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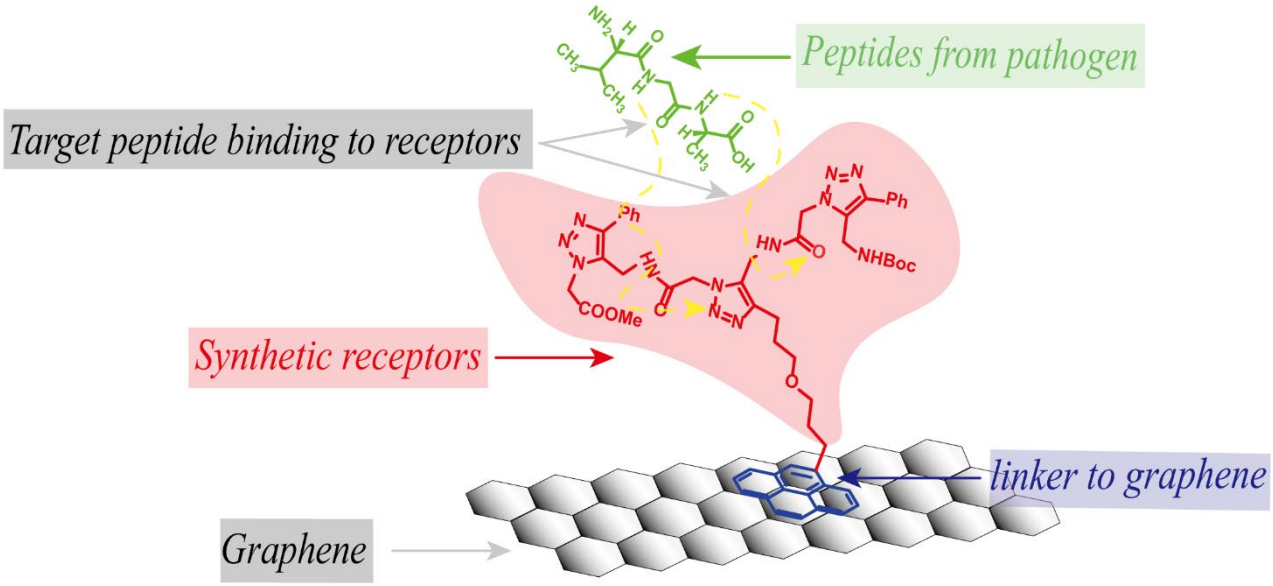

Click chemistry for the assembly of triazole-based graphene biosensors

Master's thesis in Materials Chemistry

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Abstract

Since bacterial infections cannot be easily diagnosed, this leads to misuse of antibiotics. As a direct result of a significant increase in antibiotic use, antibiotic-resistant bacteria (ARB) are becoming a threat to global health and any people die from antibiotic-resistant infections. Therefore, it is becoming increasingly urgent to develop devices that can quickly and easily diagnose common bacterial infections. Consequently, we decided to develop biosensors for detecting bacterial infections, with synthetic receptors as the core component.

This Master's thesis is a component of the PEST-BIN project funded by the European Union, which strives to create biosensors using 1,2,3-triazole-based receptors linked to graphene-coated chips. 1,2,3-Triazole oligomers are interesting materials that can be developed to mimic the properties of peptides, including folding, hydrogen bonding, and π-π-stacking. Consequently, they show potential for applications such as synthetic receptors that mimic natural peptide receptors. The objective of the present thesis is to prepare graphene-based synthetic receptors, which consist of 1,2,3-triazole trimers, that can recognize specific biomarker peptides and amino acids.

To prepare the final receptors, different organic reactions were performed, including the Sonogashira coupling, RuAAC (Ruthenium Azide-Alkyne Cycloaddition), amide coupling, as well as Williamson ether synthesis. All the 1,4,5-trisubstituted 1,2,3-triazole monomers were successfully synthesized and converted into various oligomers using amide coupling. Ultimately, I managed to synthesize six distinct trimers. Subsequently, attempts were made to functionalize graphene with the prepared trimers. The work on the trimer functionalization is still ongoing and further efforts are required to complete this phase.

Keywords: biosensors; synthetic receptors; triazole; RuAAC; oligomers.

Table of Contents

List of Abbreviations

ARB – Antibiotic resistant bacteria

CuAAC – Copper-Catalyzed Azide-Alkyne Cycloaddition

CuI – Copper(I) iodide

CVD – Chemical vapor deposition

DCM – Dichloromethane

DIPEA – *N*, *N*-Diisopropylethylamine

DMF – *N*, *N*-Dimethylformamide

ELISA – Enzyme-Linked Immunosorbent Assay

equiv – Equivalent

EtOAc – Ethyl acetate

GO – Graphene oxide

HATU – Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium

HRMS – High Resolution Mass Spectrometry

IR – Infrared Spectroscopy

ITC – Isothermal Titration Calorimetry

MeCN – Acetonitrile

NGS – Next-Generation Sequencing

NMR – Nuclear Magnetic Resonance

PCR – Polymerase Chain Reaction

PE – Pentane

rGO – Reduced graphene oxide

rt – Room temperature

RuAAC – Ruthenium-Catalyzed Azide-Alkyne Cycloaddition

TBAF – *tetra*-n-butylammonium fluoride

TFA – Trifluoroacetic acid

THF – Tetrahydrofuran

TLC – Thin layer chromatography

1 Introduction

Infections caused by pathogens pose a huge problem and a high risk for human health. Many people worldwide are affected by bacterial diseases and do not get the urgent help that they need.[1] As a result, millions of people die from these pathogens, a group of microorganisms including bacteria and viruses, which can cause harmful infections. It is therefore urgently important for scientists to develop new medical techniques for detecting pathogenic diseases. Currently, commonly employed techniques in molecular biology and diagnostics include the Polymerase Chain Reaction (PCR), Enzyme-Linked Immunosorbent Assay (ELISA), and Next-Generation Sequencing (NGS).[2] Despite their widespread use, these methods have notable drawbacks, including issues such as low sensitivity and selectivity, high costs, time-consuming procedures, and the potential for contamination. Recognizing these limitations, there is a growing need to explore and adopt new techniques and devices. Biosensors, for instance, offer a promising avenue for addressing these challenges and can potentially provide more efficient, rapid, and cost-effective solutions for various applications in diagnostics and research.[3]

The discovery of graphene has spectacularly accelerated research on fabricating low-cost electrode materials because of its unique physical properties, including high specific surface area, high carrier mobility, high electrical conductivity, flexibility, and optical transparency.[4] Graphene and its oxygenated derivatives, including graphene oxide (GO) and reduced graphene oxide (rGO), are becoming an important class of nanomaterials in the field of biosensors.

This Master's thesis is part of the EU-funded project, PEST BIN, which is dedicated to advancing the development of biosensor applications. The project is in collaboration with researchers at K and MC2 at Chalmers University of Technology, as well as at the Sahlgrenska Academy. The goal of this project is to develop a biosensor that consists of a synthetic receptor attached to an aromatic linker chain, which in turn is bound to a graphene-coated chip as shown in **Figure 1.1**. The function of the device is to produce a signal when a certain bacterial infection is encountered.

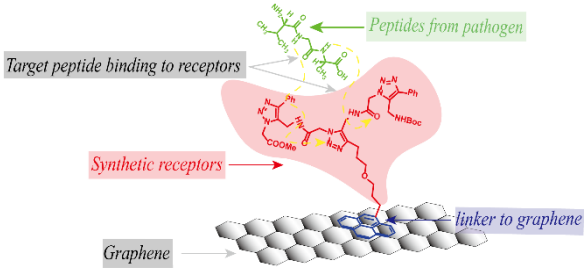


Figure 1.1 Triazole-based graphene biosensor

1.1 Problems and Objectives

This project is a pivotal component of a large research project. A biosensor isolated from *Streptococcus pneumoniae* is cleaved to smaller peptides by a research group at the Sahlgrenska Academy. Meanwhile, synthetic receptors are prepared at our group. Finally, the synthesized receptors are attached to a graphene surface and sent to the Yurgens' group in the Department of Microtechnology and Nanoscience at Chalmers where they will evaluate the binding of these synthetic receptors to a graphene chip using different analytical techniques, such as Raman Spectroscopy and Hall measurements. The purpose is to see if there are any changes in electron density before and after the biomarker is targeted by the synthetic receptor.

