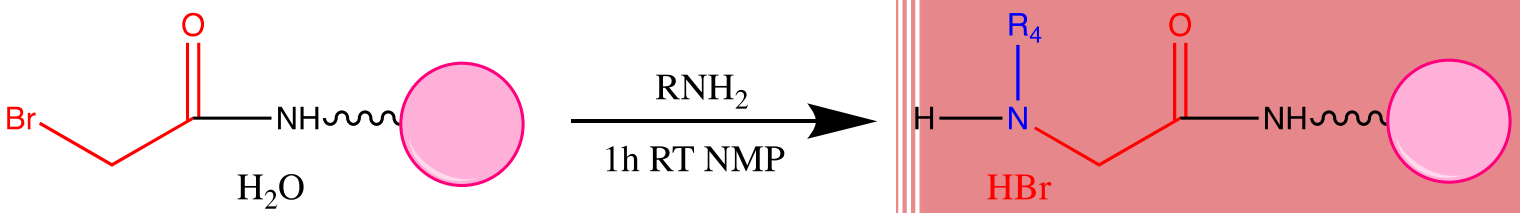
Synthesis of potential antimicro-bial peptide mimetics, peptoids



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Abstract

During the last 10years, natural macrocyclic peptides have been tested as antimicrobial drugs against E.Coli, ect, with positive results. Because peptides are naturally occurring, infectious diseases are prone to become resistant to them the same way some of them have become resistant to common antibiotics. Therefore, researchers are looking for new potential treatments for these diseases. A good candidate for a future treatment is the peptidomimetic, peptoid, due to their much more stable chemical structure. They mimic the properties of antimicrobial peptides by having the sidechains on the nitrogen instead of the chiral carbon.

In this project peptoids are synthesized by using sub-monomer solid phase peptoid synthesis. A manual protocol was followed and included activation of resin (deprotection), bromoacetylation, displacement (amine addition) and cleavage of the product from the resin. This is done to try and retrieve pure peptoids. Solid phase synthesis allows a generation of a linear structure compared to the solution phase synthesis, where cyclization of a heterocyclic precursor is involved. In this project, we synthesized 6 peptoids. To analyze the peptoids we used HPLC-MS to identify, purify and quantify the components in the sample. The peptoids were identified even though some errors occurred with the synthesis which may have affected the purity of the peptoids.

Table of contents

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1 Introduction

Today scientists all over the world are researching in new ways to expand the treatment available, especially, within the antimicrobial sector and more specific, antibiotics. It has become highly relevant for scientists to find new classes of compounds to combat bacterial infections, because there is a rising occurrence of these infections becoming resistant to common treatments i.e. antibiotics. One of the new medicinal classes that has been approvingly developed within the past years are antimicrobial and host defense peptides which have shown exceedingly potential and therefore becoming a potential class of antibiotics[1].

To make this treatment more advanced and effective its relevant to look at a peptidomimetic called peptoids. Peptoids mimic the properties of peptides but offer better stability and therefore could replace the action of peptides upon further research[2].
Peptoids have been successfully used in vitro (in lab) for inhibiting bacterial growth such as *E. Coli* [3].

Biofilms are another area where research have shown promising development. *Pseudonomas aeruginosa* is a fatal pathogen, and especially people who suffer from Cystic Fibrosis are chronically infected by this pathogen, up to 80 % in the US alone[4]. In 2010 Kapoor et al. shared positive results, that peptoids caused maximum reduction in the biofilms and therefore are peptoids a possible new treatment in this area[4]. The rapidly expending research filed on the antimicrobial activity of peptoids makes them good candidates for future potential antibiotics.

All this leads to the purpose of this study being to explore the peptidomimetic, peptoids, as we learn how to design these for a specific purpose and synthesize them with the method; solid phase synthesis.

2 Background

The following chapters will describe and compare the different principles and properties of the synthetic peptide mimetics, peptoids, and natural occurring peptides. The process of synthesizing peptides and peptoids are very similar and it will be compared and look at the differences.

2.1 Peptoids vs Peptides

Peptides are built from short monomer chains of amino acids. A peptide is any group of organic compounds containing two or more amino acids that are linked by a peptide bond (amide bond) (-COOH and -NH_2)[5].

Peptides can come in all sizes, the smallest being two amino acids joined by a substituted amide linkage, called dipeptide[6].

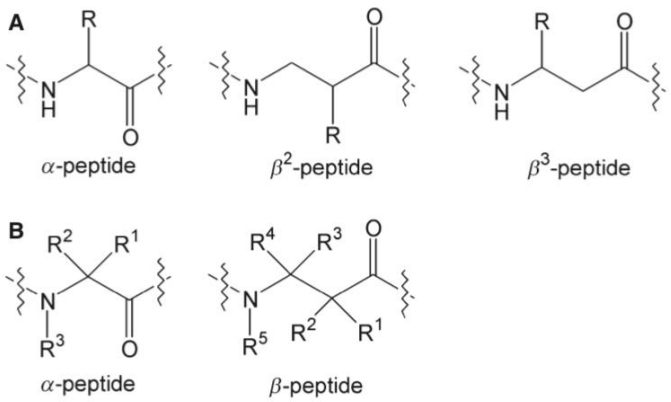


Figure 1, different backbone structures of peptide mi-

Looking at the structure of peptides, the placement of the sidechains decides whether it is an α- or β-structure which is shown on figure 1[7]. When a few amino acids are joined by peptide bonds the structure is an oligopeptide and when many amino acids are joined this way the structure is a polypeptide[6]. Looking at the simple structure of a peptide the amino acid residue at the end with the free α-amino group is the amino terminal residue (the *N*-terminal). The other end has a free carboxyl group and is called the *C*-terminal[8]. Figure 2 shows the different terminals highlighted and the groups in blue show the group that defines the terminal.

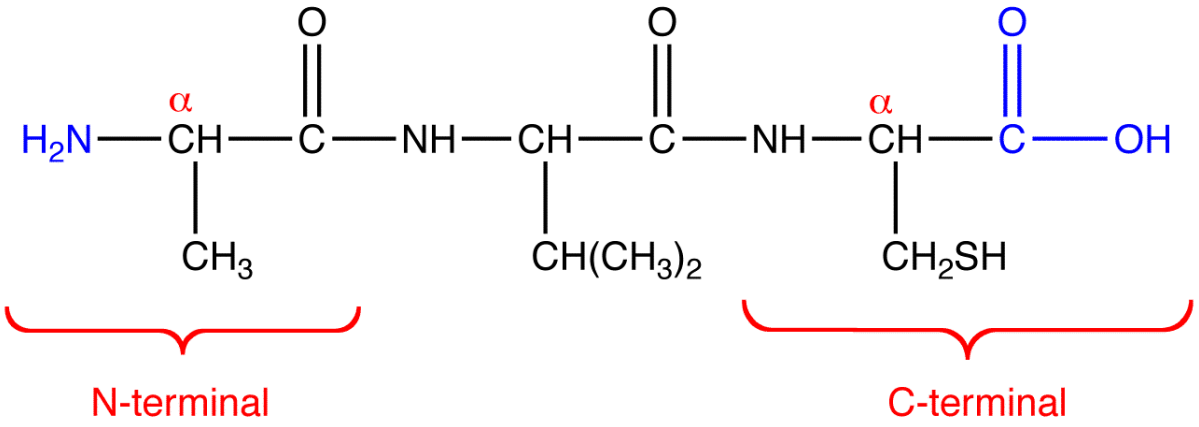


Figure 2, peptide where the terminals are highlighted (drawn in ChemSketch)

A peptoid is a peptidomimetic and is structurally very similar to a peptide[9] . The difference lies within the placement of the R-groups, the chirality at the alpha carbon monomers and the H-groups as seen on figure 3[10]. A peptoid is a manipulation of the chemical structure of a peptide in order to design a more compatible and useful structure for the desired area, an example could be to enter a cell[11]. Another difference is the chirality of the backbone. Chirality is when at a Carbon have four different substituent groups. So now when the R group of the carbon in peptides is moved to the Nitrogen then that carbon in peptoids has two hydrogens and two other bonds. This is not a chiral carbon anymore. Chirality gives the carbon the possibility to rotate and form isomers. In peptoids that only isomers are cis and trans but around the carbon atom they cannot rotate[12].

Hydrogen bonding is absent since the hydrogen on the amine is displaced with the R-group. Peptoids can therefore not form hydrogen bonds from the amine in the backbone. Due to the placement of the R-groups there is no hydrogen-bond donor, peptoids will have some of the same qualities as peptides, but it can also have many functions a peptide will no[1]
t. It can be altered the way that is necessary and since it not natural it does not have an exceeding limit[7].

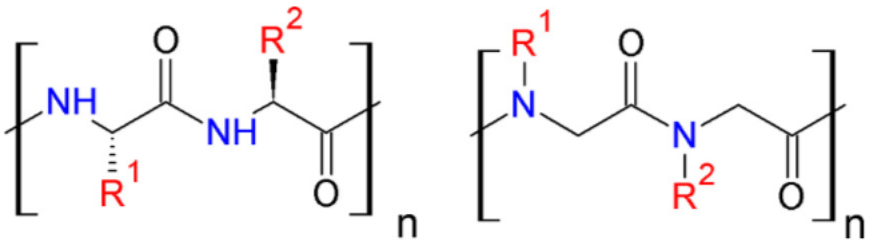


Figure 3, Chemical structures of peptide (left) and peptoid (right)1.

N-substituted glycines can be looked at as amino acids where the one sidechain is attached to the nitrogen amine instead of the α-carbon. Oligomers of these building blocks are the so called α-peptoids. Similarly, if the *N*-substitutes are β-glycines the building blocks then the peptoids are named β-peptoids just as in peptides[7].

When synthesizing a peptoid, it is important to have a chemical library of building blocks for the synthesis that is going to be used in the lab. A bigger library of building blocks result in better peptoids since then the peptoid will offer higher structural diversity and more exact ways to build the peptoid that is needed. One of the reasons to develop peptoids is that they are not as easily degradable as peptides are. This is a positive alteration since they will last longer and can fight bacteria without being degraded[12].

Peptoids have most of the same qualities and abilities as peptides, therefore when studying their function some of the theory will be the same as for peptides[11]. Scientists have shown that even though peptides and peptoids have the exact same structure, they will have different chiral properties[12,13].

2.2 Biologically active peptoids

Both peptides and peptoids have diverse biological activities. Studies have shown that they have anticancer, anti-viral, anti-fungal etc. properties. In this section we will focus our attention to the antimicrobial properties of peptides and peptoids. Other areas where peptoids have been applied for new medicine are areas within treatment of HIV, herpesvirus and influenza[14].

2.2.1 Antimicrobial peptides and peptoids

A new approach within the antimicrobial peptide (AMP) system is to try and develop AMP mimicry. This is a field where peptoids are particularly well suited, since they are easily synthesized based on solid phase synthesis, and still have access to sequences at a respectively low cost[12]. Antimicrobial peptides are a part of an innate immunity that evolved millions of years ago in all living systems[14]. The antimicrobial effect on gram-negative bacteria has been cationic peptides attaching to the organism. These naturally occurring peptides therefore be highly relevant in the antimicrobial research. Therefore, these can be highly useful in the development of antimicrobial research[14].

In the human body AMP's serve as effector molecules which are small molecules that bind to a specific protein and regulate biological activity[8] in areas such as inflammation, immune activation and healing processes[14]. One of the positive things about peptoids is, that they can be built exactly the way they are needed[15] and that any chemical functionality can be added by the given amine for the wanted effect[12].This library can consist of countless amines, functional groups and so on. An example can be seen on figure 4.

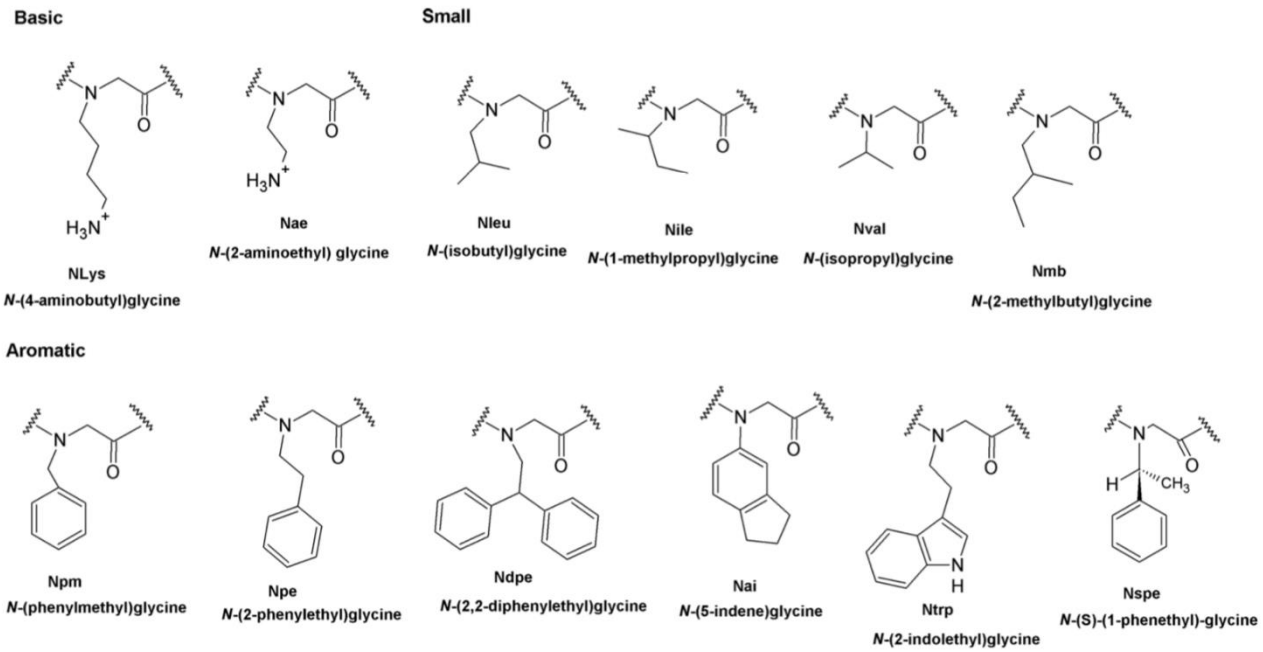


Figure 4, monomers used to build peptoids1(image adapted from (reference) with permission)

Peptoids, with their properties, represent a new generation of chemically diverse libraries of novel molecules[10].

