.

Synthesis of 5,11,17,23-tetramethyl ester-calix[4]arene: conditions 1

In a Shlenk tube equipped with a magnetic stir bar 5,11,17,23-tetracarboxylic acid-calix[4]arene (602.5 mg) was dissolved in 2 mL of methanol. It was followed by an addition of 100 µL of concentrated sulfuric acid. The reaction mixture was stirred at 60 ^{o}C for 24 hours under nitrogen conditions and monitored by TLC. After 24 hours, reaction was terminated due to the lack of reactivity.

Synthesis of 5,11,17,23-tetramethyl ester-calix[4]arene: conditions 2

In a Shlenk tube equipped with a magnetic stir bar 5,11,17,23-tetracarboxylic acid-calix[4]arene (60.2 mg, 1.0 equiv.) was placed. 0.5 mL of DMF was added and the mixture was stirred until the solid dissolved completly. It was followed by an addition of DMAP (10 mg) and methanol (1.6 mL). Reaction mixture was stirred for 5 minutes at 0 ^{o}C and DCC (82.5 mg, 4.0 equiv.) was added at that time. Then the reaction mixture was brought to room temperature and it was stirred for

24 hours under nitrogen conditions and monitored by TLC. The reaction was terminated after that time due to the lack of reactivity.

Synthesis of *5,11,17,23-tetra-trioxa-tridecane -calix[4]arene*

In a Shlenk tube equipped with a magnetic stir bar 5,11,17,23-tetracarboxylic acid-calix[4]arene (30.1 mg, 1.0 equiv.) was placed. 0.5 mL of DMSO was added and the mixture was stirred until the solid dissolved completly. It was followed by an addition of 4,7,10-trioxa-1,13-tridecane diamine (45 µL, 4.0 equiv.). Reaction mixture was stirred at 80 ^{o}C for 24 hours under nitrogen atmosphere and monitored by TLC. After that time, the reaction was terminated due to the lack of reactivity.

Synthesis of dansyl chloride-4,7,10-trioxa-1,13-tridecane diamine adduct: conditions 1

In a Shlenk tube equipped with a magnetic stir bar dansyl chloride (269.75 mg, 1.0 equiv.) and 4,7,10-trioxa-1,13-tridecane diamine (442.8 µL, 2.0 equiv.) were dissolved in 3.85 mL of dichloromethane. Reaction mixture was stirred at room temperature under nitrogen conditions and monitored by TLC for three hours. Upon completion, reaction was quenched with 10 mL of water and extracted three times with 10 mL of DCM. Organic layers were combined, then washed with brine (1x 10 mL) and dried over Na_2SO_4. After the solvent was evaporated, the crude product was purified by column chromatography using ethyl acetate and acetone. Two products were obtained from this reaction. The NMR conformed that one of the product was the desired dansyl chloride-4,7,10-trioxa-1,13-tridecane diamine product, while the major product obtained from the reaction was disubstituted with dansyl chloride at both amine ends.

Synthesis of dansyl chloride-4,7,10-trioxa-1,13-tridecane diamine adduct: conditions 2

In a Shlenk tube equipped in a magnetic stir bar dansyl chloride (134.9 mg, 1.0 equiv.) and 4,7,10-trioxa-1,13-tridecane diamine (553.5 µL, 5.0 equiv.) were dissolved in 1.9 mL of DCM. There was vigorous bubbling present during the addition of the diamine and the color of the solution changed from dark yellow to fluorescent yellow. Once the bubbling stopped, after about 5 minutes trimethylamine (66.9 µL, 0.5 equiv.) was added. Reaction was stirred at room

temperature for 24 hours under nitrogen conditions and monitored by TLC. Upon completion reaction mixture was purified by column chromatography using 1:1 to 3:1 ethanol in DCM. Only one product formed and the NMR confirmed that obtained sticky yellow liquid was the desired product.

Synthesis of biotin 4,7,10-trioxa-1,13-tridecane diamine adduct: conditions 1

In a Shlenk tube equipped with a magnetic stir bar biotin (150.0 mg, 1.0 equiv.) was placed. 30 mL of DMF was added and the mixture was stirred until the solid dissolved completly. Carbonyldiimidazole (170.0 mg, 1.7 equiv.) was then diluted in 0.75 mL of DMF, added to the mixture and it was stirred at room temperature under nitrogen conditions for 4 hours. Then 4,7,10-trioxa-1,13-tridecane diamine (521 µL, 3.9 equiv.) was added and the mixture was stirred for an additional 18 hours at the same conditions monitored by TLC. The reaction did not show any reactivity, therefore it was terminated.

Synthesis of biotin 4,7,10-trioxa-1,13-tridecane diamine adduct: conditions 2

In a Shlenk tube equipped with a magnetic stir bar biotin (244.3 mg, 1.0 equiv.) was dissolved in 1.25 mL of DMF. TBTU (385.3 mg, 1.2 equiv.) and DIPEA (209 µL, 1.2 equiv.) were added and the mixture was stirred at room temperature for 30 minutes. The mixture was then added drop wise onto a solution of 4,7,10-trioxa-1,13-tridecane diamine (550 µL, 2.5 equiv.) dissolved in 65 mL of DCM at 4^{o}C. After the addition was complete, the mixture was stirred at room temperature for 24 hours under nitrogen conditions and monitored by TLC. Observed were two products forming. The crude product was not purified.

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