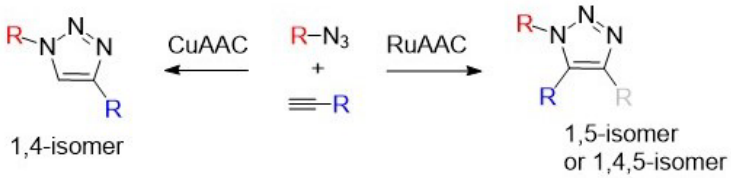
The aim of this master's thesis is shown in **Figure 1.1**. The focus of our work lies in the synthesis of synthetic receptors (depicted in red). Finally, the synthetic receptor is bound to a graphene chip (depicted in grey) and characterized using the techniques mentioned above.

Potential challenges may arise in three distinct phases of this collaborative effort. The first challenge concerns the successful isolation of peptides from bacteria with the effectiveness of bacterial and biological methods. Secondly, the synthesis of synthetic receptors and their connection with the linker represents a critical step in the project. Lastly, the detection and characterization of the synthetic receptor-graphene complex by analytical methods, particularly Raman Spectroscopy, introduce another stage where challenges may emerge. It is imperative to address and resolve issues in each of these regions to ensure the seamless progression and success of the project.

1.2 Aim and Scope

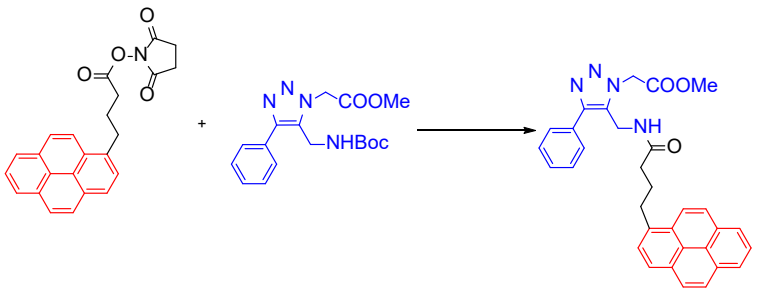
This part of the project aims to prepare synthetic receptors and evaluate their binding properties to diagnostic biomarkers in the form of peptides. The main tools to attain this goal are the use of organic and organometallic synthesis, in conjunction with methods for the characterization of organic compounds, including Nuclear Magnetic Resonance (NMR) spectroscopy, single crystal X-ray diffraction, and fluorescence spectroscopy, to study the binding of the biomarkers to the receptor. The strategy for synthesizing the receptor is to couple functionalized building blocks (monomers) into oligomers. The receptor is then connected to a graphene surface via a linker, to form a graphene-based biosensor.

In this project, triazoles are investigated as the monomers, connecting these via amide bonds, to afford suitable functionalities for biomarker binding. Different triazole monomers will be synthesized via a ruthenium-catalyzed azide-alkyne cycloaddition (RuAAC click reaction) (**Scheme 1.1**)[5] where the precursor alkynes will be prepared via Sonogashira coupling.[6] By combining these monomers in different ways, a variety of different receptors can be formed from the same monomers using a mix-and-match approach to tailor the binding to the biomarker.



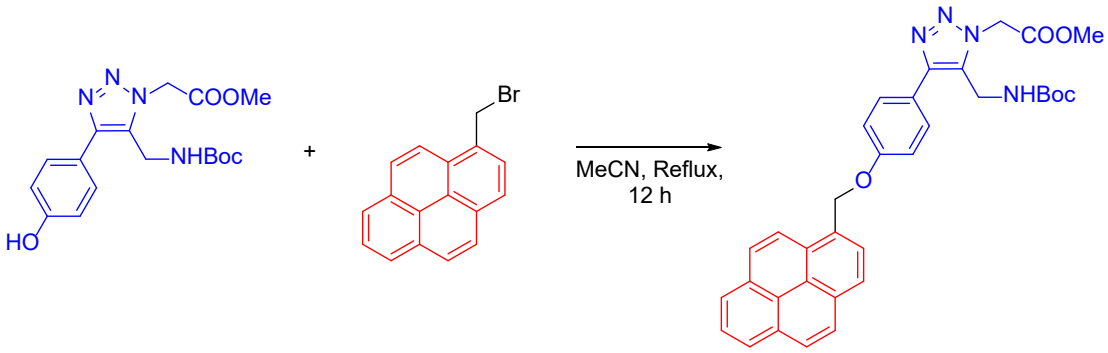
Scheme 1.1 Click chemistry for triazole formation.

In parallel with this work, different methods will be explored to attach a triazole via a linker to a graphene chip, in order to mimic the effects of the synthetic receptor. We need to ensure that such a molecule is compatible with and does not alter the properties of graphene. Non-covalent interactions are preferred, and the linker should include a polyaromatic group, such as a pyrene unit, that can bind efficiently to the graphene surface. **Scheme 1.2** shows one of the model compounds that will be prepared for this purpose.



Scheme 1.2 Connection to pyrene

Meanwhile, the Williamson ether synthesis could also be used to attach a triazole via a linker to a graphene chip. **Scheme 1.3** shows the detailed reaction of this method.



Scheme 1.3 Williamson ether synthesis

When the compounds are bound to the linker, they will be bound to a graphene chip and the functionalized graphene will then be sent to the Yurgens' Group at MC2 for characterization. The characterization of the derivatized graphene chips falls outside the scope of my Master's project and will not be included in the report. During my Master's thesis work, I have visited MC2 to see how they work in the clean room and to learn more about how the characterization is performed.

2 Background

2.1 Antibiotic Resistance and its Impact on Society

As a direct result of increasing antibiotic use, antibiotic resistant bacteria (ARB) are becoming a threat to global health, putting at risk the achievements of modern medicine. A striking example is the artemisinin-resistant strains of malaria, whose further spread could jeopardize the recent advances in medicine in malaria control.[7] The fact that high rates of resistance have been reported for bacteria associated with common and easily transmitted infections (e.g. urinary tract infection, pneumonia) gives rise to concern about their fast spread. In Europe, 35,000 deaths per year are attributed to antibiotic-resistant infections, costing €1.5 billion annually.[8]

The gaps in surveillance, the plethora of unreported cases, and the lack of data-sharing and coordination are slowing down the development control protocols, resulting in little consciousness of the problem and even faster spread. An important tool to control infections is the detection of these resistant genes in bacteria.

Conventional techniques for diagnosing bacterial infections encompass various methods, such as the use of cell cultures, the polymerase chain reaction (PCR) for nucleic acid identification, and immunological assays, such as the enzyme-linked immunosorbent assay (ELISA), based on antigen-antibody interactions.[9]

However, these traditional methods may involve substantial expense and are time-consuming, often requiring complex, multi-stage sample preparations. Current research is actively investigating innovative technological strategies to develop cost-effective and efficient alternatives for diagnosing bacterial infections.

2.2 Biosensors

Biosensors are of paramount importance, not only in biomedical applications such as clinical diagnostics, but also in the agriculture and food industries.[10] The development of highly selective, sensitive, and low-cost devices can provide efficient healthcare testing with high accuracy in the healthcare industry.[11]

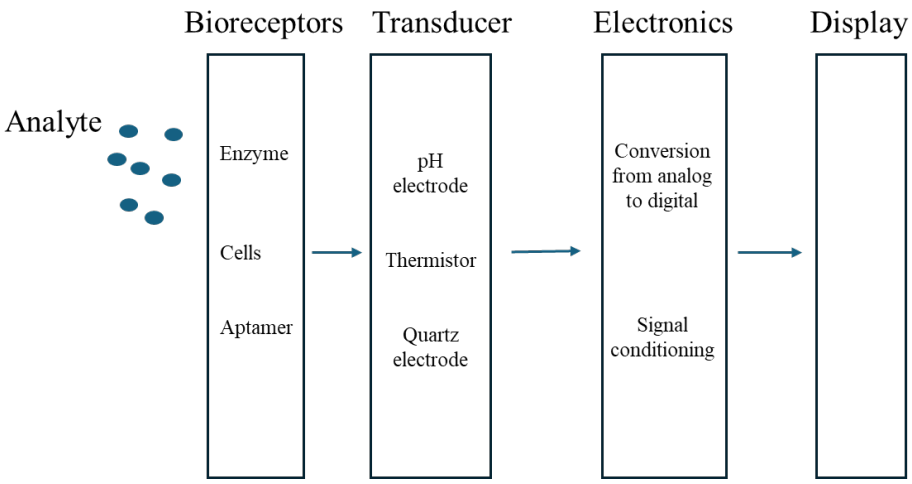


Figure 2.1 Schematic representation of a biosensor

Typically, a biosensor can be divided into five major parts: analyte, bioreceptor, transducer, electronics, and display (**Figure 2.1**).[12] The analyte is the substance targeted for detection. For instance, glucose can serve as an analyte in a biosensor designed to detect glucose levels. In this project, specific peptides isolated from the pathogen *Streptococcus pneumoniae*[13] are the analytes of interest. Bioreceptors are the specific molecules that can recognize these analytes. Cells, aptamers, and antibodies are the most common examples of bioreceptors. In this project, we will instead use synthetic receptors, consisting of tailor-made triazole oligomers, that are regarded as bioreceptors in this project. Transducers are the elements that could convert bio-recognition into a measurable signal, i.e. change of resistance. The signal strength depends on the number of interactions between analytes and bioreceptors. Electronics typically involve complex electronic circuits that perform signal conditions and amplify signals. The display unit of the biosensor then quantifies the processed signals. Displays are usually treated as a user-interpretation system, which makes it easy and understandable for users to interpret the data.[12]