Even though there has been a great success in developing novel antimicrobial compounds, the resistance continues to spread worldwide. It has been shown that a significant number of human AMP's have a universal structure familiar to human defensives (cat-ionic protein) and cathelicidin (polypeptides stored in the lysosome). And a manipulation of these universal structures show a promising new way to design new drugs to prevent and treat systemic and tropical infections[14].

These libraries that are developed several places are used to map out epitopes for different qualities like, antibody bonding, discern ribonucleotide sequences that have specific catalytic or binding activity and to provide initial leads in receptor based assays[10]. The advantages with the peptoids are that there is a limitless diversity in the production of these based on their modular structure and qualities, and they can easily be synthesized and sequenced and furthermore they inherit biological relevance[15].

2.2.2 Design of antimicrobial peptoids

When an antimicrobial peptoid is designed it is important to analyze the peptoids itself but also what it is needed for. So, when the peptoid must enter a gram-negative cell, charges in the

sidechains are needed so that the peptoid is able the enter the hydrophilic cell membrane, but it also must be able to live in the hydrophobic environment inside the membrane. Therefore, the design of the peptoid needed is done by where the peptoid is applied. A peptoid is thereby not given done, the person researching is making his/her own peptoids along the way.
When designing the synthesis for a peptoid it is important to know where it should be used, what environment (negative/positive) and what functional groups there should be a part of this peptoid[16].

Gram-negative bacteria consist of a negatively charged outer membrane with lipopolysaccharides on the extracellular membrane and phospholipids on the intracellular membrane. This type of bacteria has also a cytoplasmic membrane only consisting of phospholipids. Antimicrobial peptides and peptoids can inhibit the growth of this group of bacteria by entering through the both membranes and creating pores in the membrane while inhibiting membrane reconstruction in the cell. The cell therefore starts leaking and ends up lysing.[17] This process is shown in figure 5.

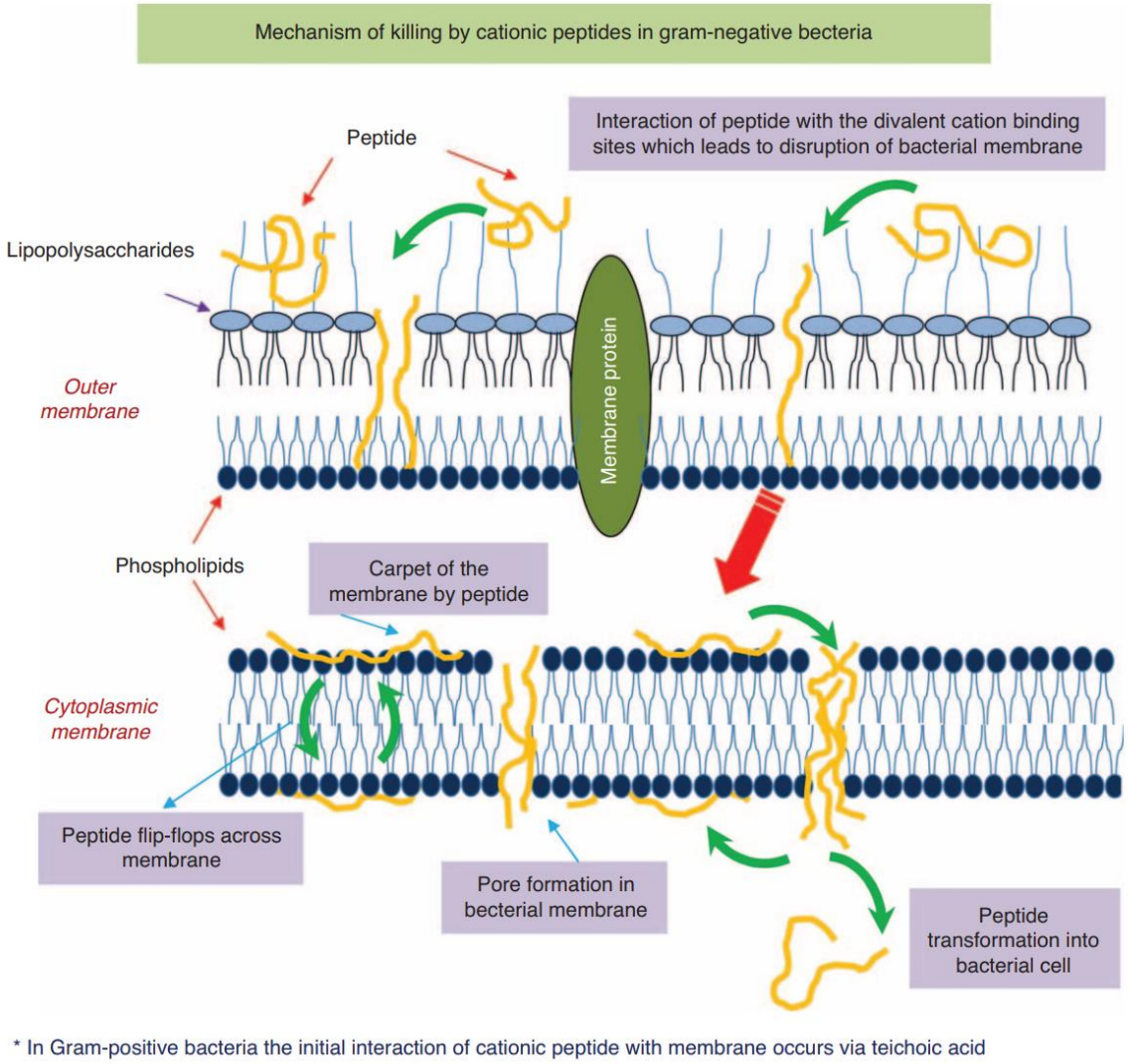


Figure 5, illustrates the process in which an antimicrobial peptoid enter the cell membranes of a gram-negative bacteria and kills it my creating pores in said membranes[17].

The reason for using antimicrobial peptoids is that the AMPs are met with resistance due to their lack in metabolic stability. One thing the peptoids are good for is their persistency against degradation by proteases[7].

One of the reasons why antimicrobial peptoids are important in the biological world is because as by right now there are many antimicrobial groups that are becoming resistant due to overuse. If a person receives too much antibiotics or other similar drugs, the infection can develop a resistant towards that specific drug, and next time they will receive another treatment. This process keeps repeating itself and, in the end, there will not be any treatment left. Therefore, a new way to develop new drugs is very important.

In this study the focus is to synthesize peptoids with antimicrobial properties against gram-negative bacteria with the following properties.

Charge

When creating a peptoid that attack gram negative bacteria it should have a positive charge to react with the negative charge of the membrane of gram-negative bacteria. By having this charge, it will not react to membranes without charge or gram-positive bacteria. This is a way to limit the search to exactly the bacteria we wish to kill and therefore avoid damaging healthy cells[14].

Aromatic and hydrophobicity

The aromatic group will affect the cells proteolytic stability, which can result in the breakdown on peptides in the cell and therefor inhibits the cell from rebuilding the membrane[17]. The group can also affect the solubility of the peptoid in the body and keeps it from folding and ruining the structure of the peptoid. Aromatic compound can also be hydrophobic. Microbial membranes have a hydrophobic inner character and it is for small molecules to penetrate through and interact with the hydrophobic core, there for it need to be hydrophobic[18].

2.2.3 Development in Antimicrobial peptoids as drugs

Since the discovery that peptoids can be applied for further development against many known bacteria, recent discovery has shown that infections caused by *E. Coli* can be treated by a valid antimicrobial agent[3] [19]. These discoveries are still undergoing further research, but the researches keep getting closer and closer to new medicine for already known infections that are close to- or already resistant[20].

But research in developing peptoids to drug is not only relevant in the human world. Looking at new ways to find cures for animal diseases such as Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP)[21] is one of the new areas where peptoids can be found useful.

2.3 Synthesis of peptoids

There are several ways to synthesize peptides and peptoids. One of the synthesis protocols that is used in this project utilizes the use of solid phase chemistry. Synthesis of a peptoid share similar principles with synthesis of peptides but since the chemical structure is different a lot of the solutions will be as well.

Figure 6 provides an overview on the different synthesis protocols of peptoids, and the following chapter will elaborate on how the different types of synthesis work.

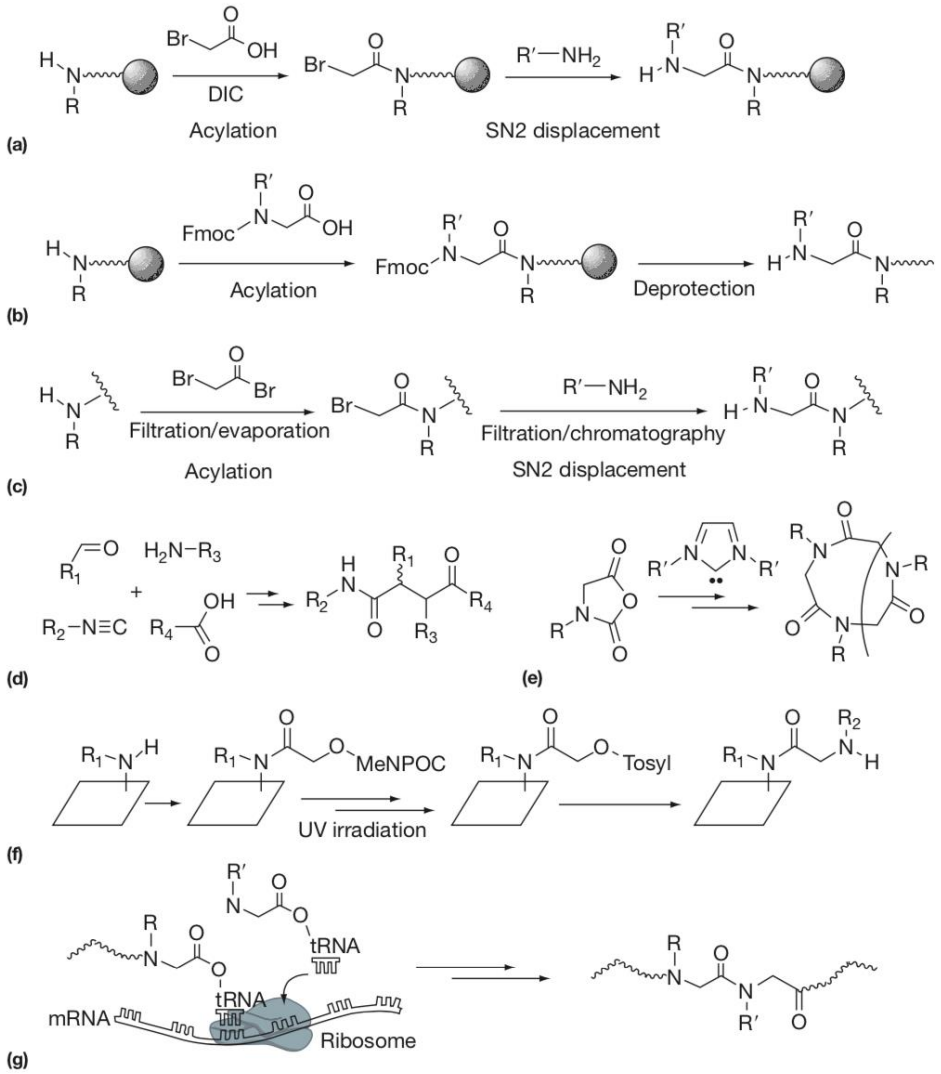


Figure 6, overview on different ways to synthesize peptoids. (a) Solid-phase synthesis: sub-monomer method. (b) Solid-phase synthesis: Fmoc-method. (c) Solution-phase synthesis: bromoacetylbromide-method. (d) Solution-phase synthesis: Ugi four-component reaction. (e) Solution-phase synthesis: amine-initiated ring-opening polymerization. (f) Photolithographic synthesis. (g) In vitro ribosomal synthesis[18].

2.3.1 Solid-phase synthesis vs solution phase synthesis

Solid-phase peptide synthesis (SPPS) [22], has been used to add an attachment step which links the peptide chain to an insoluble polymeric ingredient, resin. To synthesize a peptide, a bond between two amino acids must been formed.

A comparison of the two synthesis can be seen on figure 7 below. The reactions in SPPS are driven using the soluble reagents and can be removed by filtration and washing steps without any recommendable lose. When the chain elongations are done and completed, the peptide will be released from solid support, the resin[22].

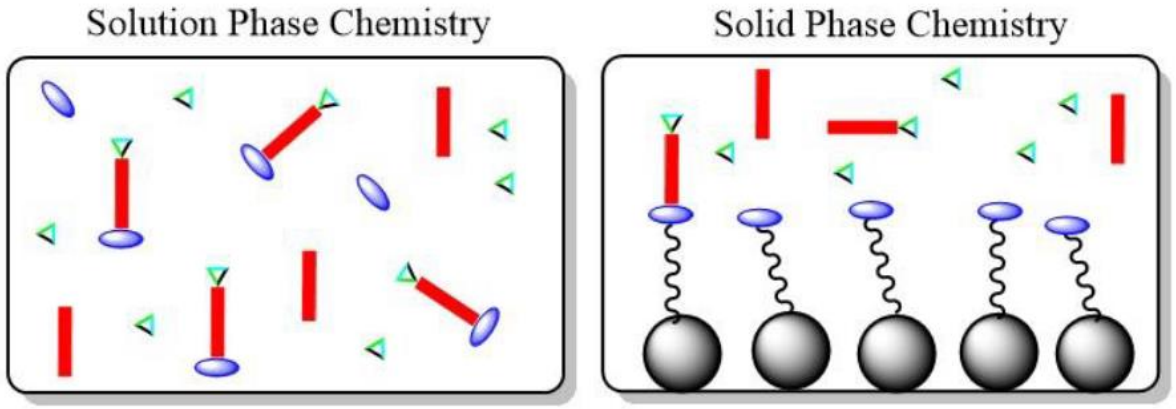


Figure 7, comparison of solution phase synthesis and solid phase synthesis[11]

Solution-phase synthesis is interesting since there are more reactions optimized in this synthesis. First, it was used for easily synthesized compound classes such as amides and urea [23]. Today it has more interest because some of advantages such as there is more reaction which is itemized in solution phase. And there are no limitations of the thermal of the chemical stability of the linker or the resin. The synthesis consists of 1 or 2 steps and the reaction in the solution needed considerably less time. Something which is different from the other synthesis, is that the reaction volumes are smaller in relation to the amount of the product[18].

2.3.2 Resin

A resin is normally used in Solid-phase Peptide Synthesis and have a matrix of polystyrene which is cross-linked with divinylbenzene 1%. The resin can be a natural or a synthetic organic compound which is consisting of viscous liquid substances or a non-crystalline.

The compound is insoluble in organic solvents which is why it swells in solvents like dimethylformamide, DMF or di-chloromethane, DCM. The swelling step for the resin in the experiment is a very important step because the reaction kinetics will be diffusion controlled. If a resin will swell too much it will have a higher diffusion rate of reagents into the core of the matrix which results in a shorter reaction time and there for a more complete chemical conversion[23]. Natural resins can be classified as oil- and spirit soluble. Not all resins are similar, but something common for all of them is the reactive site loading level. The resin used in this synthesis is ring amine MBHA resin with a Fmoc protecting group, see figure 8 and have a loading capacity of 0.4mmol/g.

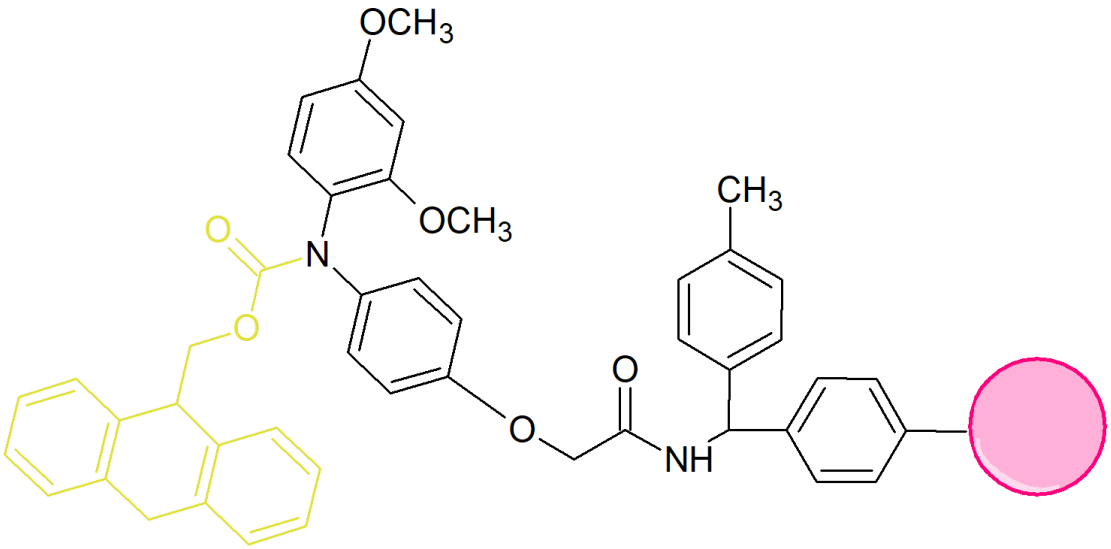


Figure 8, Rink Amide MBHA Resin drawn in ChemSketch with the protective Fmoc group in yellow.

For the rink mine resin is it important to know that the resins are extremely acid-sensitive support. Therefor the protected peptoid acid fragments can be obtained. They can be obtained without losing any sidechain protection functionality by using acetic acid in DCM[24].

A parameter that can have an effect on the synthesis is the solvent because the solvent not only determines the swelling of the resin, but it also influences the accessibility of the reactive sites. And therefore, directly affect the kinetics of the coupling reaction. The resin is swelled in DMF, which is important to create accessibility of coupling sites on the resin[24].

2.3.3 Protective groups

Fluorenylmethyloxycarbonyl (Fmoc) is a protecting group and often used in organic synthesis as an important base labile N-protecting group. The load of the carrier resin is determined by coupling an Fmoc amino acid and determining the resulting conversion. Fmoc is acid-stable which means it can withstand cleavage of butoxycarbonyl(Boc), protecting group[24]. The structure and mechanisms of the two protection groups used in this study can be seen in figure 9.

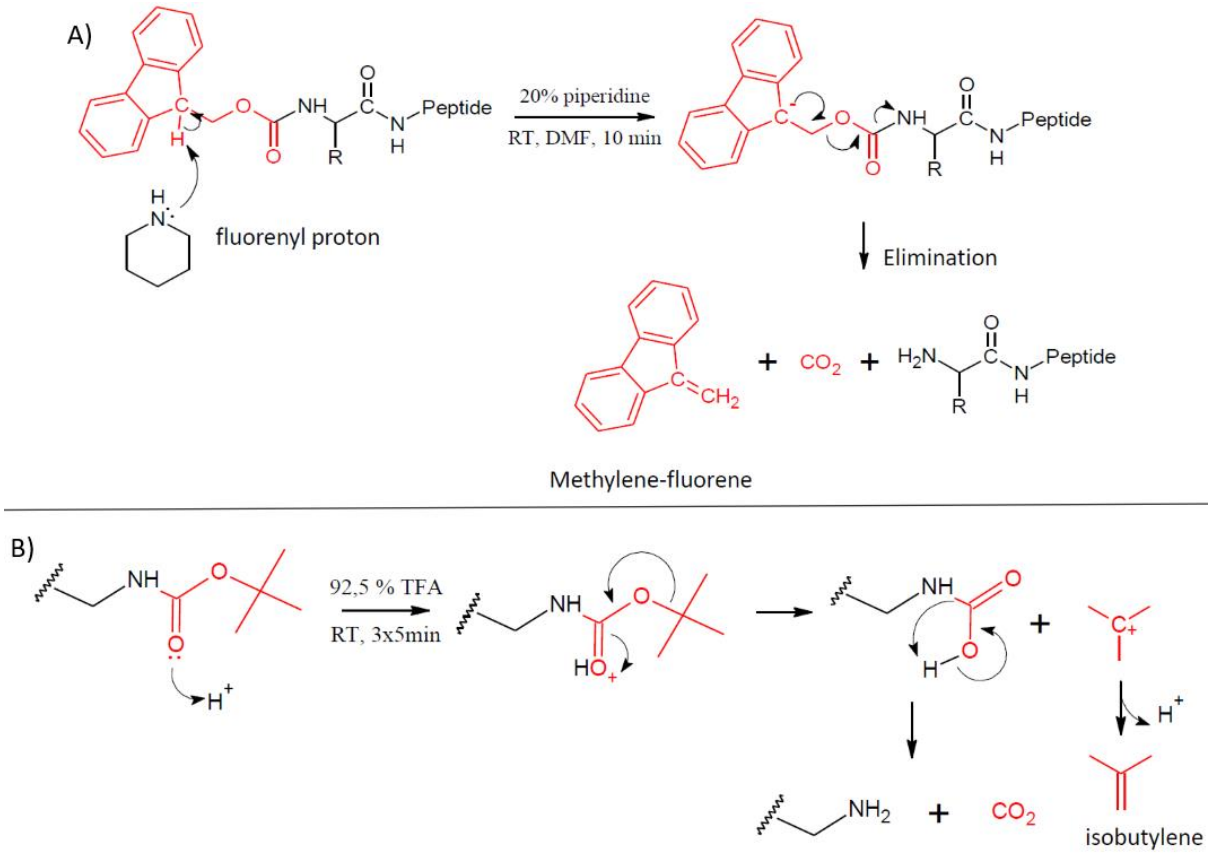


Figure 9 Shows the structure and deprotecting mechanism of Fmoc(a) and Boc(B)[11].

Rink amide resins has a good detachment of peptide or peptoid amides and can easily be cleaved by TFA. Therefore, the rink amide resin is preferable to use in the synthesis[24].

2.3.4 Difference in synthesis methods

A peptoid can easily be synthesized via sub-monomer solid-phase synthesis. This method was invented in 1992 and is called the sub-monomer method because each off the cycle monomer additions consists of an acylation and nucleophilic displacement step[25]. For this reaction there is some side reactions that may be unique to the synthesis of the peptoids. The side chains in the sub-monomer method can bear a nucleophile 3-4 atoms from the amino-N. This means that there it is possible for cyclization after the bromo-acetylation step. There are two methods for synthesis of a peptoids; monomer and sub-monomer. The monomer method is similar to the SPPS-method. First, the protected monomers for a peptoid synthesis must be synthesized. The sub-monomer method eliminates the need of protected monomers. These two methods are described more detailed in section 2.4.4 The peptoids can also be synthesized by a solution phase synthesis like peptides but the sequences is limited to >10 residues[26] for solution phase peptoid synthesis.

The method in solution phase peptoid synthesis is a sub-monomeric and is done by using bromoacetylbromide[26]. In this method the first step is the acylation reaction for the N-terminus, then filtration and evaporation. The solution-phase synthesis is useful in large production of peptides for example for industrial purposes.

2.3.5 Sub-monomer solid-phase peptoid synthesis

A monomer is poor soluble and can led to a low coupling efficiency. Therefor the time of cleavage is extended causing the acid induced degradation. The degradation is coming from the mixed peptoid [27].

The synthesis is used to synthesize specific biopolymers which can be synthesized directly on a solid support, resin. The first step in the synthesis is deprotection of the resin or resin activation. followed by bromoacetylation and coupling of the amine or bromine displacement step. Here the amine acts as a nucleophile. The coupling yield is very high and after the coupling where the reactions are coupled to resin, the reagents must be drained and washed. Those two steps are done so the reaction is ready to the following step. In this step the time of the cleavage depends on the variety and how many protecting groups are present in the sequence[27].

The final step is the analysis where the analytical HPLC-MS method is used to determine the purity of the product and to analyze if the theoretical molecular weight is the same as the measured.

Resin activation/Fmoc deprotection

To deprotect the Fmoc group, a solution of 20% 4-dimethylpiperidine in DMF is added and incubated twice, first two minutes then 12 minutes. A short prewash is done afterwards followed by the cleavage reaction. The deprotection of MBHA resin can be seen in figure 10.

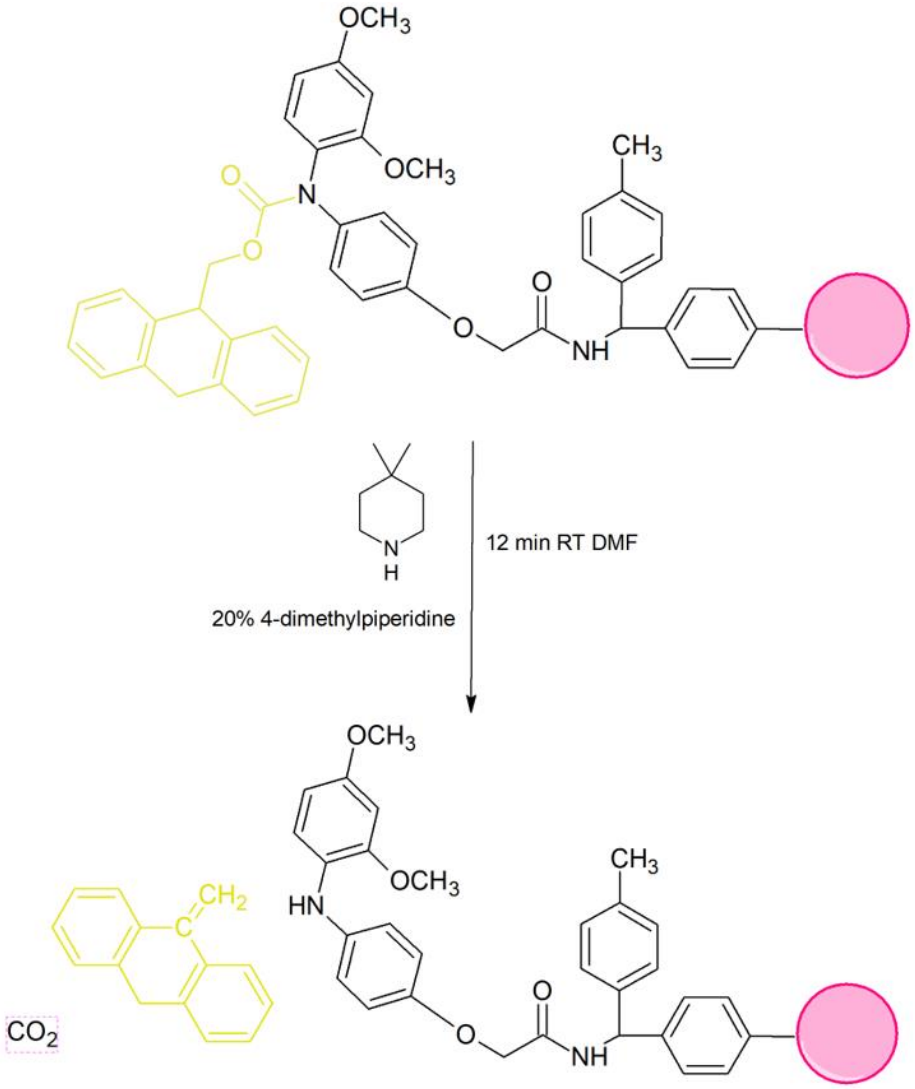


Figure 8, illustration made in Chemdraw and shows the resin activation with the Fmoc protecting group in yellow.