Biosensors could be classified into two main categories, 1) catalytic biosensors and affinity or 2) non-catalytic biosensors. For catalytic biosensors, the interaction between analytes and bioreceptors results in the formation of new compounds. This type of biosensor comprises enzymes, microorganisms, tissues, and so on.[14] For non-catalytic biosensors, the analytes and bioreceptors bind irreversibly and there are no new compounds formed in the binding process.

Several requirements must be considered to develop a highly effective and capable biosensor system. To create an optimal biosensor, factors such as selectivity, sensitivity, stability, response time, stability, and reproducibility are crucial.[15] Selectivity concerns the ability of biosensors to detect a target analyte within a mixture of many unwanted contaminants. Sensitivity is essential for detecting the minimum number of analytes, determining the fewest required steps, and identifying the lowest concentration. Stability gauges the biosensor's susceptibility to environmental disturbance, with degradation over time being a significant influence factor. Response time measures the duration it takes for the biosensors to react after interacting with the analytes. Reproducibility involves assessing whether the biosensor can consistently yield the same results after multiple tests and determining how closely the measured results align with the actual values.[12]

2.3 Graphene-Based Biosensors

Graphene biosensors can be divided into several categories: nucleic acid-functionalized graphene biosensors, antibody-functionalized graphene biosensors, peptide-functionalized graphene biosensors, and other biomolecule-functionalized graphene biosensors.[16]

Detection using nucleic acid-functionalized graphene biosensors relies on interactions causing structural changes and signal output. Researchers are also turning to graphene biosensors with antibodies as recognition molecules for pathogenic bacteria and virus detection. Meanwhile, the design of peptide-recognizing graphene-based biosensors for target detection mainly relies on two principles. The first is that the cleavage of specific peptide chains by targets results in a change of signals. The second strategy is based on the separation of peptide chains from a graphene surface or the attachment of analytes on peptides-graphene complexes because of the potent force between the peptide and target.[17]

2.4 Synthetic Receptors

A synthetic receptor refers to a man-made molecule designed to mimic the recognition properties of natural receptors. Natural receptors, such as those found in cells, are

proteins or other molecules that specifically bind to substances, triggering a biological response. Synthetic receptors are created through chemical synthesis and are often designed to mimic the recognition and binding capabilities of natural receptors.[18]

These synthetic receptors can be tailored for various applications, including molecular sensing, drug delivery, and catalysis. They are engineered to selectively bind to specific target molecules, acting as molecular recognition tools. Synthetic receptors can be advantageous in certain situations, where natural receptors are impractical or unavailable. Their design allows for precision and customization, making them valuable in fields such as chemistry, biology, and materials science. Examples of synthetic receptors include molecularly imprinted polymers, host-guest complexes, and various supramolecular structures designed for specific molecular recognition tasks.[19]

In our work, the biosensors are synthetic receptors based on triazole oligomers, intended to detect small peptides and amino acids. The detection strategy should be the binding of a molecule by a change in the electron mobility.

2.5 Graphene

Graphene is an interesting material with exceptional properties. It is the thinnest and strongest material known. Its charge carriers exhibit giant intrinsic mobility, have zero effective mass, and can travel for micrometers without scattering at room temperature. Graphene can sustain current densities six orders of magnitude higher than that of copper and shows record thermal conductivity and stiffness, remains impermeable to gases, and reconciles such conflicting qualities as brittleness and ductility.[20]

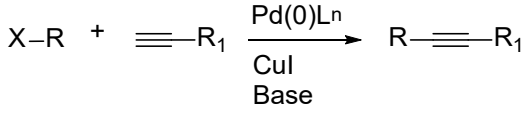
There are several different methods to produce graphene sheets. The mechanical exfoliation method produces graphene sheets through a peeling process from graphite specimens. Dry etching creates mesas, which, when peeled off using Scotch tape, yield thin flakes. These flakes, containing monolayer or few-layer graphene, are washed and transferred to a substrate.[21]

Chemical vapor deposition (CVD)[22] is another promising method for large-scale production of mono- or few-layer graphene using high-temperature metal surfaces. In a typical CVD process, carbon dissolves in a metal substrate, and upon cooling, it

precipitates on the substrate. The deposition process controls graphene thickness and crystalline ordering by adjusting the cooling rate and carbon concentration in the metal.[23]

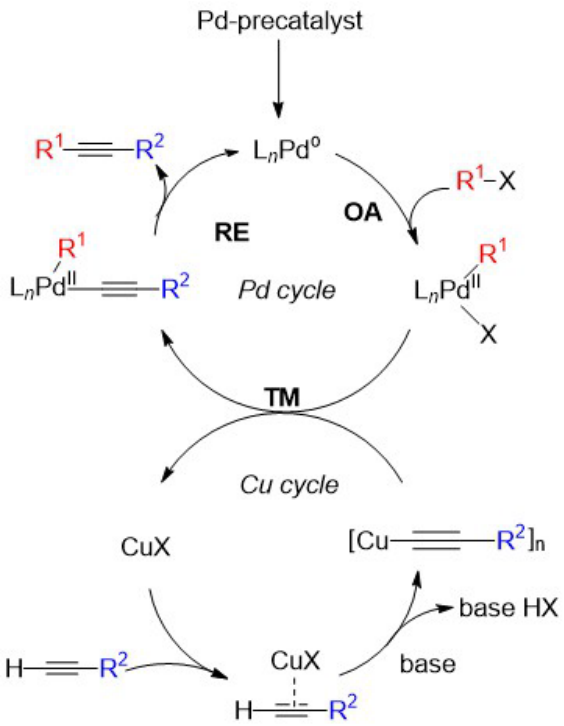
2.6 The Sonogashira Coupling Reaction

The Sonogashira reaction is a cross-coupling technique involving the coupling of terminal alkynes and aryl or vinyl halides to form carbon-carbon bonds, and is typically catalyzed by palladium.[24] The resulting products find diverse applications, spanning from pharmaceuticals to nanomaterials. In 1975, Heck and Cassar[6, 25] independently reported the Pd-catalyzed arylation and alkenylation of alkynes, using organic or inorganic bases at temperatures around 100 °C. Following this, Sonogashira and Hagihara[26] demonstrated that the addition of a catalytic amount of copper(I) iodide significantly enhanced the alkynylation rate, enabling cross-coupling even at room temperature. The cornerstone of the Sonogashira–Hagihara protocol, commonly known as the Sonogashira coupling, has evolved into a widely accepted method. In this reaction, Pd and Cu co-catalyze the construction of sp–sp^2 bonds, involving terminal alkynes and aryl or alkenyl halides or triflates (**Scheme 2.1**).[27] Since its discovery, the Sonogashira coupling has undergone extensive studies to elucidate its mechanism and explore various catalysts, ligands, and additives.



Scheme 2.1 The Sonogashira coupling

The precise mechanism of the palladium/copper-catalyzed Sonogashira reaction remains inadequately understood, primarily due to the intricate analysis required for deciphering the combined action of the two present metal catalysts.[27] It is generally postulated to occur through two independent catalytic cycles (**Scheme 2.2**).