Washing stage

The resin is washed with DMF because it is a solvent most of our amines and biproducts are very soluble. This step is repeated multiple times to make sure every biproduct is washed out of the cartridge and therefore cannot react in the other steps of the synthesis[28].

Bromoacetylation

A solution of 0,6 M bromoacetic acid in DMF and 86 µl of N, N´-diisopropylcarbodiimide (DIC) is used for this step. The DIC is coupling reagent added to the solution while the bromoacetic acid is a part of the peptoid chain by reacting with the group on the

resin/amine and creating water as a biproduct[15]. The acetylation is illustrated in figure 11 below.

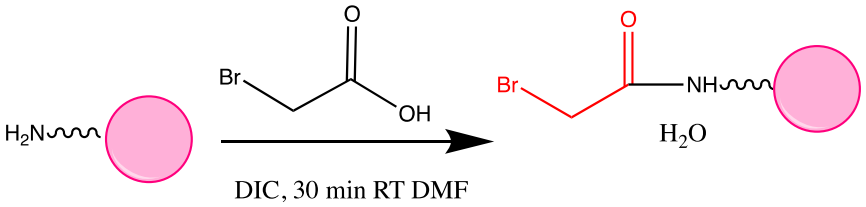


Figure 9, illustration made in Chemdraw and edited in OneNote

Displacement

For the displacement step a solution of amine in NMP is prepared. In this step various amines with different physiochemical properties are incorporated into the polypeptoid chain. Amines are readily soluble in solvents compatible with the solid phase synthesis methods such as DMF and NMP. NMP is a better solvent because it has shown to dissolve protected amines and amino acids better than DMF and it has been shown that it leads to leaner products[25].

The displacement is illustrated in figure 12 below.

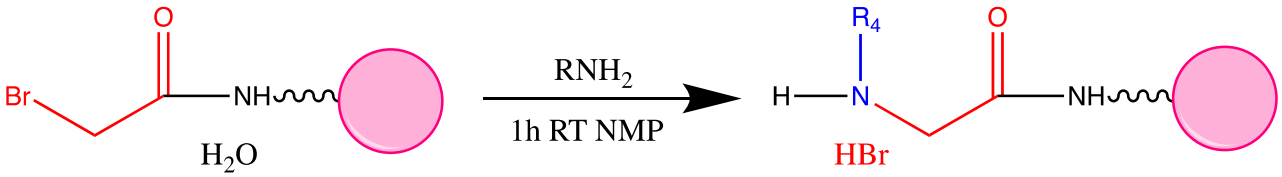


Figure 10, Illustration made in Chemdraw and edited in OneNote

Cleavage

For the cleavage step of the (amino acid/peptide acid) from the resin requires strong acidic condition, which is why trifloorumethanesulfonic acid/trifluoracetic acid (TFA) is used with this peptoid. The acid is needed because of the choice of the resin we have used. This means that different resins require different cleavage conditions. By using MHBA resin means that it is acid labile, which lead us to use a strong acid to cleave it. In this cleavage step concentrated TFA (95%) is the standard reagent to perform the final cleavage of the peptoid for the resin. We also use scavenger TIPS and water which gives us a cocktail. Also, the side chain protecting groups are removed in this step. The purpose of the cleavage step is to separate the peptoid from the support and removing the protecting groups from the side chain[25].

3. Method and materials

3.1 Amines and calculations

Abbreviation	Side chain name	Structure	molecular weight (g/mol)	density (g/mL)	c2 (mol/mL)	v2 (mL)	c1 (mol/mL)	v1 (mL)	mL NPF
Nlys	N-Boc-1,4-butanediamine		188.27	0.989	0.001	2.2	0.005253	0.419	1.781
N2ap	2-Aminopentane		87.16	0.734	0.001	2.2	0.008421	0.261	1.939
Nspe	(S)-(-)-a- Methylbenzylamine		121.18	0.94	0.001	2.2	0.007757	0.284	1.916
Nba	Butylamine		73.14	0.74	0.001	1.2	0.010118	0.119	1.081
Nha	Hexylamine		101.19	0.766	0.001	1.2	0.007570	0.159	1.041

Figure 11, amine library with concentrations and volumes for the 1M amine solution and the corresponding volume of NPF added to the amine to make the solution.

3.2 Synthesis

The lab manual followed throughout the lab work can be seen in appendix 1 and the specifics of the syntheses is written here. The specific calculations of volume a concentration can be seen in appendix 2.

For all syntheses we used 100mg resin which was swelled in 2mL DMF for 10 min. While the bromoacetylation was incubating, the amine solution was prepared for the following displacement. All the displacements were incubated for an hour.

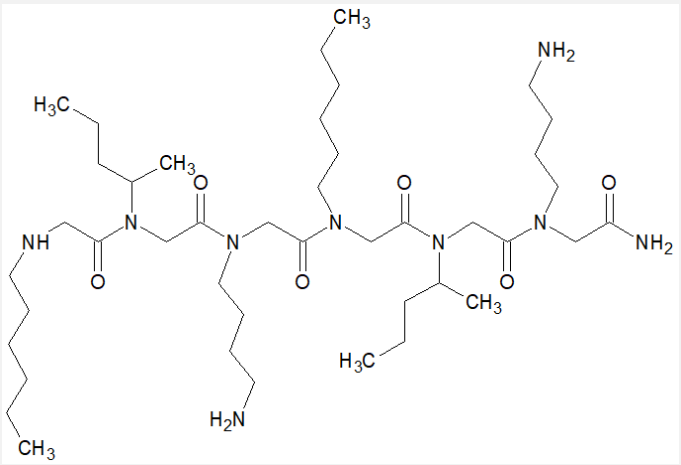
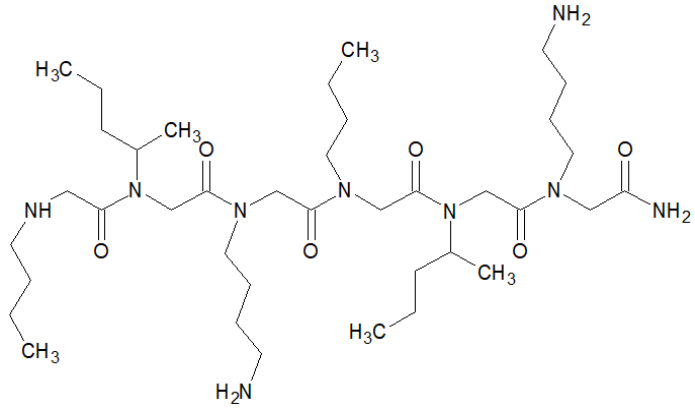
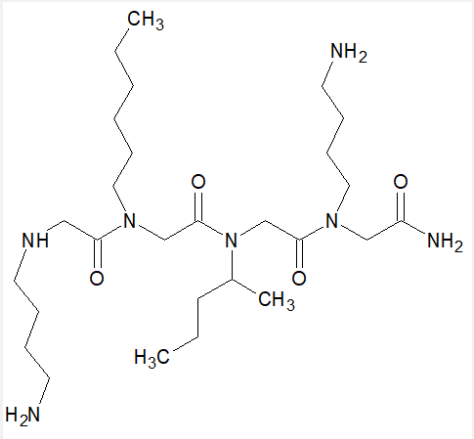
Displacement 1: NLys. Displacement 2: Nspe but for the first two syntheses Nspe was replaced with N2ap. Displacement 3 is where the two peptoid chains are different because one peptoid has Nha and the other has Nba. For displacement 4 to 6 we repeated displacement 1 through 3 with the same amines. Our second synthesis was cleaved after the displacement 4. When continuing a paused synthesis, the resin was swelled for 40 minutes to make sure it was completely swelled and room temperature. During cleavage the vial was shook for 60 minutes. The TFA was then evaporated by blowing a stream of nitrogen until just the crude oil was left in the vial.

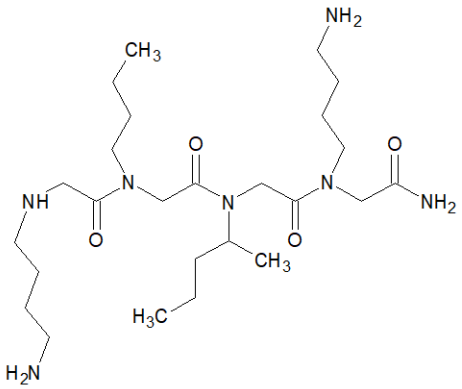
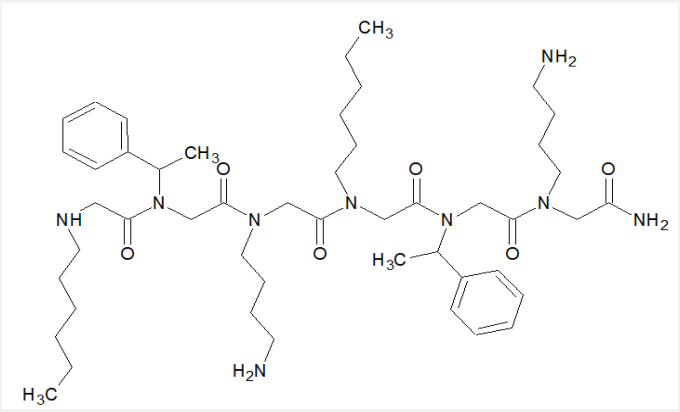
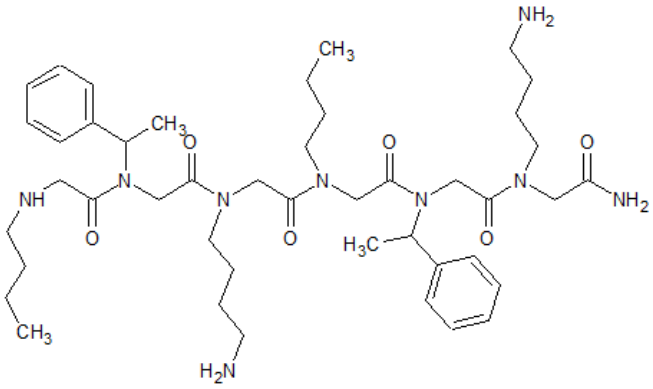
During the evaporation we prepared an acetonitrile/water (1:1) solution. We then dissolved the crude oil in acetonitrile/water (1:1) with additional acetonitrile added to completely dissolve the peptoids.

As the solution was looking brown and sticky after lyophilizing, we then dissolved the contaminated peptoids in ether and left to evaporate over a couple of days. The freeze-drying was then repeated. The product was still looking brown and sticky and therefore we could not weight the final yield of the product.

3.3. Synthesized peptoids

It was possible to synthesize a total of six peptoids. In the table below the structures, molar weight, formula and sequences of the synthesized peptoids can be seen. The first number is from which synthetization it was synthesized, and the second number is whether it contains Nha or Nba as the 3rd and 6th amine in the chain.

Peptoid number	mer	Structure	Molar weight(g/mol)	Formula and sequence N-C
1.1	6		810.16512	$C_{42}H_{83}N_9O_6$ Nlys-Nha-N2ap-Nlys-Nha-N2ap-Nlys
1.2	6		754.0588	]$C_{38}H_{75}N_9O_6$ Nlys-Nba-N2ap-Nlys-Nba-N2ap-Nlys
2.1	4		541.7701	$C_{27}H_{55}N_7O_4$ Nlys-Nha-N2ap-Nlys

2.2	4		513.71694	$C_{25}H_{51}N_7O_4$ Nlys-Nba- N2ap-Nlys
3.1	6		878.19756	$C_{48}H_{79}N_9O_6$ Nha-Nspe- Nlys-Nha- Nspe-Nlys
3.2	6		822.09124	$C_{44}H_{71}N_9O_6$ Nba-Nspe- Nlys-Nba- Nspe-Nlys

3.4 HPLC + MS

High pressure liquid chromatography mass spectroscopy (HPLC-MS) is used in this project to analyze the data retrieved in the lab.

This technique is used to identify, and access how pure it is. Also to quantify components in the retrieved sample[29]. The HPLC machine forces the solvent through a closed column containing very fine particles[30].