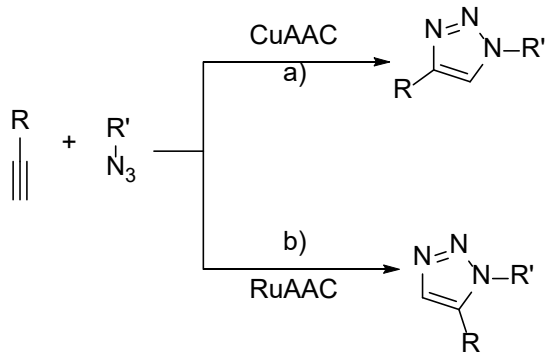
Scheme 2.2 Proposed mechanism of the Sonogashira coupling

The initial 'palladium cycle' is conventional in C–C cross-coupling formations and initiates with the catalytically active species $Pd(0)L_2$.[28] This species can be colloidal or a low-ligated Pd(0)-species stabilized by the present ligands, including base or solvent molecules. When phosphanes serve as ligands, negative-ion electrospray ionization mass spectrometry has detected the corresponding bis(phosphane)-palladium, along with other involved species, in the gas phase. The $[Pd(0)L_2]$ complex can be formed from Pd(0) complexes like $Pd(PPh_3)_4$ or generated from Pd(II) complexes such as $PdCl_2(PPh_3)_2$. The latter route involves the formation of a $[Pd(II)L_2(C{\equiv}CR_2)_2]$ species, leading to $[Pd(0)L_2]$ after reductive elimination by forming a diyne. This route becomes significant in the presence of an oxidant like molecular oxygen. Additionally, amines may also reduce Pd(II) to Pd(0) through the formation of iminium cations. There is also structural evidence of the reduction mechanism of Pd(II) to Pd(0) by inorganic bases.[29]

2.7 Ruthenium-Catalyzed Azide Alkyne Cycloaddition

The Ruthenium-Catalyzed Azide Alkyne Cycloaddition (RuAAC)[30] affords 1,5-disubstituted 1,2,3-triazoles in one step and complements the more established copper-catalyzed reaction providing the 1,4-isomer. The RuAAC reaction has quickly found its

way into the organic chemistry toolbox and found applications in many different areas, such as medicinal chemistry, polymer synthesis, organocatalysis, supramolecular chemistry, and the fabrication of electronic devices.[30]

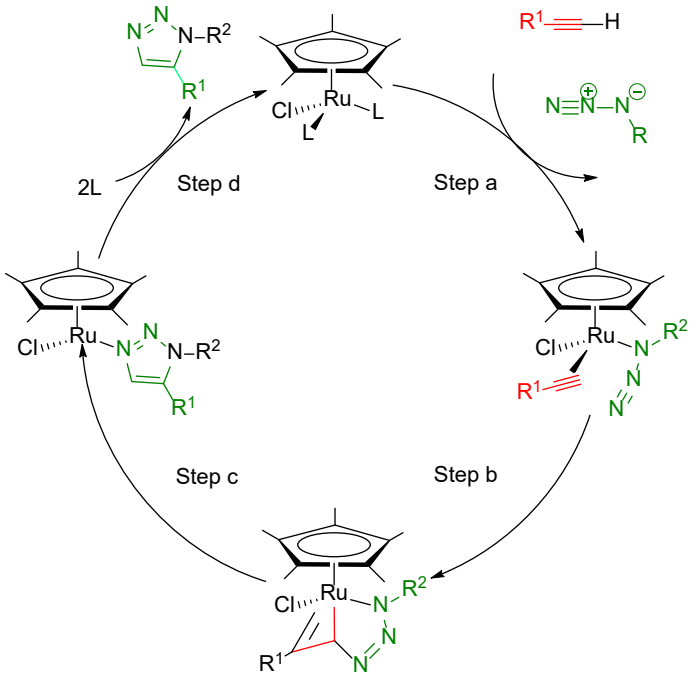


Scheme 2.3 Metal-catalyzed Azide Alkyne Cycloadditions

a) CuAAC (Copper-catalyzed Azide Alkyne Cycloaddition)

b) RuAAC (Ruthenium-catalyzed Azide Alkyne Cycloaddition)

The discovery of direct methods for synthesizing 1,4-disubstituted 1,2,3-triazoles in a single step from an organic azide and an alkyne, utilizing a Cu(I) catalyst, marked a significant breakthrough (reaction a), (**Scheme 2.3**).[30] This approach has gained widespread application across diverse domains. Subsequently, another advancement emerged, introducing a method to form 1,5-disubstituted 1,2,3-triazoles by substituting copper with ruthenium (reaction b), Scheme 2.3.[31] The choice of different catalysts influences the isomeric distribution, yielding a different substitution pattern on the triazole scaffold. These advancements contribute to the versatility and utility of triazole synthesis, allowing tailored approaches for specific applications.

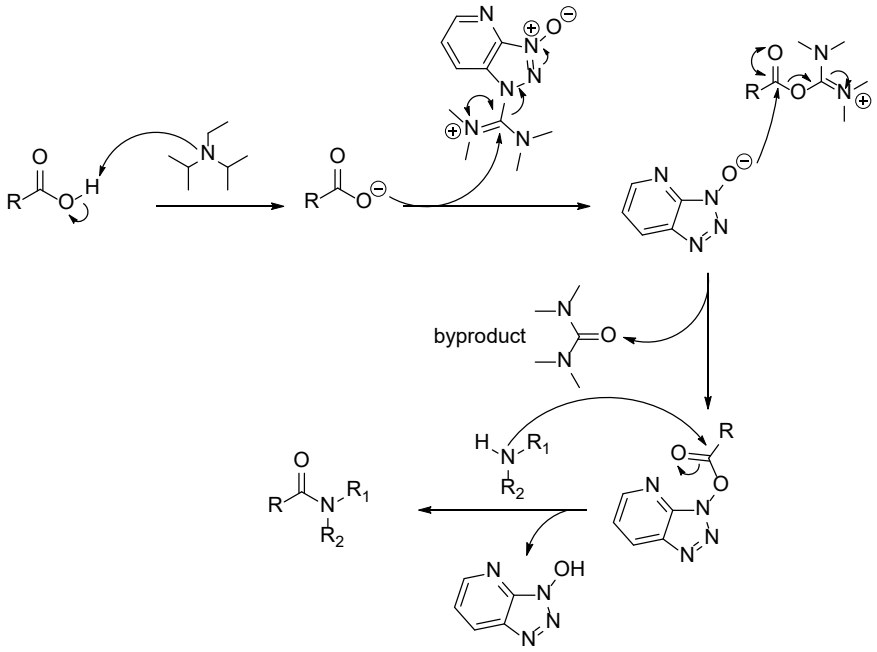


Scheme 2.4 Proposed Mechanism of RuAAC

The mechanism of RuAAC is shown in **Scheme 2.4**. The displacement of the spectator ligands (step a) produces the activated complex, which is converted, via the oxidative coupling of an alkyne and an azide (step b), to the ruthenacycle (the intermediate at the lower end of the catalytic cycle). This step controls the regioselectivity of the overall process. The new C-N bond is formed between the more electronegative and less sterically demanding carbon of the alkyne and the terminal nitrogen of the azide. The metallacycle intermediate then undergoes reductive elimination (step c) releasing the aromatic triazole product and regenerating the catalyst (step d).[5]

2.8 HATU-mediated Amide Coupling Reactions

HATU (Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium) is an important reagent used in peptide coupling chemistry to generate an active ester from a carboxylic acid. HATU is always used together with Hünig's base (*N, N*-diisopropylethylamine, DIPEA),[32] or triethylamine to form amide bonds.