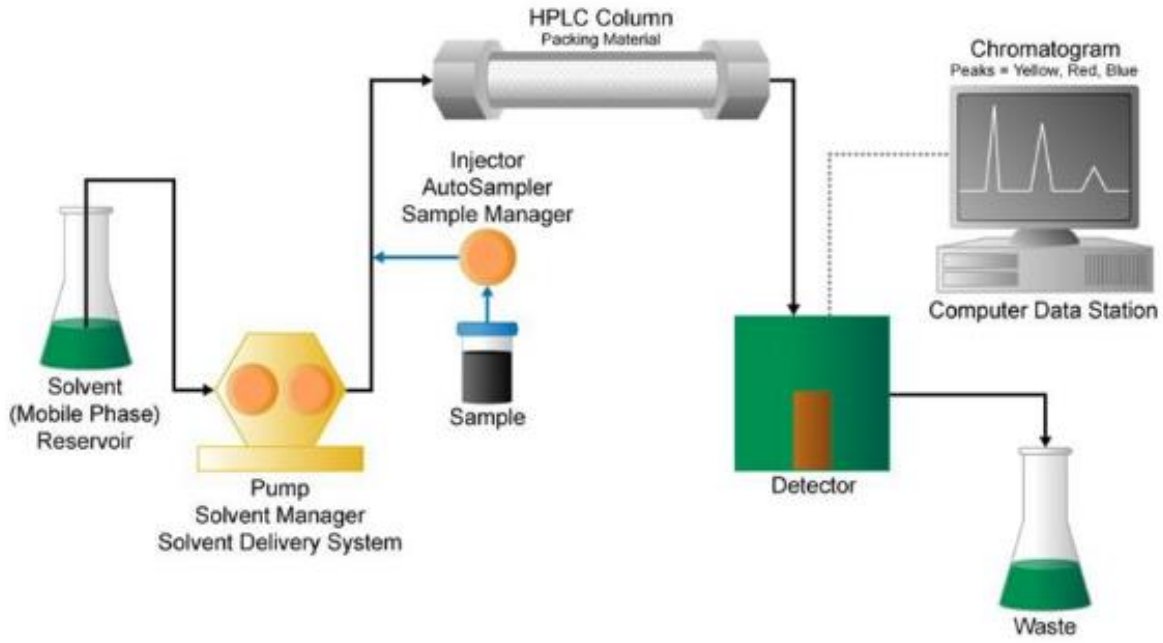


Figure 12, components in HPLC analyzes[29].

When a mixture of different analytes is passed through the column the analytes will interact differently with the beads in the column dependent on their property. The peptides are usually run on C18 columns. Therefore, the analytes will come out in an order where the analytes that interact the least with the beads come first and those that interact the most are eluted the last[31]. The mass spectroscopy (MS) is the part after HPLC where the molecular mass of the sample is analyzed[32]. This is necessary in order to see the purity of the crude and the calculate where potential mistakes could be[29]. This technique separates ions according to their mass-to-charge and this ions are trapped and detected by the MS detectors[30]. In the MS adducts can be seen e.g. M+H, M+Na, M+K etc. These adducts change the mass per charge of the product equal to the molecular weight of the adduct.

4 Results and data analysis

Analyzing these results from the HPLC-MS method it can occur that the signals from both M+ and M+2 will be in the same chromatogram. The signal will be equivalent which means that the M+2 signal will be M+/2+2. This will be indicated in the table below with either an M+ or M+2.[33]

Before our sample was run in the HPLC-MS we ran acetonitrile as a blank to test if the column is impure. All samples were run on a gradient 15-65 % acetonitrile water with 0,1 % formic acid, on a C18 column, 2.6 um/100 Å (particle/pore size). The chromatogram has time on the x-axis and relative abundance on the y-axis. A spectrum was created for each of the peaks in the chromatogram to inspect the mass per charge of the molecules. The spectra have mass per charge of the x-axis and relative abundance on the y-axis. To get a better overview of the peaks in the chromatogram we have plotted base peak ranging from 250-2000. The base peak of the blanks can be found in appendix 3.

Peptoid 1.1

The blank had no peaks fitting our results.

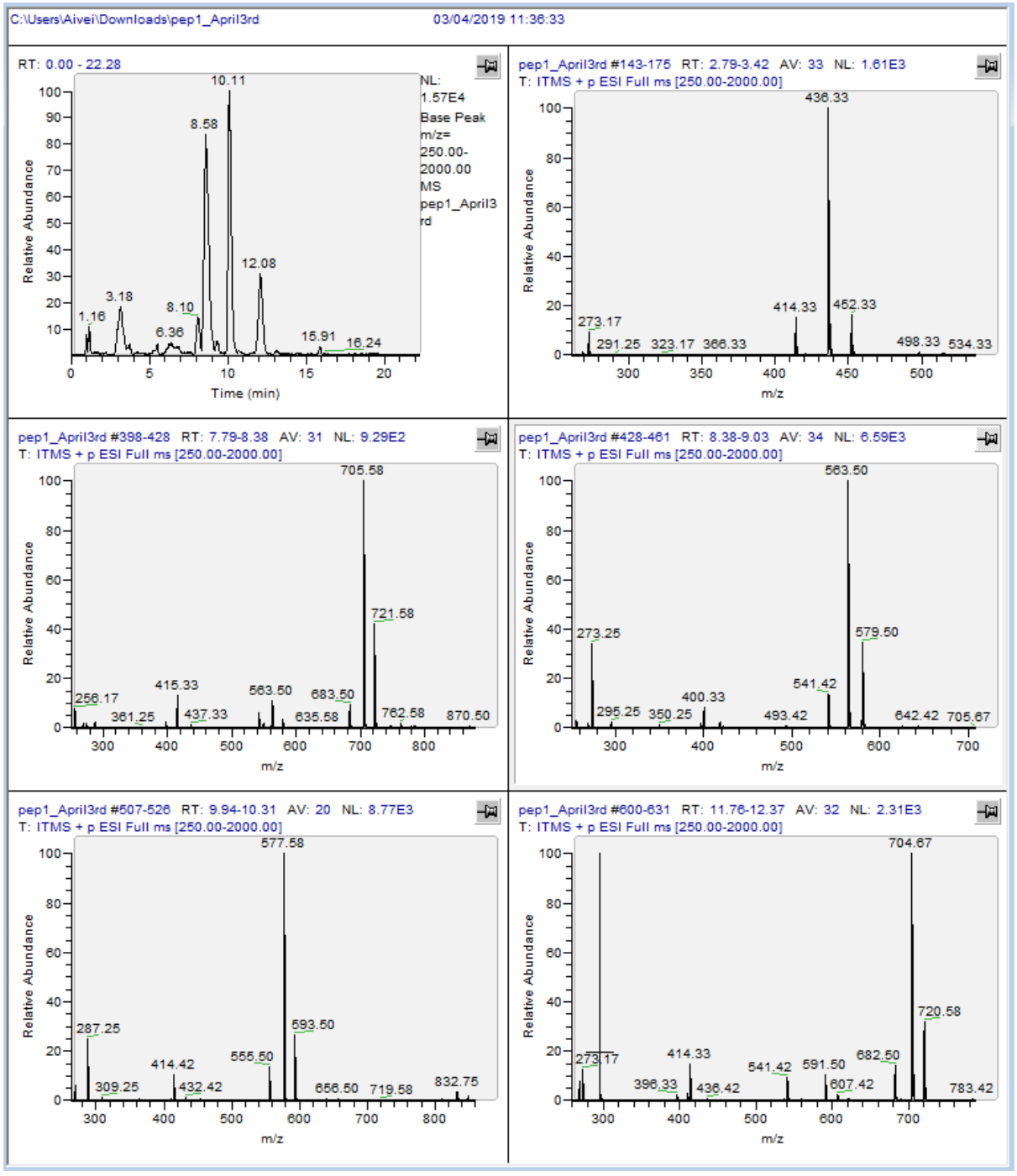


Figure 13 shows the HLPC-MS results of peptoid 1.1. with five peaks in the base peak chromatogram after 2 minutes runtime.

Peptoid 1.1 (6-mer)									
Amine name	Nha	N2sp	Nlys	Nha	N2sp	Nlys			
Molar weight (g/mol)	101,19	121,18	188,27	101,19	121,18	188,27			
M+ Calculated	810.165								
M+ Observed	414,42								
RT of peak	9,88-10,49								
Result	The M+H+NH4 result observed shows that this entire peptoid is synthesized as planned								

Peptoid 1.2

The blank for this test is the same as for peptoid 1.1 and is figure 1 in appendix 3.

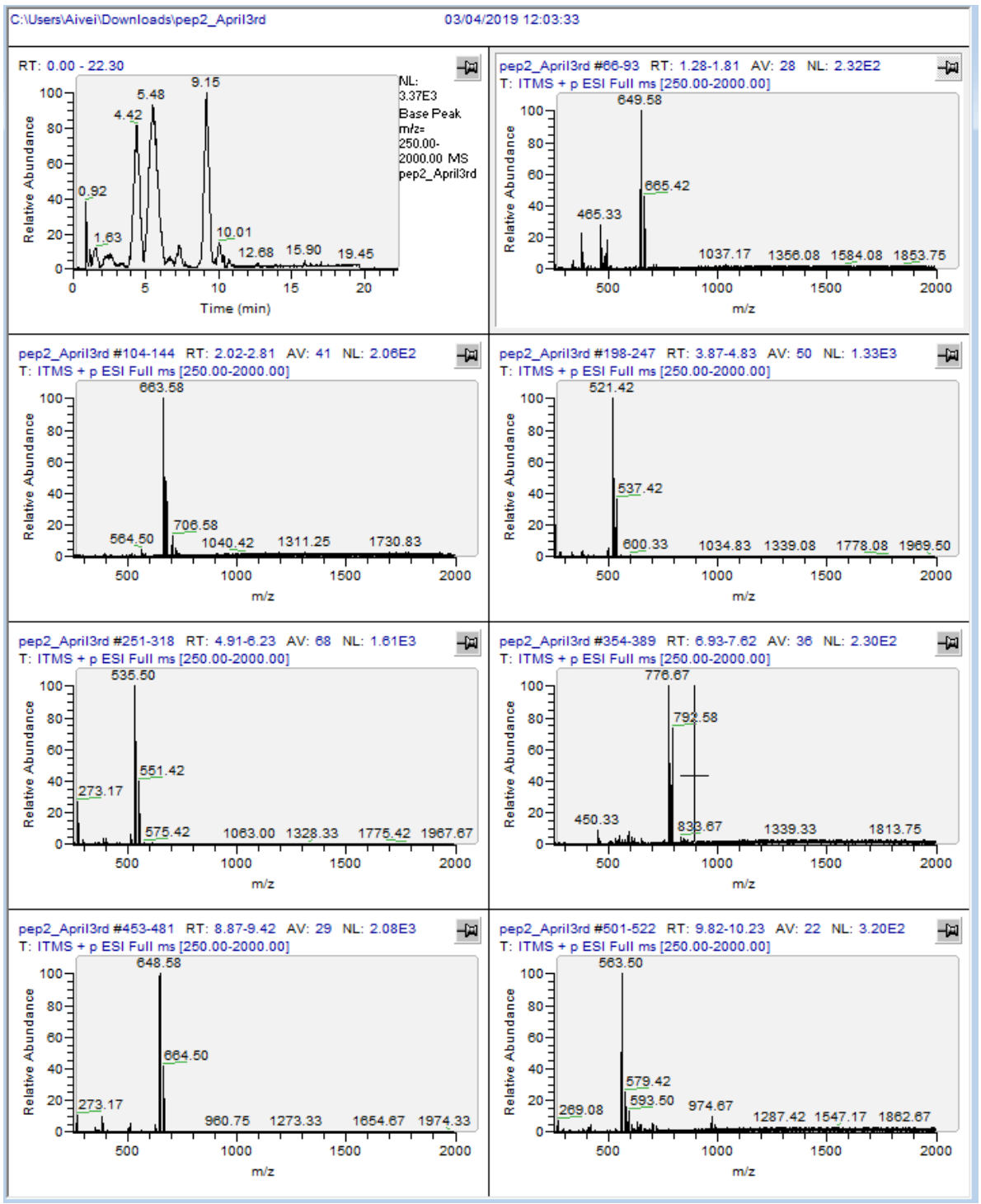


Figure 14 shows the HLPC-MS results of peptoid 1.2 with seven peaks in the base peak chromatogram after 1-minute runtime.

Peptoid 1.2 (6-mer)									
Amine name	Nba	N2ap	Nlys	Nba	N2sp	Nlys			
Molar weight (g/mol)	73,14	87,16	188,27	73,14	87,16	188,27			
M+ Calculated	754.059								
M+ Observed	648,58								
RT of peak	8,87-9,42								
Result	The M+ value shows possibility of the peptoid being synthesized as planned (subtracting 19 from the M+)								

Peptoid 2.2

A blank was run before our sample and fits a peak in our results. The data for the blank can be seen in appendix 3 figure 2.