Scheme 2.5 Mechanism of N-acylation using HATU

The mechanism of the HATU-mediated coupling reaction is illustrated in **Scheme 2.5**. The most commonly and commercially available iminium isomer is included in the mechanism. The reaction begins with the base DIPEA (diisopropylethylamine) attacking the acid, leading to the formation of an unstable O-acyl(tetramethyl)-isouronium salt. Subsequently, the OAt anion rapidly attacks the isouronium salt, resulting in the formation of the activated ester, and also the byproduct tetramethyl urea. Ultimately, a nucleophile, for instance, an amine, participates and adds to the OAt-activated ester, yielding the final amide product.[33]

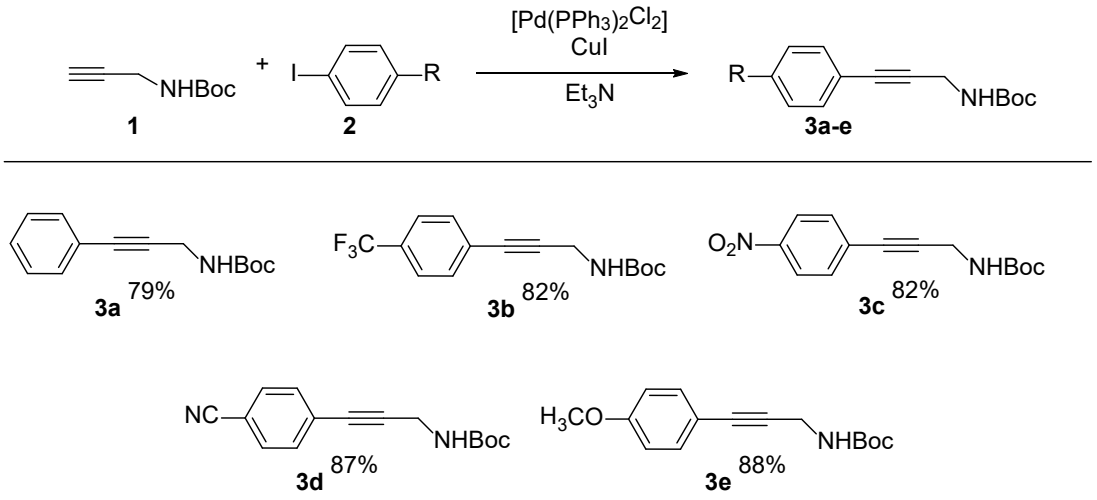
3 Methods

Several different methods for chemical synthesis and analysis were used in this project. For all the steps of the synthesis and purification, a Schlenk line (for working under an inert atmosphere), thin-layer chromatography, extraction, rotary evaporation, and either automated (Biotage Selekt) or manual flash chromatography were used. Nuclear Magnetic Resonance (NMR) (1D and 2D) was used for the characterization of the compounds that are obtained after purification. Infrared Spectroscopy (IR), and High-Resolution Mass Spectrometry (HRMS) will also be used for the characterization of the final compounds in the future. 1D and 2D NMR, fluorescence spectroscopy and possibly also ITC (Isothermal Titration Calorimetry) will be used for initial studies of the receptor binding to amino acids and small peptides.

4 Results and Discussion

4.1 Sonogashira Coupling Reactions

Sonogashira coupling reactions were carried out at the start, as this is the first reaction step in the synthetic sequence. The aim was to prepare some internal alkynes for use in the next step, i.e. the Ruthenium-Catalyzed Azide Alkyne Cycloaddition (RuAAC). Several different aryl halides, **2** (**Table 4.1**) were used as the reactants, including iodobenzene, 1-iodo-4-methoxybenzene, 4-iodophenol, 1-iodo-4-(trifluoromethyl) benzene, 4-iodobenzonitrile, and 1-iodo-4-nitrobenzene. The objective was to assess whether there are any alterations in the properties of molecules with distinct substituents. Following the reactions, the resulting mixtures underwent purification by automated flush chromatography and were then subjected to ^{1}H NMR characterization. The final alkyne products were subsequently employed in RuAAC reaction steps.



Reagents and conditions: iodoaryl compound (1.04 equiv.), [Pd(PPh$_3$)$_2$Cl$_2$] (2.0 mol%), CuI (2.3 mol%), triethyl amine (2.07 equiv.), acetonitrile. rt, 1 h.

Table 4.1 Sonogashira Coupling Reactions

Each reaction afforded products in high yields (**Table 4.1**). Notably, the reaction involving the aromatic ring devoid of any substituents, **3a**, produced the lowest yield, approximately 80%. This outcome could be attributed to this being my initial start in organic reactions, potentially leading to operational challenges that affected the yield. Conversely, the product with the methoxyl group, **3e**, exhibited the highest yield at 88%. This discrepancy may be attributed to the manual column purification method

employed for this reaction, as opposed to the automated chromatography utilized for the other reactions. Despite being more time-consuming, the manual column approach appears to contribute to a higher yield.

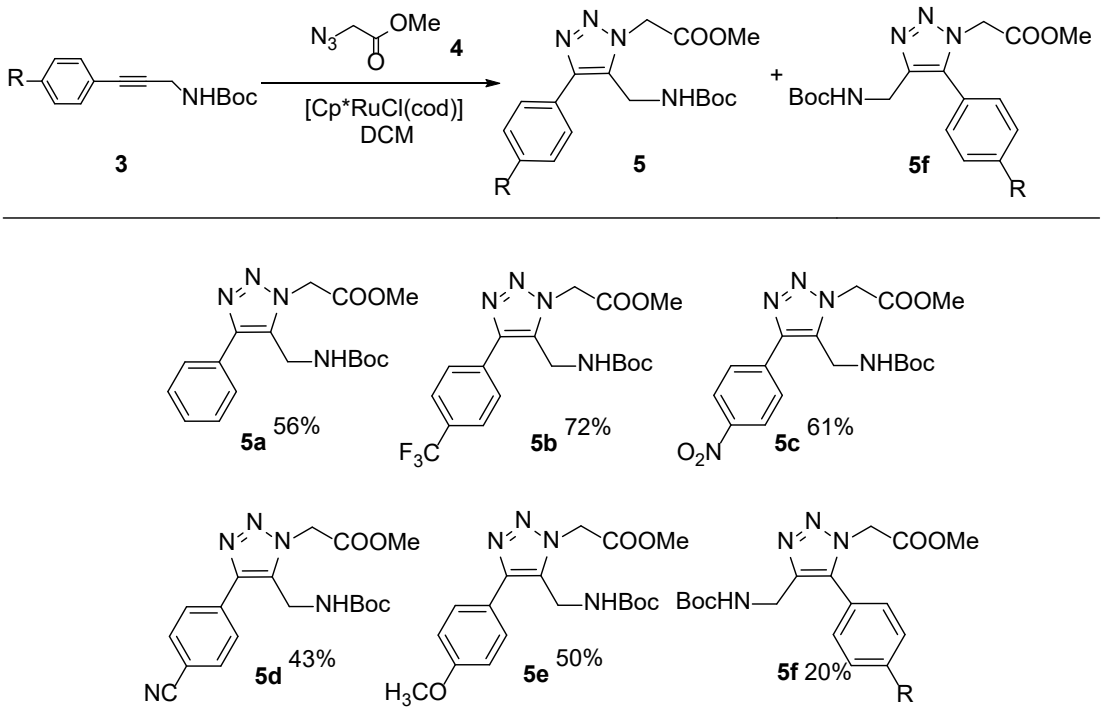
In summary, Sonogashira coupling reactions consistently yielded high results throughout the entire project.

4.2 Ruthenium-catalyzed Azide-Alkyne Cycloaddition (RuAAC)

The purified products obtained from the Sonogashira Coupling Reactions were subsequently subjected to the next reaction step, the Ruthenium-Catalyzed Azide-Alkyne Cycloaddition (RuAAC) reaction. This step aimed to produce several triazoles. The goal was to generate triazole monomers for further utilization in the formation of triazole dimers and trimers.

Following the RuAAC reactions, the resulting mixture consistently contained two 1,4,5-trisubstituted isomers. However, effective purification was achieved through flash chromatography. The purified isomers were then stored separately, and specifically, isomer I (**5a-e**) was selected for use in subsequent coupling reactions. Isomer II (**5f**) is the positional exchange of the aromatic group and -NHBoc group. The differentiation between the two isomers was achieved through the utilization of 2D NMR spectra analysis.

The synthesis of triazoles is outlined in **Table 4.2** and was conducted under a nitrogen atmosphere in the presence of the ruthenium catalyst [Cp*RuCl(cod)], dissolved in dichloromethane (DCM). The reaction involved the sequential addition of the internal alkyne **3** and azide **4** to the reaction mixture, and the mixture was stirred at room temperature for 30 minutes. Notably, the reaction mixture underwent a rapid color change, turning dark brown within a few minutes, and reaching completion after 30 minutes.



Reagents and conditions: Azide (1 equiv.), [Cp* RuCl(cod)] (5 mol%), DCM, rt, 30 min.