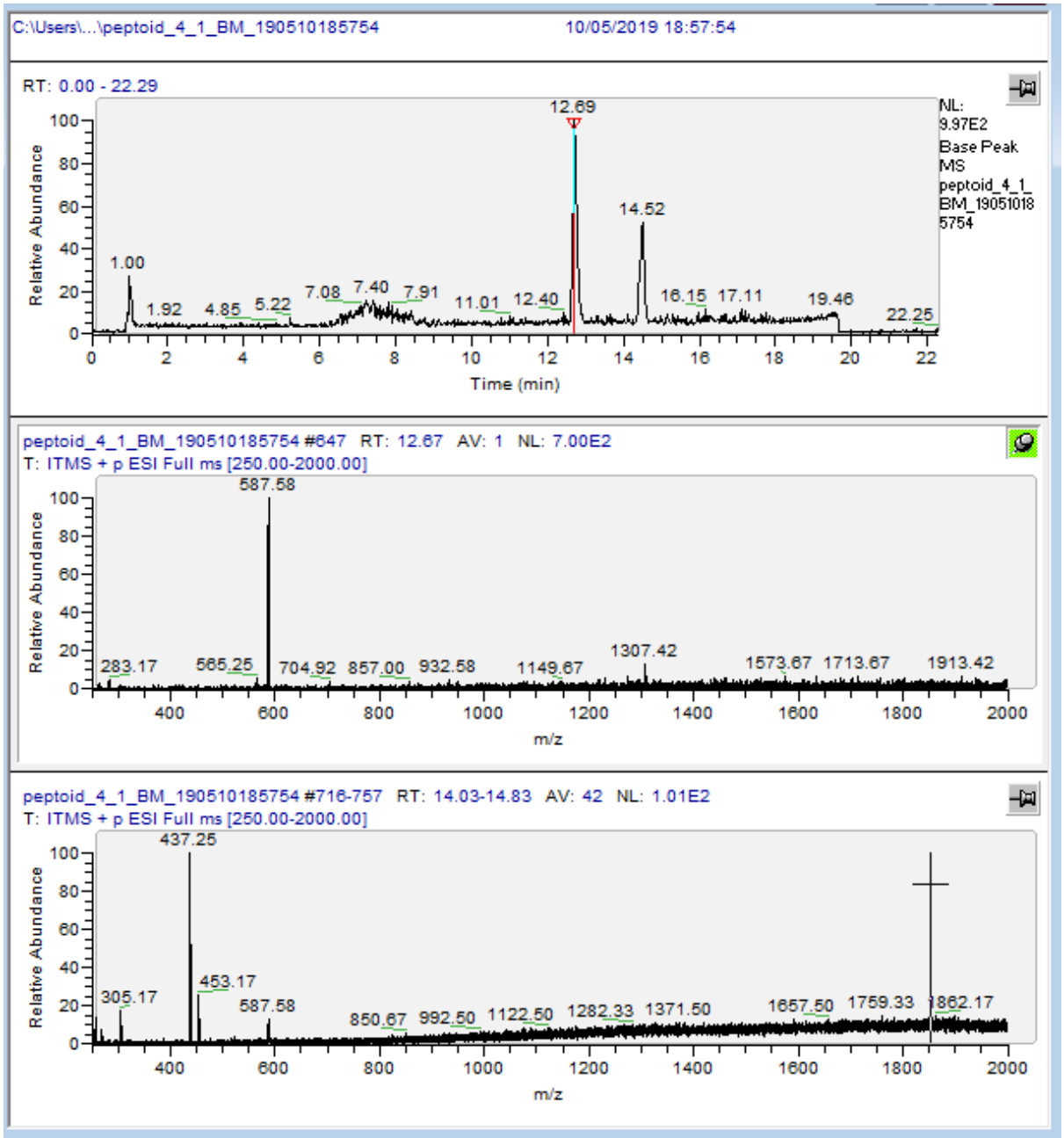


Figure 15 shows the HLPC-MS results of peptoid 2.2. with two peaks in the base peak chromatogram after 2 minutes runtime. The peak at 12.69 has the same mass par charge as the peak in the blank test (appendix 3 figure 2), so we don't expect this peak to be our product.

Peptoid 2.2 (4-mer)									
Amine name	Nlys	Nba	N2ap	Nlys					
Molar weight (g/mol)	188,27	73,14	87,16	188,27					
M+ Calculated	513.717								
M+ Observed	437.17								
RT of peak	14.03-14.83								
Result	The M+ value indicates a difference in 76.547 which could mean, that the Nba has not been attached properly to the peptoid.								

Peptoid 3.1

A blank was run before our samples and have a peak fitting our results. The data of the blank can be found in appendix 3 figure 3.

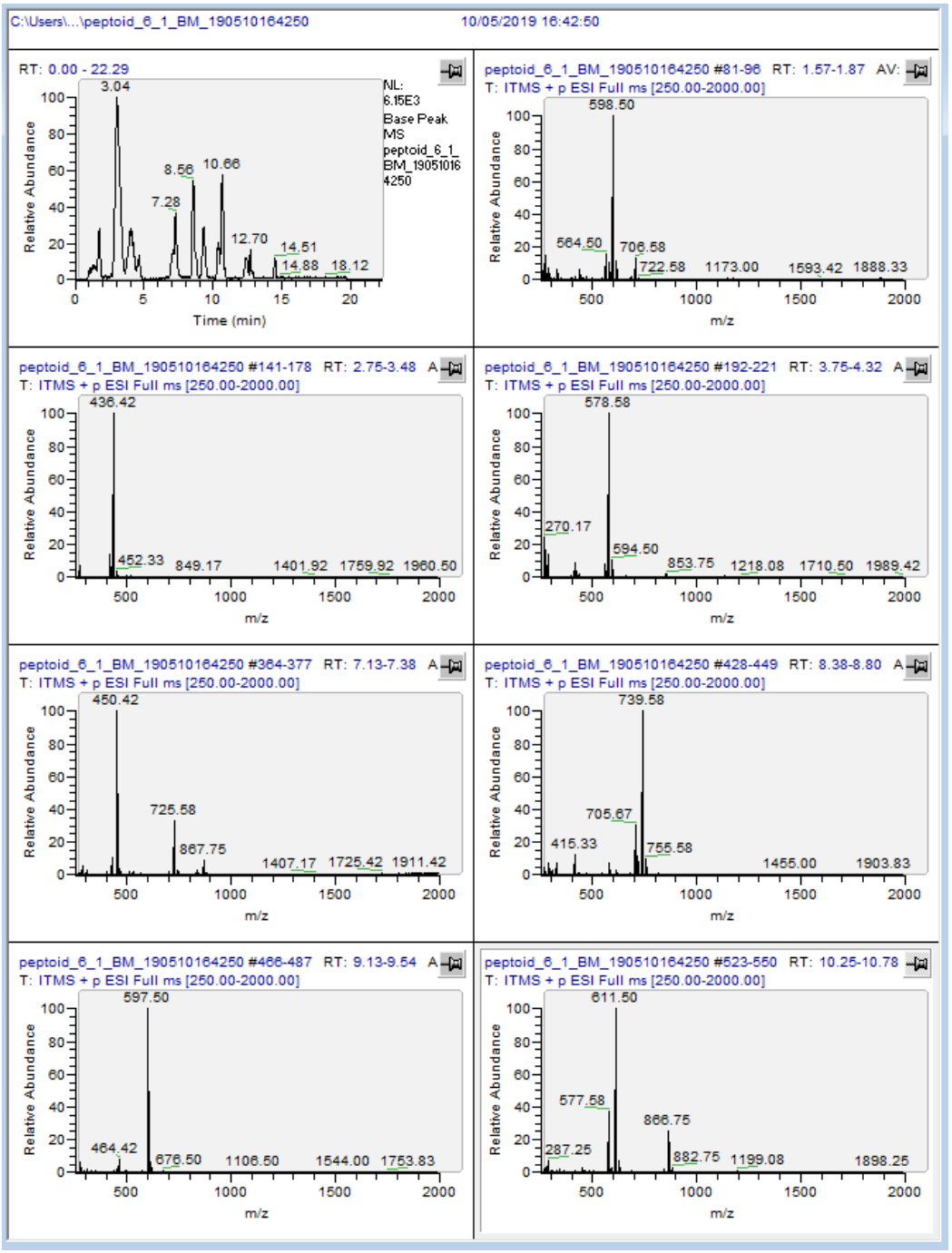


Figure 16 shows the HLPC-MS results of peptoid 3.1. with seven peaks in the base peak chromatogram after 1-minute runtime. The peak at 12.70 had the same mass par charge as the peak in the blank test (appendix 3 figure 3), so we don't expect this peak to be our product.

Peptoid 3.1 (6-mer)									
Amine name	Nha	Nspe	Nlys	Nha	Nspe	Nlys			
Molar weight (g/mol)	101,19	121,18	188,27	101,19	121,18	188,27			
M+ Calculated	878,198								
M+ Observed	739								
RT of peak	8,38-8,80								
Result	The M+ value shows possibility of the peptoid being synthesized without one of the Nspe. (subtracting 19 from the M+).								

Peptoid 3.2

The same blank as for peptoid 3.1 was used.

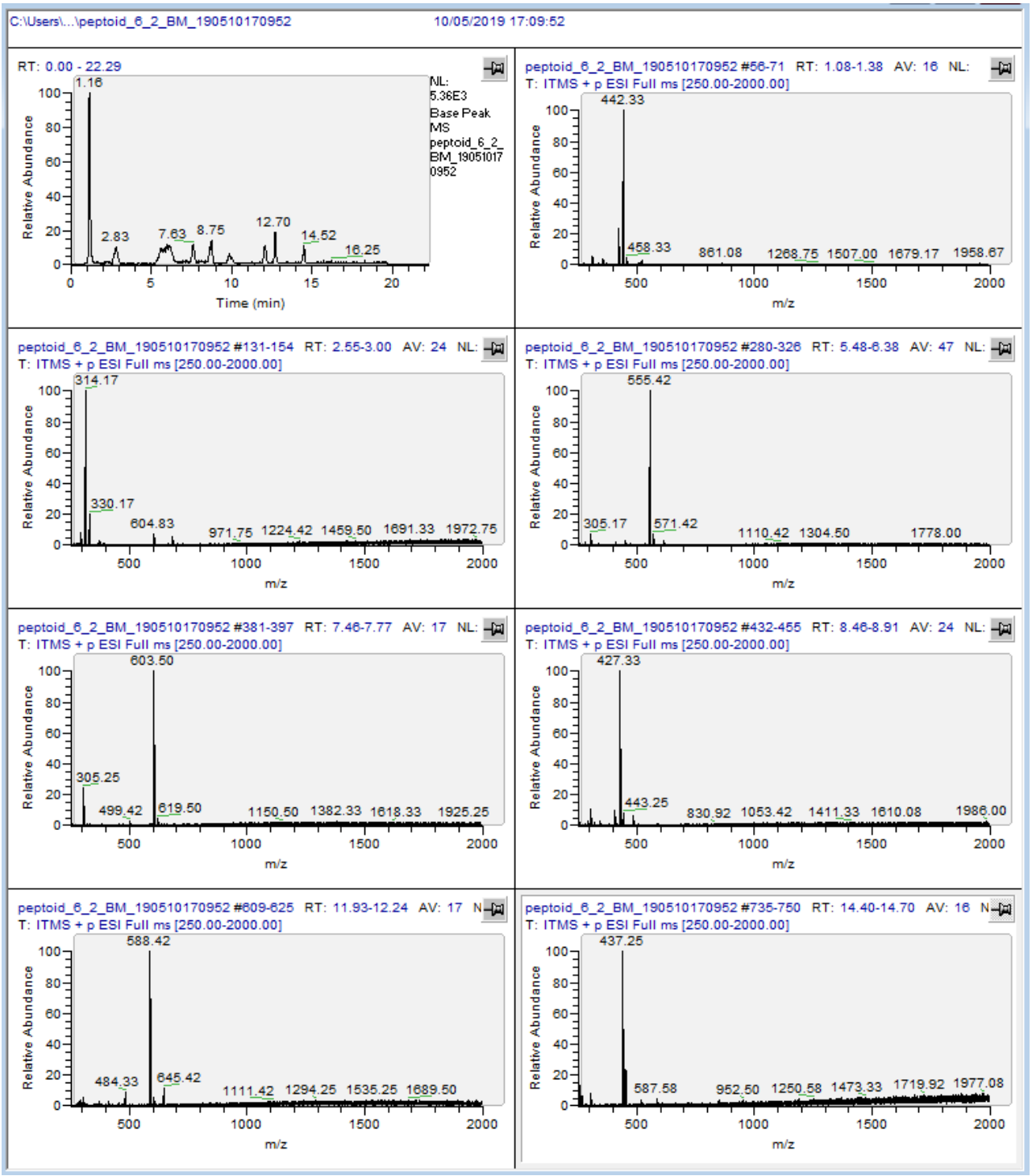


Figure 17shows the HLPC-MS results of peptoid 3.1. with seven peaks in the base peak chromatogram after 1-minute runtime. The peak at 12.70 had the same mass par charge as the peak in the blank test (appendix 3 figure 3), so we don't expect this peak to be our product.

Peptoid 3.2 (6-mer)									
Amine name	Nba	Nspe	Nlys	Nba	Nspe	Nlys			
Molar weight (g/mol)	73,14	121,18	188,27	73,14	121,18	188,27			
M+ Calculated	822,091								
M+2 Observed	442,33								
RT of peak	0,77-1,65								
Result	The peak indicates that an M+2 could be the entire peptoid in this case. The calculations are a bit off, so either this or the peptoid has not been synthesized properly.								

5 Discussion

During the laboratory work the second synthesis was stopped after four sub-monomers due to accidently using N2ap in the second displacement instead of the wanted Nspe. After this mistake was recognized, the synthesis was stopped according to protocol and stored at -20C^{o} for a week prior to cleavage. When cleaving the peptoid 2.1 cartridge contained no resin which justifies why no tests were performed on that peptoid. It cannot be determined whether the resin was dropped in the fume-hood or if it has been tampered with, yet the mistake is accounted for.

5.1 Results

The first synthesis performed, resulted in a white powder and had acceptable results in the HPLC-MS, but the other crude products were brown, sticky and generated inconclusive HPLC-MS results. An improper Fmoc deprotection(activation) of the resin at the start of the synthesis, would lower the yield of the product significantly because the resin needs to be activated otherwise product will not be formed on it. A low yield of product makes the product hard to detect in the HPLC-MS over impurities in the solution. In the solution there could be sub-monomeric sequences different from the wanted peptoid which results in shorter and very different peptoids in one solution. A mixture of many structures makes it arduous to determine the exact structures of the peptoids in the solution, but possible since we know the mass. The poor result of the actual "powder" could be because the cleavage solution hadn't completely evaporated and therefore polluted the solution during the first lyophilizing. Even after evaporating over the weekend and re-lyophilizing, the results still did not look promising. If TFA was left in the solution it explains that the HPLC-MS results had a bigger peak than normal in the chromatogram, since TFA are small molecules that run through the column quickly. The TFA left in the solution could be causing the poor results of the crude yet the results were only somewhat improved after the second evaporation which indicates that other factors have had additional impact. It is plausible that the solution is polluted by solutions used during the synthesis, e.g. DMF, DIC, amine residues, etc., which would create very impure solution and adducts in the HPLC-MS results.