Table 4.2 Ruthenium-catalyzed Azide Alkyne Cycloaddition

However, the crude product consistently contained residual catalyst and two product isomers. While removing the catalyst was a straightforward task, separating the isomers posed a challenge. The proximity of the two isomers on the column chromatography led to their simultaneous elution, necessitating careful adjustment of the solvent system gradient and column length. Additionally, it is noteworthy that the quantity of isomer I consistently appeared to be approximately 10 times higher than that of isomer II. Due to the presence of byproducts, the yields in the triazole synthesis are not consistently high when compared to the Sonogashira reactions, ranging from the highest yield of 72% for **5b** to the lowest of 43% for **5d**. The existence of byproducts, as well as the purification challenges, likely contributes to variations in the overall yields of the reactions.

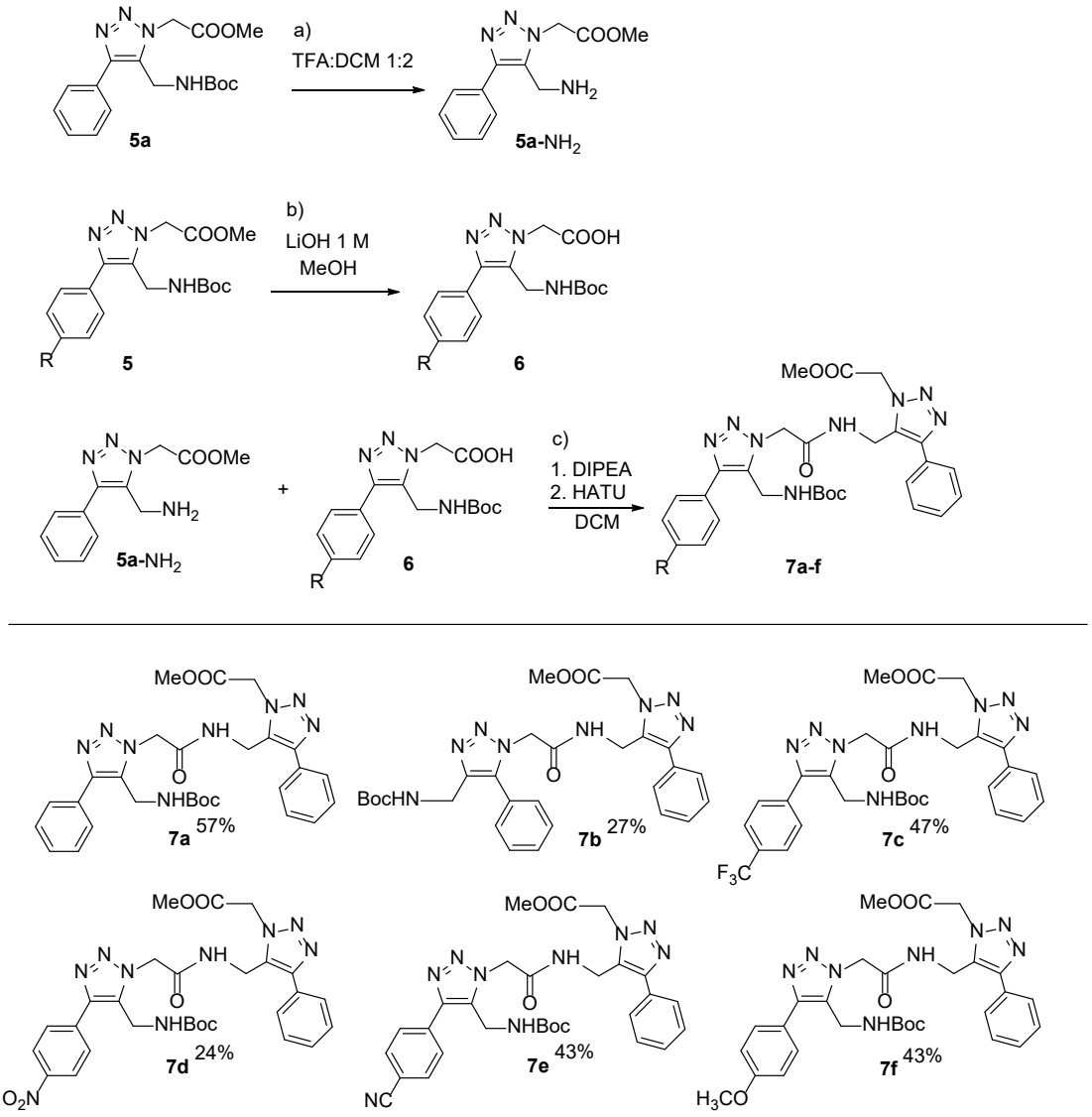
4.3 HATU-mediated Amide Coupling Reactions

4.3.1 Synthesis of Triazole Dimers

Following the synthesis of various isomers, the *tert*-butoxycarbonyl (-Boc) and ester groups underwent distinct deprotection processes, utilizing trifluoroacetic acid (TFA)

for amine formation and lithium hydroxide (LiOH) for acid formation. This deprotection resulted in the creation of acid and amine functionalities. Subsequently, acid-amine coupling reactions were carried out to synthesize the triazole dimers. The coupling reaction needed a meticulous approach. Firstly, the acid was introduced into the flask and dissolved in dichloromethane (DCM). Subsequently, the base DIPEA was added and the mixture was stirred for several minutes. Following this, HATU was introduced to facilitate the formation of the activated ester. Finally, the amine was added to complete the synthesis of the triazole dimer. This stepwise procedure ensures the controlled and efficient progression of the coupling reaction.

The synthesis of triazole dimers is detailed in **Table 4.3**. The deprotection of the Boc group consistently proceeded smoothly. The TLC results after 1 hour confirmed the completion of Boc deprotection. Also, the only workup required was to place the reaction flask on the rotary evaporator at 60°C for 15 minutes for effective work-up. On the other hand, the deprotection of the ester group posed challenges, occasionally leaving some starting materials even after 1 hour. To address this, additional LiOH was sometimes necessary, and in the work-up step, equimolar amounts of HCl were needed to neutralize the base.



Reagents and conditions: a) TFA (11.6 equiv.), DCM, rt, 1 h; b) LiOH 1 M (1.56 equiv.), MeOH, 1 h; c) DIPEA (4.00 equiv.), HATU (1.05 equiv.), DCM, rt, 5 h.

Table 4.3 Synthesis of triazole dimers

The coupling reactions were carried out under a nitrogen atmosphere, yet yields remained consistently low. The highest yield observed was for **7c** at 47%, while the lowest was for **7d** at 24%. Possible explanations for these low yields include incomplete reaction durations, as evidenced by TLC results showing residual starting materials even after 5 hours. Moreover, active hydrogens in the acid and amine may form hydrogen bonds with the silica gel during flash chromatography, complicating the separation process and leading to lower yields. Additionally, ^{1}H NMR spectra indicated the presence of impurities in the final product after column chromatography.

In the context of HATU-mediated coupling reactions, the byproduct tetramethyl urea is always present after column chromatography, proving difficult to eliminate. However, by integrating a washing step into the extraction process, the byproduct does not appear in the NMR spectra. Therefore, we have to conduct an additional washing step into the work-up procedures.

Additionally, in the reaction step, the need for acid (HCl) to neutralize the base (DIPEA) after overnight stirring is a common point. Instead of adding an equivalent amount of base, which might degrade during the reaction or have been imprecisely added previously, it is advisable to verify the pH value using pH paper. $NaHCO_3$ can be added to fine-tune the pH to approximately 7, thereby reducing product loss in the subsequent washing and extraction phases.