During all the syntheses the syntheses has been paused and then continued one or two days later. This could affect the yield

In section “Cleavage and Side-Chain Deprotection”, of the Fmoc protocol in appendix, the first step is to transfer the dried resin to a scintillation glass vial and add the cleavage solution to the vial. After cleavage, the solution is then transferred to a new fritted cartridge to filter off the resin ensuring only the product and cleavage solution is left in a new pre-weighted vial. This results in lost product from the multiple transfers. One solution to this may be to perform the cleavage in the same cartridge as the synthesis. This is done by adding the cleavage solution to the cartridge containing the dried resin and do the cleavage directly in that cartridge. By doing these two transfers are removed from the process rather than transferring resin to vial and then back to cartridge for filtering. This way it is still possible to wash the cartridge while the product and cleavage solution will come directly through the filter and into the pre-weighted vial. From our results we are unable to pinpoint where exactly in the synthesis a mistake has been made. If the synthesis should be redone, avoid this by takeout samples of the product after displacements, cleave them and test them with the HPLC-MS to see if it corresponds to the molecular weight of the planed product. This can be done after the first displacement and after the third but not after the second because a chain of two monomers can cyclize when cleaved. This is done by performing the synthesis as described earlier but after the first displacement, take out a small sample of the resin and cleave it as the protocol says to do with the finished product. It is then tested with HPLC-MS and the results are saved. Skip doing this for the second displacement but continue taking samples of the third and fourth and so on. Doing this during a synthesis will lower the yield of the finished product but it makes it possible to test the yield of each coupling. When you know the efficiency of each step we get a clearer image of the entire synthesis process.

All numbers the following example and in figure 20 are fabricated and does not illustrate theoretical efficiency of the coupling. This can be illustrated by figure 20 where we gather 100% coupling at first displacement but since its preposterous to expect 100% efficiency, to illustrate reality we hypothesize that for each following displacement the coupling is 98% for this example. As mentioned in 2.4.2 the loading capacity of 100 mg MBHA resin is 0.04-0.09 mmol. In the illustration below, we gather that we would get 0.05mmol of product, if all steps were 100% efficient. The second displacement is only 98% efficient which leave us to get 0.049 mmol product after the second displacement. Because the third displacement also have a yield of 98% then the amount of product after the third displacement will be 98% of 0.049 mmol which is

0.04802 mmol. This means that after the sixth displacement there will not be a product yield of 98% but a yield of $\frac{0.05}{0.04519604} * 100 = 90.39\%$ instead.

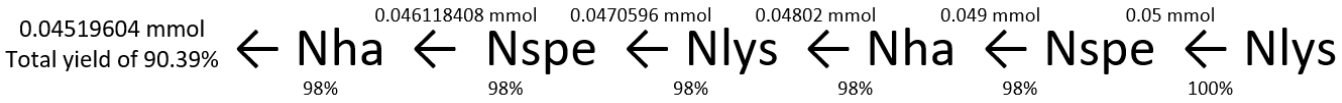


Figure 18 illustrates the hypothetical yields of each displacement in a peptoid synthesis. Illustrated using OneNote.

We can calculate the theoretical yield if our synthesis were 100% efficient. Because of the loading capacity of the resin being imprecise, we can only calculate the theoretical weight as being between 0.04 mmol and 0.09 mmol. Below is a table where the theoretical weight is calculated for each of the peptoids synthesized in the project.

Peptoid	**Molecular weight** g/mol	**0.04 mmol** g	**0.09 mmol** g
1.1	810.16512	0.0324066	0.072914861
1.2	754.0588	0.0301624	0.067865292
2.1	541.7701	0.0216708	0.048759309
2.2	513.71694	0.0205487	0.046234525
3.1	878.19756	0.0351279	0.07903778
3.2	822.09124	0.0328836	0.073988212

The result of testing during the synthesis process with reveal weak steps in the synthesis and therefore we can optimize the process of these steps. How to optimize these steps depends on the results of the tests and may require multiple synthesis with samples taken throughout the process. We can then redo the synthesis with the optimized steps and hopefully get much better results and better yield of the product.

Since the results obtained overall look somewhat usable, the numbers and peaks where hard to analyse due to low purity of the solvent. Several reasons for these have been discussed, and further on the laboratory work should be done more confidently.

6 Conclusion

To synthesize antimicrobial agents, it is necessary to mimic the structure of antimicrobial peptides. The peptoids hydrophobicity and the length are some of the important factors in designing a useable peptoid. Some of the important properties of the final peptoid for example the ability to enter a Gram-negative bacterium was highly prioritized.

As a conclusion of our experiment, the peptoids could not be purified, even though the protocol for solid phase synthesis was followed, which could be due to an error during the synthesis.

A mistake could have been done in the lab, or some mistakes in the preparation of the solution during the synthesis. Something that is worth noticing is that the wrong amine was used.

By analyzing the peptoid with HPLC-MS, it can be concluded that the method of the synthesis was correct, just with some mistakes. We synthesized some peptoids but not the expected ones. One mistake has not coursed our results, but a combination of all mistakes taking into consideration has resulted in no usable peptoid for further development.

7 Future work in biology

Peptoids have shown to be highly relevant in today's research in the antimicrobial world. Positive results have made it possible to take laboratory work to higher levels and not only work with the synthesis in chemistry but move over to biology as well. This makes is possible to keep developing peptoids to specific matters, and therefore for us it would be possible to keep working with the method to have better results. Once our results have become sufficient it will be possible to work with them in the biology lab, trying to learn how to design the most sufficient peptoids for the desired use.

Members of this group would like to keep working in this field and potentially write their BA to get the connection in both areas, both chemistry and biology.

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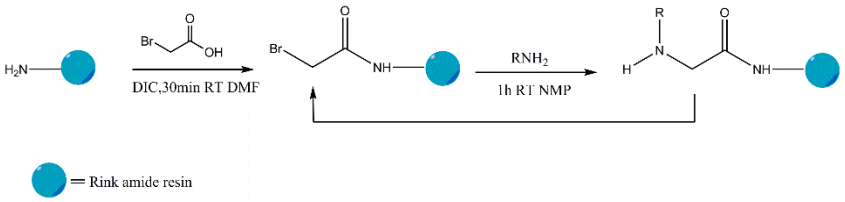
10 Appendix

Appendix 1 Fmoc protocol



Setup:

1. Fritted reaction vessel (syringe)
2. Pump for waste suction
3. Mixing plate

Synthesis:

1. Add (100 mg) **200 mg** of **Rink amide resin MBHA**
2. Swell the resin by adding 2-**4 ml of N-methylpyrrolidinone (NMP) or DMF** Shake for 10 min up to 1 hour and drain the solution by vacuum to isolate the swelled resin
3. Add 1 ml of **20 % 4-dimethylpiperidine** in **DMF** to deprotect the Fmoc group by 2 min agitation and drain. Repeat with a 12 min incubation.
4. Rinse the resin by adding **2 ml of DMF**, agitate for 15 sec and drain. Repeat 3 X
5. **Bromoacetylation:** Add **1 ml of 0,6 M bromoacetic acid** (0,6 mmol) in **DMF** and **86 ul of N,N'-diisopropylcarbodiimide (DIC)** (0,56 mmol). Incubate by gentle bubbling for 30 min. Drain and rinse with **2 ml of DMF** (repeat 4 x) (**repeat this step once more 30min if synthesis didn't work).**
6. **Displacement:** Add **1 ml of 1-2 M amine** in **N-methyl pyrrolidinone (NMP** note: it goes through gloves) or **DMSO, DMF**. Incubate with bubbling for 30-**120** min. Drain and rinse with **DMF** (4 x 2 min).
7. Repeat steps ***5.*** and **6.** to grow the peptoid chain.
8. After the final displacement is done, rinse with 2 **ml of DMF** (repeat 4 x), then **2 ml of dichloromethane (DCM)** (repeat 3 x).
9. Cap and store the reaction vessel (syringe) until cleavage!
10. **Pause in synthesis (optional):** To pause during peptoid synthesis, finish the displacement reaction and continue with **step 8**. To continue the growing peptoid chain, restart

the synthesis by re-swelling the dried resin as in **step 2** and repeat the submonomer cycle as in **step 5 and 6**. The resin can be dried and stored after any displacement except the 2nd displacement because the resin-peptoid conjugate may form a **cyclic diketopiperazine** side product.

Cleavage and Side-Chain Deprotection

1. Transfer all of dried resin to a **20 mL** scintillation glass vial (white cap).

2. Working inside a hood and using proper personal protective equipment, add 4 **mL of trifluoroacetic acid (TFA)** cleavage cocktail1 (e.g. **95% aq. TFA, 2.5 % triisopropylsilane**, **2.5 % water**) to the scintillation glass vial and cap tightly. Shake for 10 minutes to 2 hours at room temperature.

3. **Collect** the TFA cleavage solution by filtering the resin through a disposable, PP fritted cartridge into a new, pre-weighed **20 mL** scintillation glass vial. A disposable, PP pipette is convenient to transfer the cleavage cocktail solutions.

4. Add **1 mL of fresh cleavage cocktail** to rinse the resin and collect any residual peptoid. (Repeat 2 x)

5. Evaporate **TFA** by blowing a gentle stream of nitrogen or by using a Biotage V10 evaporator.

6. Redissolve the crude oil in **6 mL of acetonitrile/water 1:1** (v/v) for HPLC. Freeze and lyophilize. Repeat

7. Record the weight of the crude product. **Store as a dry powder at -20 °C**.

8. Test cleavage (optional): A test cleavage on 0.5% of the resin can be performed to quickly determine the purity and mass of the synthesized peptoid and whether correct cleavage conditions were chosen. Test cleavages are especially useful to monitor the progress of the synthesis.

Characterization and Purification of the Polypeptoid

1. Through a combination of analytical HPLC, electrospray LC-MS, and/or MALDI-TOF, determine the purity of the crude product and whether the desired molecular weight is present.

2. Prepare a **˜5-10 mg/mL** solution of the dry peptoid powder in **water** with minimal **acetonitrile** as needed for solubility. Filter clear solution of crude peptoid product with **0.45 µm syringe filter** to remove dust and particles.

3. **Analytical HPLC and electrospray LC-MS**: Prepare a **˜ 20 µg/mL** crude peptoid solution. Filter **200 µL** with a **0.45 µm filter** and inject **20 µL**.

4. MALDI: Mix 1 µL of ˜20 mg/mL peptoid with 1 µL matrix. Spot 1 µL on MALDI plate and allow to air dry. Matrix and acquisition mode are dependent on sample (Fig. 5).

5. Purify the crude peptoid mixture with reverse-phase prep HPLC. Choose the gradient and column (C4 or C18) based on the hydrophobicity of polypeptoid. Combine purified fractions, freeze, and lyophilize, resulting in a fluffy white powder. Record the weight of the final product.

6. Formation of HCl salt (optional): Re-dissolve the lyophilized powder in 100 mM HCl (aq.) with minimal acetonitrile. Transfer to pre-weighed glass vial. Freeze and re-lyophilize. Repeat 2 x. Reweigh to determine mass of peptoid powder.

This procedure has been edited from:

Video Article

Solid-phase Submonomer Synthesis of Peptoid Polymers and their Self-Assembly into Highly-Ordered Nanosheets

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URL: http://www.jove.com/video/3373/

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Keywords: Bioengineering, Issue 57, Biomimetic polymer, peptoid, nanosheet, solid-phase synthesis, self-assembly, bilayer,

Date Published: 11/2/2011

Appendix 2 Calculations

0.6 M bromoacetic acid

$$m = M * n = 138.948 \frac{g}{mol} * 0.6 = 83.3688g * 0.025L = 2.08422g$$

To make 25mL 0.6M solution we need to add 2.08g and fill to 25mL.

Cleavage solution

TFA cleavage solutions contain 95% TFA, 2.5% water and 2.5% triisopropylsilane.

To make enough cleavage solution for 2 syringes (12 mL) we added 11.4 mL TFA, 0.3 mL water and 0.3 mL triisopropylsilane to a tube with lid.

To make enough cleavage solution for 3 syringes (18 mL) we added 17.1 mL TFA, 0.45 mL water and 0.45mL triisopropylsilane to a tube with lid.

Amine solutions

All amine solutions were made in TPP tubes of fitting size.

The general equations for calculating the amine solutions can be seen below:

$$c_1 * v_1 = c_2 * v_2$$

Where the initial concentration of the amine is calculated with the density over molar weight given in the table above:

$$c_1 = \frac{\rho\left(\frac{g}{mL}\right)}{Mw\left(\frac{g}{mol}\right)} = M\left(\frac{mol}{mL}\right)$$

The final concentrations of the amine solutions are 1M. All the individual calculations for each amine solution can be seen in chapter 3.1 figure 13. For amine solutions needed for both two different peptoid-chains, we made a total of 2.2 ml to make sure we wouldn't miss any in the synthesis in case some got stuck to the walls of the tube.

N-Boc-1,4-butanediamine (NLys):

$$\frac{0.989 \frac{g}{mL}}{188.27 \frac{g}{mol}} = 0.00527 \frac{mol}{mL}$$

$$v_1 = \frac{0.001 * 2.2}{0.00527} = 0.421\ mL$$

The 2.2 mL 1M solution is made by adding 0.421 mL to 1.779 mL N-Methylpyrrolidinone (NMP).

2-Aminopentane (N2ap):

$$\frac{0.736\frac{g}{mL}}{87.16\frac{g}{mol}} = 0.00844\frac{mol}{mL}$$

$$v_1 = \frac{0.001 * 2.2}{0.00844} = 0.261mL$$

The 2.2 mL 1M solution is made by adding 0.261 mL to 1.940 mL NMP

(S)-(-)-a- Methylbenzylamine (Nspe):

$$\frac{0.94\frac{g}{mL}}{121.18\frac{g}{mol}} = 0.00776\frac{mol}{mL}$$

$$v_1 = \frac{0.001 * 2.2}{0.00776} = 0.284\ mL$$

The 2.2 mL 1M solution is made by adding 0.284 mL to 1.914 mL NMP.