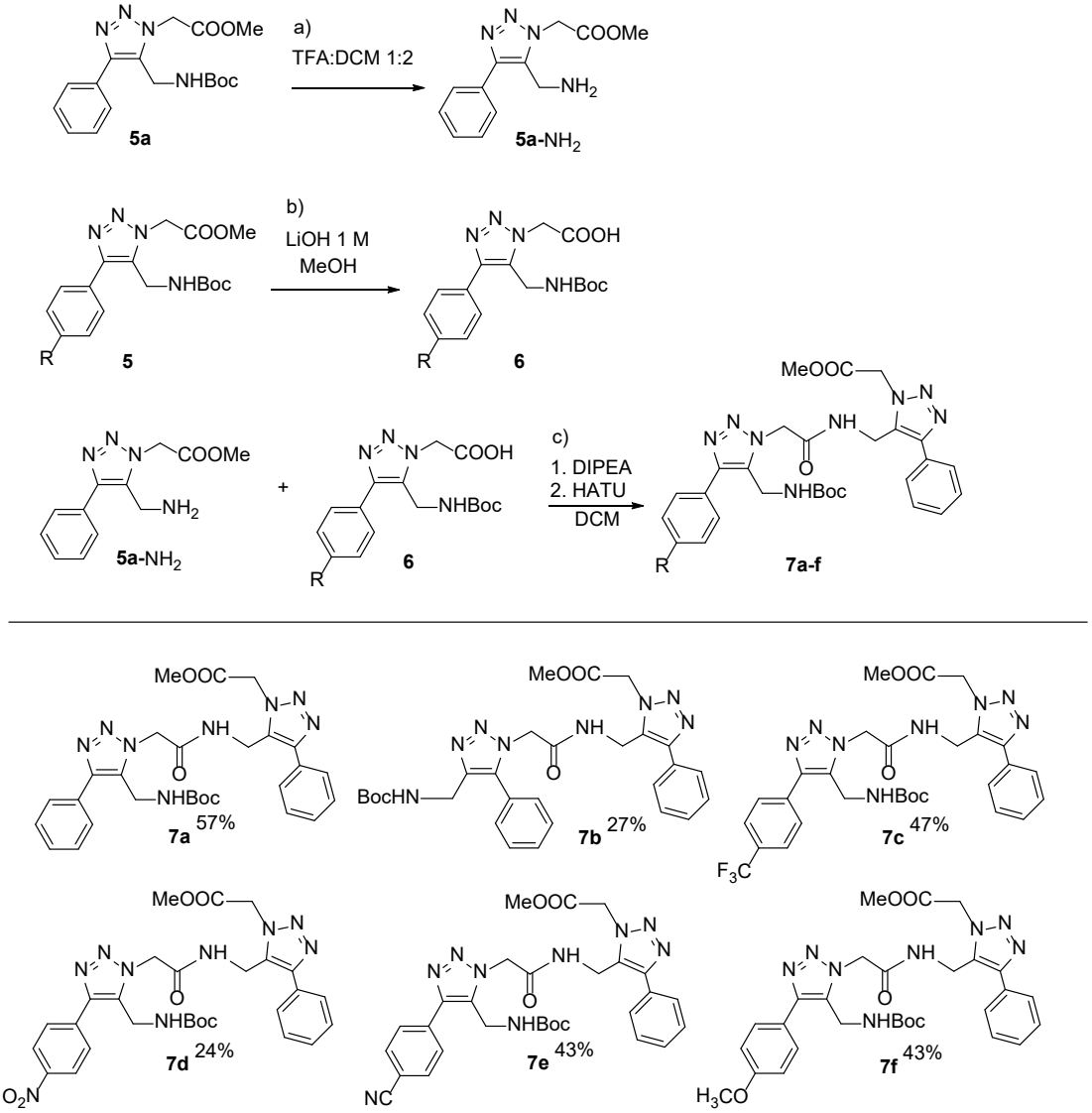
4.3.2 Synthesis of Triazole Trimers

After several dimers were synthesized, *tert*-butoxycarbonyl (-Boc) and the ester groups were deprotected separately, generating the corresponding acid and amine. Then, the acid-amine coupling reactions were carried out to synthesize the trimers. The deprotection of the Boc group was always conducted on the dimer, since fewer steps are required for the work-up in this case. This will also help to minimize the loss of product during the work-up. Also, the deprotection of the ester group should be carried out on the triazole monomer. Every procedure used was the same as for the synthesis of triazole dimers.

The synthesis of triazole trimers is detailed in **Table 4.4**. Similar challenges were encountered in the deprotection process as observed in the synthesis of dimers. The yields, unfortunately, consistently remained low, with the highest observed for **9c** and **9e** at 37%, and **9b** at 16%. This could be attributed to potential interaction between the product and silica gel via hydrogen bonds, as well as incomplete reactions. It is reassuring to note that the NMR spectra confirm the high purity of each triazole trimer, despite the challenges encountered with the overall yield.

Moreover, the polarity of the triazole trimer significantly increased, prompting the use of a DCM/MeOH 10:1 solvent system for TLC analysis, and a DCM/MeOH 20:1

solvent system for column chromatography. This adjustment was likely necessary to achieve optimal separation and visualization of the compounds.



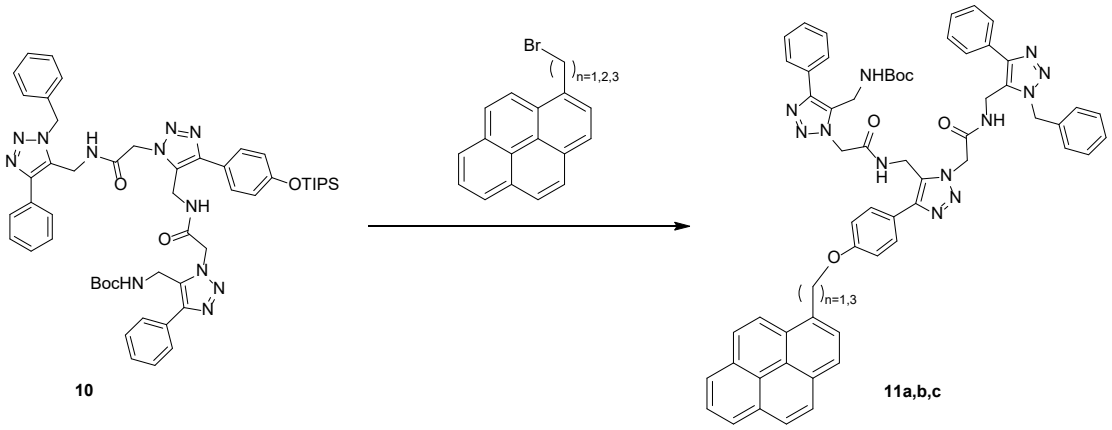
Reagents and conditions: a) TFA (11.6 equiv.), DCM, rt, 1 h; b) LiOH 1 M (1.56 equiv.), MeOH, 1 h; c) DIPEA (4.00 equiv.), HATU (1.05 equiv.), DCM, rt, 5 h.

Table 4.4 Synthesis of triazole trimers

4.4 Functionalization of Graphene

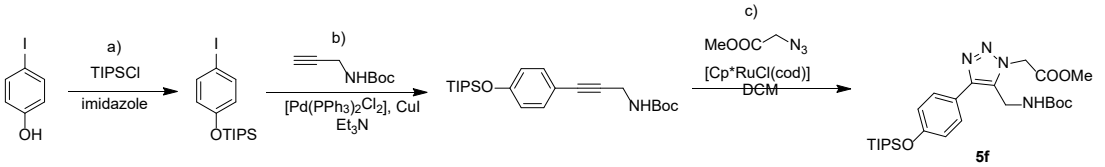
We would like to functionalize the synthesized dimers and trimers with a polyaromatic structure, both to enhance the UV/vis and fluorescence signals, but also to evaluate the interaction of the triazole trimers with a graphene surface. To pursue this idea, we aimed to functionalize the trimers by appending to them a molecule that can serve both purposes, i.e. a pyrene. Pyrene is known to interact with graphene itself via π-stacking[34]

allowing the detection of electron density changes in an eventual final graphene-based sensor. To synthesize such a target molecule, we planned to functionalize commercially available 1-(bromomethyl)-pyrene and 1-(bromopropyl)-pyrene with trimer **10** to obtain the molecules **11a**, **11b**, and **11c** (n=1,2,3).



Scheme 4.1 Functionalization of bromomethyl/bromoethyl/bromopropyl pyrene with triazole based trimer

To achieve this, we initiated the synthesis of trimer **10**. We first synthesized the three different monomers required. Monomer **5a** was synthesized according to the procedure in **Table 4.2**. The syntheses of monomers **5f** and **5g** are shown in Schemes **4.2** and **4.3**, respectively. For monomer **5f,** we started with TIPS protection of the hydroxy-functionality in 4-iodophenol. After obtaining the desired internal alkyne **12** via a Sonogashira coupling, we performed a RuAAC reaction, yielding triazole **5f** in a 60% yield.