Butylamine (Nba):

$$c_1 = \frac{0.74\frac{g}{mL}}{73.14\frac{g}{mol}} = 0.01011\frac{mol}{mL}$$

$$v_1 = \frac{0.001 * 2.2}{0.01011} = 0.119\ mL$$

The 1.2 mL 1M solution is made by adding 0.119 mL to 1.081 mL NMP.

Hexylamine (Nha):

$$\frac{0.766\frac{g}{mL}}{101.19\frac{g}{mol}} = 0.00757\frac{mol}{mL}$$

$$v_1 = \frac{0.001 * 2.2}{0.00757} = 0.159\ mL$$

The 1.2 mL 1M solution is made by adding 0.159 mL to 1.041 mL NMP.

Appendix 3 HPLC-MS blanks

ACN april 3rd

The blank had no peaks fitting our results.

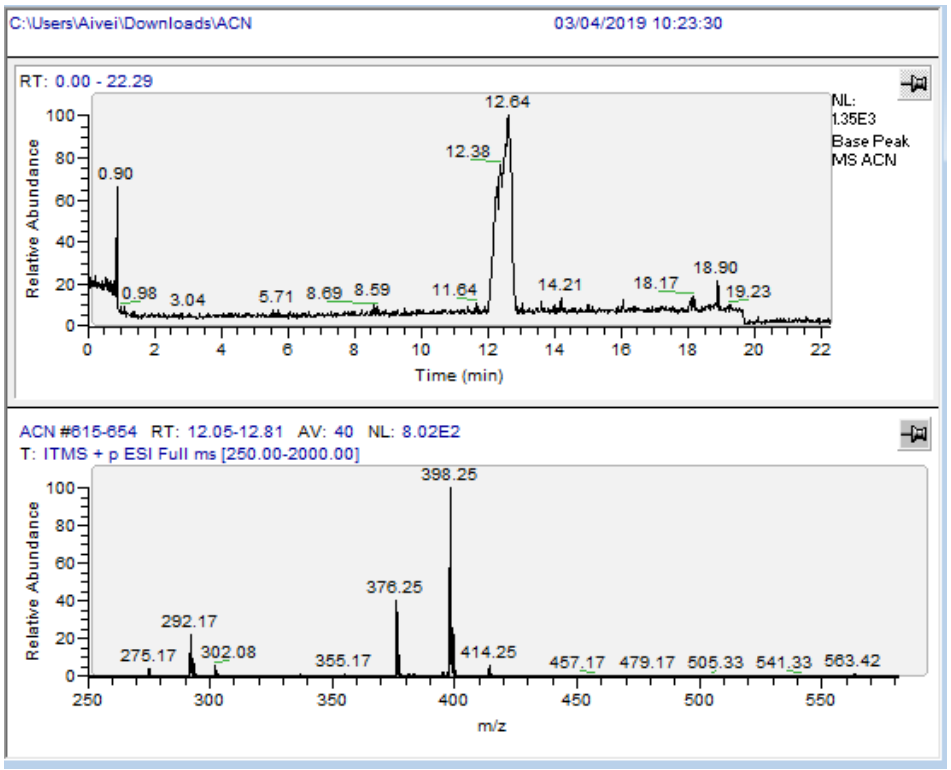


Figure 19 shows the HLPC-MS result of a blank test performed with pure acetonitrile before our samples (peptoid 1.1 and 1.2) were tested. It shows a spike at RT 12.05-12.81 which does not interfere with our sample.

A blank was run before our sample and fits a peak in our results.

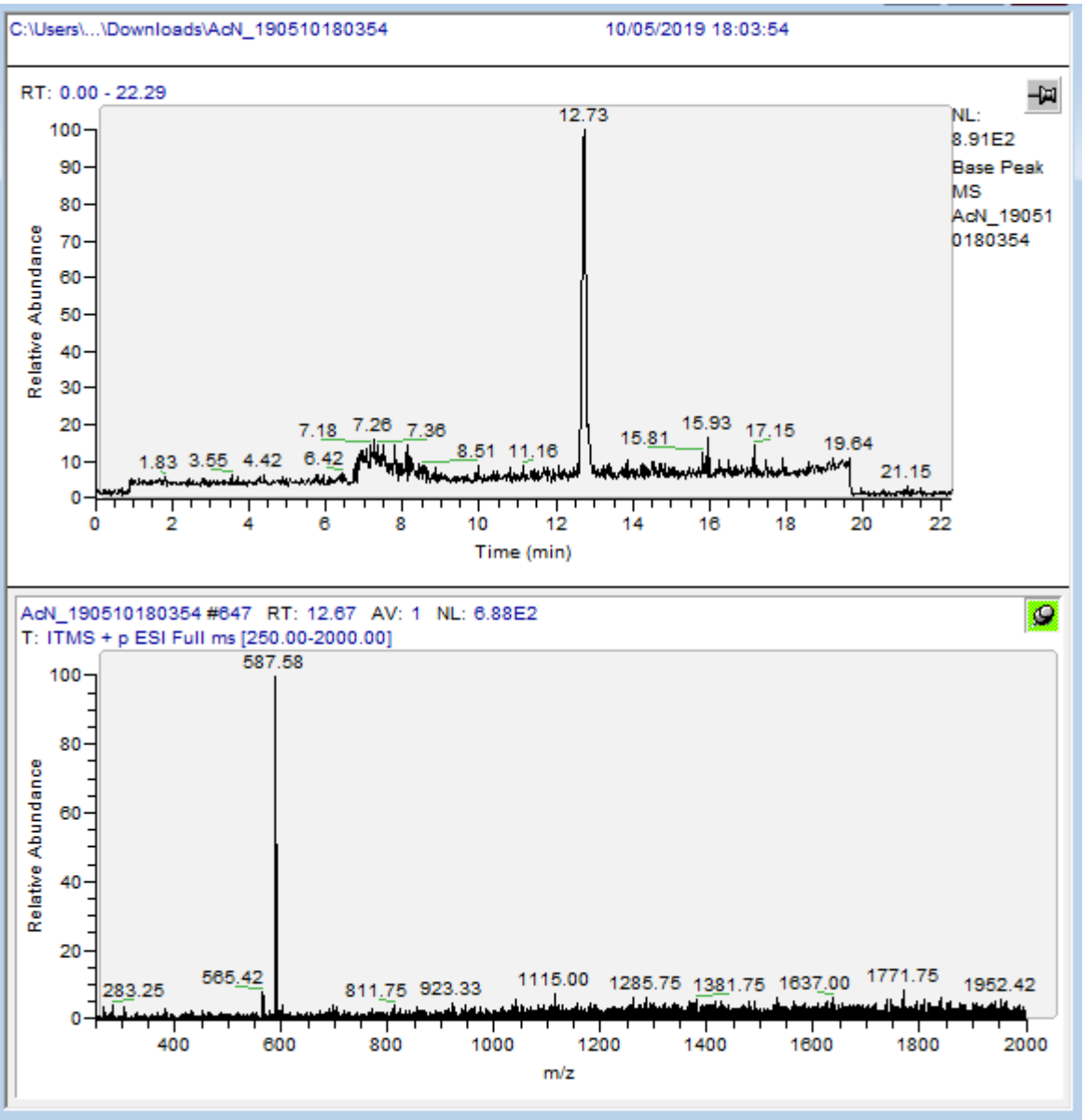


Figure 2 shows the HLPC-MS result of a blank test performed with pure acetonitrile before our sample (peptoid 2.2) were tested. It shows a spike at RT 12.69 which does interfere with our sample.

A blank was run before our samples and have a peak fitting our results.

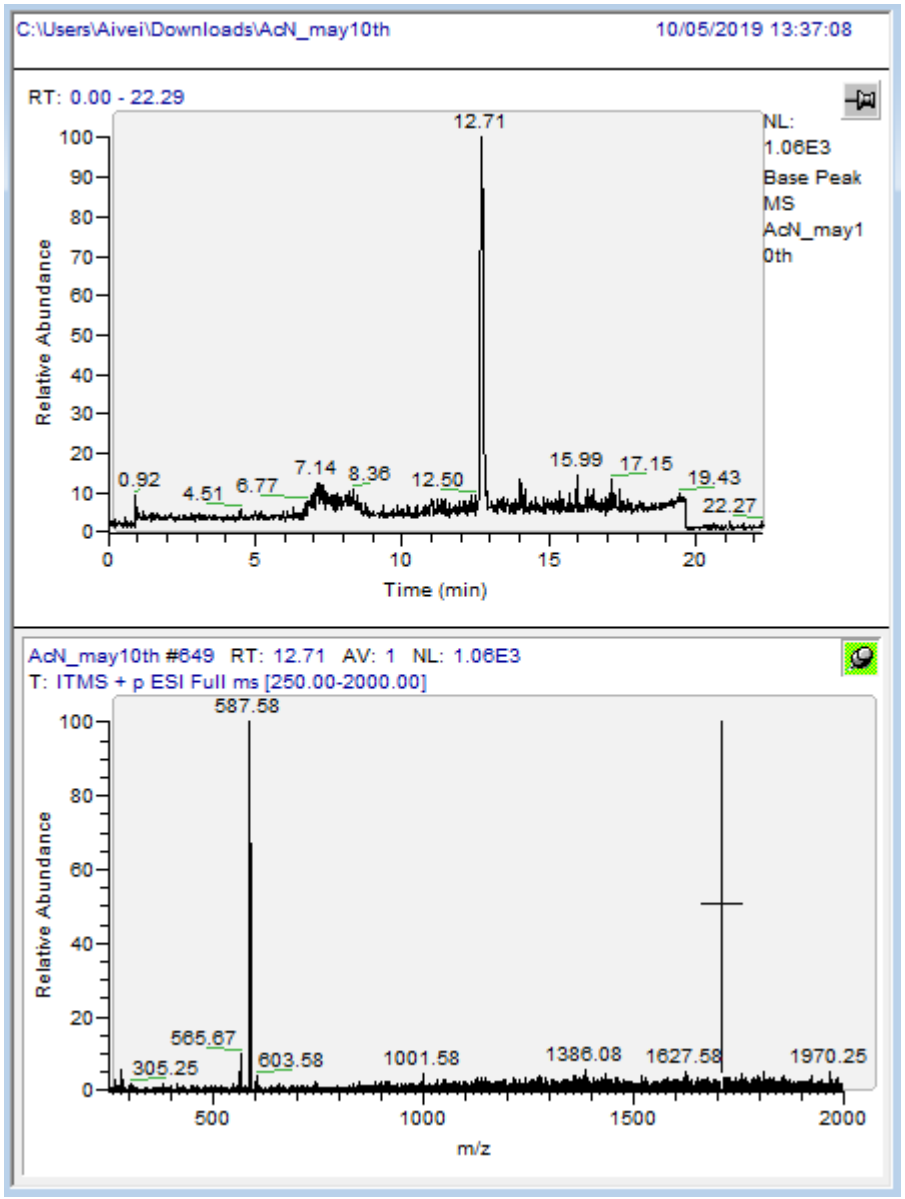
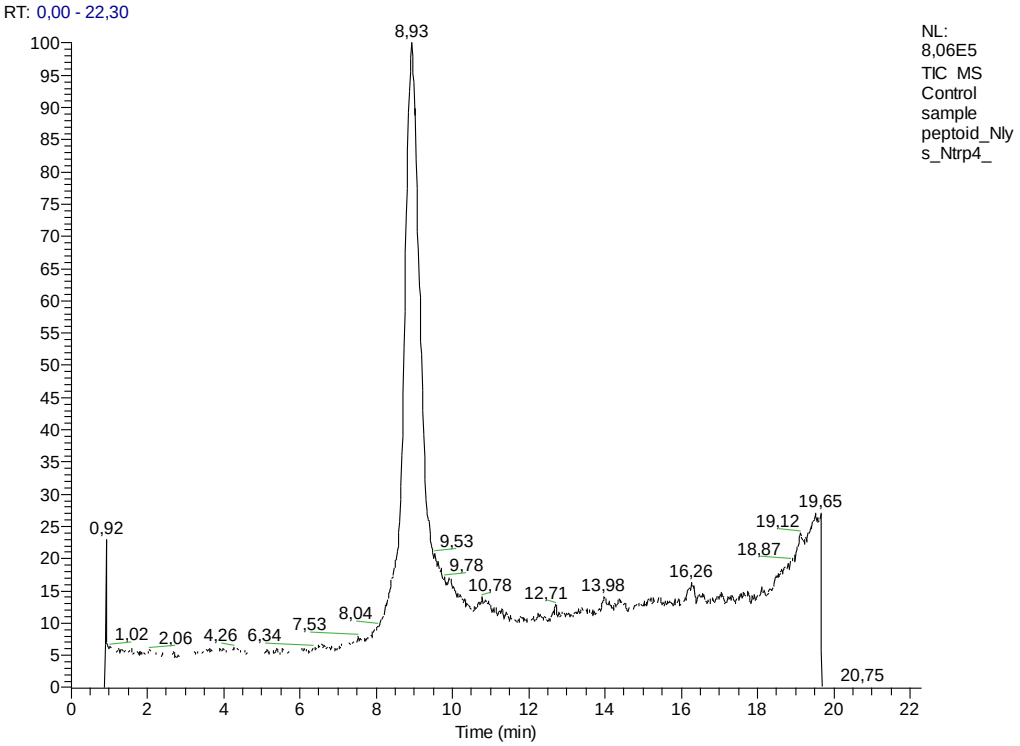


Figure 3 shows the HLPC-MS result of a blank test performed with pure acetonitrile before our samples (peptoid 3.1 and 3.2) were tested. It shows a spike at RT 12.71 which does interfere with our sample. The spike has the same mass per charge as the spike in appendix 3 figure 2.

Control Nlys, Ntrp4



Figur 1, Chromatogram of control of Nlys and Ntrp4 (the sample has been run on a gradient 15-65 % acetonitrle water with 0,1 % formic acid, on a c18 coloumn (phenomenex)).