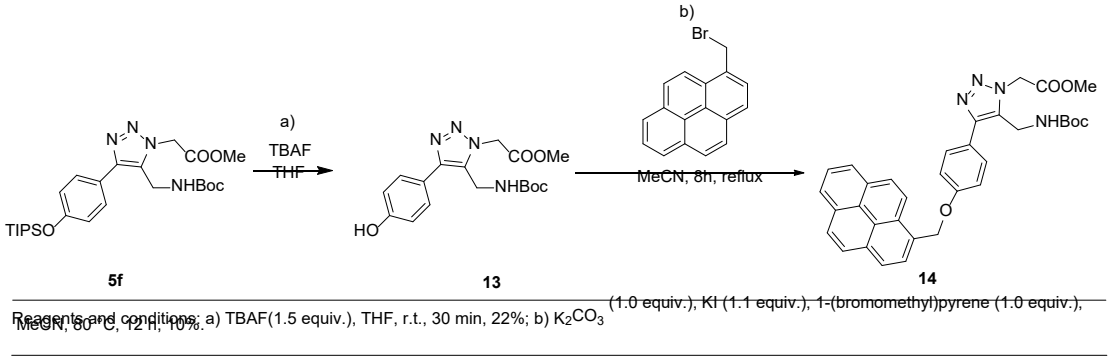


Reagents and conditions: a) TIPSCl (1.00 equiv.), imidazole (2.27 equiv), DCM, rt, 16 h, 68%; b) iodoaryl compound (1.00 equiv.), $[Pd(PPh_3)_2Cl_2]$ (2.0 mol%), CuI (2.3 mol%), triethyl amine (2.07 equiv.), acetonitrile, r.t., 1 h, 70%; c) Azide (1.00 equiv.), [Cp*RuCl(cod)] (5.0 mol%), DCM, r.t., 30 min, 56%;

Scheme 4.2 Synthesis of triazole monomer **5f**

After obtaining triazole **5f,** we tried to couple it with 1-(bromomethyl)-pyrene, both to try the reaction and to explore the fluorescence of the final molecule, to have an idea of what to expect with the subsequent attachment of the trimer to the pyrene (**Scheme 4.3**). To do that, we deprotected the phenol with TBAF and performed a Williamson

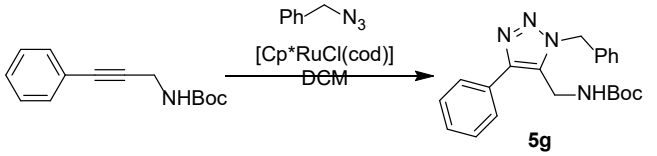
etherification on 1-(bromomethyl)-pyrene, which allowed the formation of compound 14a in 10% yield.



Scheme 4.3 Coupling of triazole **5f** with 1-(bromomethyl)-pyrene

We are currently investigating the reasons behind the low yields in the deprotection step (22%) and in the etherification (10%). For the etherification, we attribute the results to the fact that the reaction as yet has only been run once, using a very small amount of starting material. On the other hand, the deprotection of the TIPS group with TBAF is what raised more doubts on our behalf, given that it should proceed smoothly and with almost quantitative yield. After a thorough NMR analysis of the crude product, we suspect that the TBAF favored not only the deprotection of TIPS, but also the hydrolysis of the methyl ester substituent in position on the triazole. Even if this is very unusual, we found an earlier example in the literature where TBAF was able to catalyze the deacylation of cellulose ester.[35] Mechanistic studies on this novel transformation have shown that the fluoride ion released from TBAF can deprotonate the acyl moiety, giving a ketene intermediate that can then react with water to afford the acid, with the source of the water coming from adventitious water in the solvent or from the hydration of the TBAF.[36] Having considered this as a possible side reaction, we decided to continue with the synthesis of the trimer and we chose triazole **5g** as the third monomer, where we substituted the methyl ester moiety with a benzyl group.

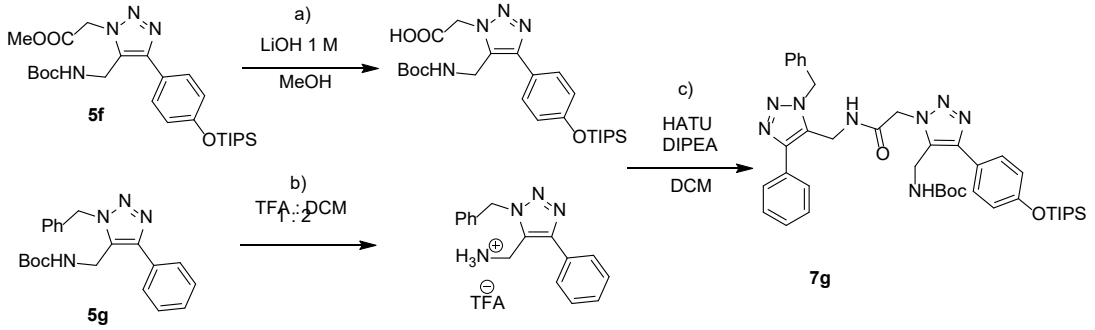
For compound **5g**, the starting alkyne was obtained following **Table 4.1** and subsequently used in the RuAAC reaction involving benzyl azide, affording the final triazole **5g** in 57% yield (**Scheme 4.4**).



Reagents and conditions: Azide (1.0 equiv.), [Cp*RuCl(cod)] (5 mol%), DCM, rt, 30 min, 57%.

Scheme 4.4 Synthesis of triazole **5g**

Having all the building blocks at hand, we are now currently pursuing the synthesis of the corresponding trimer. First, we hydrolyzed the methyl ester of monomer **6a** and deprotected the amine of monomer **5f** to access dimer **7g** after coupling (Scheme **4.5**). The obtained yield was 20% and this is probably due to the fact that this reaction has only been performed once, as this part of the project is still ongoing. Even with such a low yield, thanks to the fact that we started from a large amount of starting materials, we were able to continue with the trimer synthesis.



Reagents and conditions: a) TFA (11.6 equiv.), DCM, r.t., 1 h; b) LiOH 1 M (1.56 equiv.), MeOH, r.t., 1 h; c) DIPEA (4.00 equiv.), HATU (1.05 equiv.), DCM, r.t., 5 h, 20%.

Scheme 4.5 Synthesis of dimer **7g**

The next step in our synthesis will be to couple dimer **7g** and monomer **5a**. This step is currently ongoing. After finishing the synthesis of the trimer, we will perform the TIPS deprotection and finally the attachment to both 1-(bromomethyl)-pyrene and 1-(bromopropyl)-pyrene to access compounds **11a**, **11b**, and **11c**.

5 Conclusions and Outlook

In conclusion, we have developed an entirely new class of triazole-based trimers as potential synthetic receptors. These molecules are currently being investigated in solution for their interactions with selected biomolecules and metal ions. Additionally, we are working on the functionalization of pyrene with trimer **10**. If it is successful, this could yield a compound with potential sensing abilities, marking a significant step toward developing a biosensor.

Once we have synthesized the target molecules **11a**, **11b,** and **11c**, we will attempt to functionalize a graphene surface with these compounds. After selecting an appropriate solvent (typically acetonitrile or methanol), we will use the CVD graphene chips (provided by the Yurgens group at MC2 and produced in the clean room) for several hours in a 0.01 M solution of **11a**, **11b,** or **11c**. These chips will then be collected and characterized at MC2. Initially, we will examine how the π-stacking of our molecule affects the conductance of graphene, which should remain unchanged after binding to ensure optimal sensing performance and efficient detection. At a later stage, the synthetic receptor molecules **11a**, **11b**, and **11c** on graphene will be used to identify a biomarker derived from the antibiotic-resistant bacterium *Streptococcus pneumoniae.* The results obtained will be compared with those from another sensor that uses graphene functionalized with antibodies. Additional characterization efforts, such as 2D-NMR, High-Resolution Mass Spectroscopy (HRMS), and Single Crystal X-ray Diffraction (SCXRD), are warranted for the synthesized triazole trimers and the resulting biosensors.

6 Challenges and Solutions

In synthetic routes, purification consistently stands out as the most challenging and time-consuming aspect. There are instances where products remain impure even after column chromatography, necessitating additional purification steps. In the context of this project, while purification is generally manageable, the synthesis of trimers posed difficulties. The NMR spectra always exhibited impurities, presenting challenges in achieving a high degree of purity.

Adding to the complexity, an automated column chromatography system experienced a serious malfunction within the first month of starting the project, and replacement of the instrument took some time. However, an unexpected turn of events revealed that the trimer purity significantly improved when manual column chromatography method was employed instead. Subsequently, recognizing the effectiveness of the manual column, it became the preferred purification technique for the trimer synthesis, as well as for the separation of triazole isomers after the RuAAC reaction. Sonogashira coupling reaction, however, the automated column chromatography is still the best solution for purification, and it saves a lot of time.

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